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**INTERACTION STROMALE-ÉPITHÉLIALE DANS LE CANCER MAMMAIRE**

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## RÉSUMÉ COURT

La formation et la progression tumorale s'accompagnent d'une réaction stromale comportant la matrice extracellulaire ainsi qu'une variété de cellules hôtes comme des macrophages, fibroblastes et cellules endothéliales. Le rôle de ces cellules stromales est mal connu. Cette thèse visait à tester l'hypothèse selon laquelle les cellules stromales peuvent moduler et influencer l'invasion des cellules cancéreuses en produisant, en présence de stimulations cancéreuses, des protéases qui participent à la dégradation de la matrice extracellulaire puis à l'invasion tumorale. À l'aide d'un modèle *in vitro*, nous avons étudié l'influence de cellules cancéreuses mammaires sur l'expression stromale de stromélysine-3 et de cathepsine D et l'influence des fibroblastes associés à la tumeur sur la migration des cellules cancéreuses dans un essai d'invasion. Les résultats indiquent que les cellules stromales associées à la tumeur participent activement à la progression tumorale mammaire mais que les cellules cancéreuses n'ont pas le même pouvoir d'induire la production de protéases et que les cellules stromales répondent de façon différente à une même stimulation selon l'hôte. Ces résultats suggèrent que cette interaction entre cellules cancéreuses et stromales pourrait être une cible intéressante pour des essais thérapeutique anticancéreux.

## RÉSUMÉ LONG

Le cancer mammaire, comme d'autres formes de carcinomes, n'est pas seulement composé de cellules tumorales. Outre les cellules cancéreuses, le stroma tumoral est composé de différents types cellulaires de l'hôte (fibroblastes, cellules endothéliales, cellules immunitaires) enchâssés dans une matrice extracellulaire. La littérature actuelle suggère que ces deux compartiments sont interdépendants dans le développement de carcinome invasif humain. En effet, les cellules hôtes activées par les cellules cancéreuses produisent de nombreuses cytokines, chémokines, facteurs de croissance, protéines de la matrice extracellulaire, de nombreuses protéases et inhibiteurs de ces protéases. Des études de cancers humains et en expérimentation animale *in vivo* et *in vitro* démontrent l'importance des interactions entre les cellules cancéreuses, les matrices extracellulaires et les cellules stromales de l'hôte dans le processus de la formation et la progression tumorale. Dans le carcinome mammaire humain, la plupart des protéases participant à la dégradation protéolytique sont d'origine stromale et appartiennent à différentes familles qui interagissent entre elles. La tumeur, en effet, représente un écosystème complexe. Les cellules tumorales stimulent sélectivement l'activation de gènes par les cellules hôtes en périphérie des cellules malignes. Ces activations géniques sélectives aboutissent à l'expression stromale de diverses protéases dont la cathepsine D, stromélysine-3, gélatinase et uPA étudiées dans cette thèse. L'analyse de la valeur pronostique confirme leur contribution dans la progression de carcinome mammaire. La présence de cellules transformées induit la production de ces protéases par ces cellules stromales associées à la tumeur. Contrairement à la cathepsine D, l'expression de stromélysine-3 est cancer-spécifique. Elle est surtout produite par les cellules stromales à proximité des cellules cancéreuses suggérant un effet de contact direct cellule-cellule. Les cellules stromales associées à la tumeur sont une population réactionnelle ou activée. Elles contribuent non seulement à la production stromale des protéases, mais favorisent aussi la migration et l'invasion des cellules cancéreuses mammaires. Il est possible donc de concevoir de nouvelles stratégies anti-tumorales ciblées sur les éléments stromaux de l'hôte qui favorisent la cancérisation mammaire.

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## LISTE DES ABBRÉVIATIONS

|                               |                                                         |
|-------------------------------|---------------------------------------------------------|
| <b>9C-RA</b>                  | <b>9-cis-retinoid acid</b>                              |
| <b>BCTC</b>                   | <b>Bioengineered cancer tissue construct</b>            |
| <b>CD</b>                     | <b>Cathepsin D</b>                                      |
| <b>CGN</b>                    | <b>cis-Golgi</b>                                        |
| <b>DR1</b>                    | <b>Directly repeated consensus hexameric half-sites</b> |
| <b>ECM</b>                    | <b>Extracellular matrix</b>                             |
| <b>EE</b>                     | <b>Early endosome</b>                                   |
| <b>EGF</b>                    | <b>Epidermal growth factor</b>                          |
| <b>ER</b>                     | <b>Réticulum endoplasmique</b>                          |
| <b>FGF</b>                    | <b>Fibroblast growth factor</b>                         |
| <b>HGF</b>                    | <b>Hepatocyte growth factor</b>                         |
| <b>IGF</b>                    | <b>Insulin-like growth factor</b>                       |
| <b>LE</b>                     | <b>Late endosome</b>                                    |
| <b>M6P</b>                    | <b>Mannose-6-phosphate</b>                              |
| <b>MMPs</b>                   | <b>Matrix metalloproteinases</b>                        |
| <b>MSF</b>                    | <b>Migration stimulating factor</b>                     |
| <b>MT-MMP</b>                 | <b>Membrane-type matrix metalloprotease</b>             |
| <b>PAI-1</b>                  | <b>Plasminogen activator inhibitor</b>                  |
| <b>PDGF</b>                   | <b>Platelet-derived growth factor</b>                   |
| <b>RARE</b>                   | <b>Retinoic acid-responsive element</b>                 |
| <b>SG</b>                     | <b>Granule sécrétoire</b>                               |
| <b>ST3</b>                    | <b>Stromélysine-3</b>                                   |
| <b>TGF</b>                    | <b>Transforming growth factor</b>                       |
| <b>TGF<math>\alpha</math></b> | <b>Transforming growth factor-<math>\alpha</math></b>   |
| <b>TGN</b>                    | <b>trans-Golgi</b>                                      |
| <b>TIMP-2</b>                 | <b>Tissue inhibitor of metalloproteinases</b>           |
| <b>t-RA</b>                   | <b>All-trans-retinoid acid</b>                          |
| <b>UPAR</b>                   | <b>Urokinase activator receptor</b>                     |

## **CHAPITRE I**

### **INTRODUCTION**

Le cancer est une édification cellulaire persistante, résultant de la croissance anormale de ces cellules nées sur place, et qui prolifèrent en faisant preuve d'autonomie biologique par rapport à l'organisme qui le porte et qu'on appelle l'hôte.

Deux processus intriqués participent à la formation d'un tissu cancéreux : la prolifération des cellules néoplasiques proprement dites dont l'ensemble constitue le parenchyme tumoral, d'une part; l'individualisation d'un stroma tumoral, c'est-à-dire d'un tissu conjonctif servant à la fois de charpente et de système de nutrition d'autre part (Gagne F. *et al.*, 1980).

Le stroma cancéreux se définit comme l'ensemble des structures mésenchymateuses modifiées qui supportent le tissu cancéreux. Il accompagne toute prolifération néoplasique et représente la réaction du tissu conjonctif qui subit diverses modifications au contact du parenchyme tumoral. Il comprend des éléments préexistant de ce tissu et d'autres éléments néoformés mais non tumoraux. Le stroma contient des éléments cellulaires, c'est-à-dire des cellules stromales (fibroblastes, macrophages, histiocytes, péricytes, etc.) et la matrice extracellulaire qui est un complexe dynamique de glycoprotéines et protéoglycans. Le stroma possède en outre et surtout, une vascularisation sanguine et lymphatique qui tient une place importante dans la vie locale de la néoplasie.

La majorité des tumeurs humaines sont des carcinomes, c'est-à-dire d'origine épithéliale. Initialement, les altérations génétiques multiples entraînent une prolifération anarchique des cellules qui restent néanmoins confinées dans leur compartiment épithélial d'origine. L'absence d'infiltration et la continuité de la membrane basale sous-épithéliale caractérisent le carcinome in situ.

L'invasion tumorale et la métastase sont les principales causes de la morbidité et le décès des patients porteurs de cancer. Le mécanisme exact responsable de la formation des métastases n'est pas complètement connu à ce jour. Cependant, certains principes ont été élucidés. L'événement critique du processus tumoral qui initie la cascade métastatique est la rupture de la membrane basale par les cellules cancéreuses avec l'invasion du stroma (Fidler and Radinsky, 1990; Liotta *et al.*, 1991a). Le stroma représente un obstacle essentiel que les

cellules tumorales doivent franchir pour se transformer en un carcinome infiltrant (Siegal *et al.*, 1981). Pour envahir les tissus et devenir une tumeur infiltrante et ainsi de former la métastase à distance, les cellules tumorales doivent pénétrer la matrice extracellulaire et atteindre le réseau vasculaire pour être transportées jusqu'au site d'implantation secondaire. L'invasion des cellules tumorales implique trois étapes (Liotta *et al.*, 1986) soit :

- l'adhésion sur les composants de la matrice extracellulaire;
- la protéolyse de la matrice extracellulaire;
- la migration sur les éléments de la matrice extracellulaire ou sur leurs fragments.

L'ensemble de ces événements dépend de mécanismes impliquant les molécules de la matrice extracellulaire (laminine, fibronectine, collagènes etc.), les récepteurs de ces molécules et, enfin, des enzymes protéolytiques.

Le stroma cancéreux est, en réalité, un tissu conjonctif de support, et les cellules stromales dans un tissu cancéreux étaient souvent et simplement considérées comme ayant un rôle passif (Diamandopoulos G.Tr. and Meissner W.A., 1985). Le stroma a été considéré comme l'expression locale d'une réaction de défense de l'organisme vis-à-vis de la prolifération tumorale. Le nom de réaction stromale a été donné à l'ensemble des phénomènes aboutissant à l'élaboration et aux modifications de ce stroma que l'on croyait destiné à limiter l'expansion du cancer. Par exemple, dans le carcinome médullaire du sein, on croyait l'infiltration lymphocytaire pure ou prédominante responsable du pronostic meilleur de cette tumeur (Gagne F. *et al.*, 1980). En conséquence, la plupart des études ont étudié isolément le rôle des cellules cancéreuses dans le processus de la croissance, de l'invasion et de la métastase des cancers. Cependant, le stroma est également nécessaire pour la formation tumorale et la croissance tumorale. À témoin le fait que la croissance tumorale, à partir d'une certaine taille, a besoin d'une formation persistante de nouveaux vaisseaux pour assurer sa croissance et que cette angiogenèse se tient justement dans le stroma (Hanahan and Folkman, 1996). En réalité, l'élément stromal dans un tissu cancéreux est une forme tissulaire spécialement associée à la prolifération et à la croissance des tumeurs épithéliales, et reçoit les signaux biologiques des cellules cancéreuses, des nutriments, des facteurs de croissance et de l'angiogenèse inversement. Les cellules stromales peuvent moduler le phénotype de cellules épithéliales cancéreuses (Atula *et al.*, 1997), favoriser la prolifération des cellules cancéreuses (Cullen *et*

*al.*, 1991; van Roozendaal *et al.*, 1996), accélérer la croissance tumorale et produire des protéases favorisant l'invasion tumorale (Noël *et al.*, 1994). Par rapport aux nombreuses études ciblant les cellules cancéreuses seules, relativement peu d'études ont été rapportées pour élucider le rôle clinique des cellules stromales et des interactions épithéliales-stromales dans des tumeurs humaines.

La cathepsine D, une endoprotéase acide et lysosomiale, a été identifiée dans le cancer du sein comme facteur pronostique potentiel (Rocheft, 1990). Cependant, alors que par méthodes biochimiques, la cathepsine D semble bien prédire un mauvais pronostic, des résultats fort contradictoires ont été obtenus par immunohistochimie alors que la plupart des études ciblaient les cellules cancéreuses (Henry *et al.*, 1990; Thorpe *et al.*, 1989). En 1993, le groupe du Dr Têtu a séparément analysé l'expression de la cathepsine D par les cellules stromales et les cellules cancéreuses dans le cancer mammaire, et conclu que seulement l'expression stromale de la cathepsine D prédisait un mauvais pronostic (Têtu *et al.*, 1993). Il en résulte une hypothèse que les cellules stromales pourraient jouer un rôle actif dans l'interaction stromale-épithéliale cancéreuse en favorisant la formation, l'invasion et la métastase tumorale. Nous avons donc cherché à mieux comprendre par cette étude le rôle de ces cellules stromales et des protéases qu'elles produisent dans des cancers du sein chez l'humain.

### **1. Les cellules stromales et le cancer mammaire.**

Comme d'autres tumeurs du tissu épithélial, le carcinome mammaire a une organisation tissulaire dans laquelle, les éléments épithéliaux sont entourés d'une prolifération stromale. Ces éléments stromaux contiennent des fibroblastes, des cellules inflammatoires comme macrophages, une matrice extracellulaire et des éléments vasculaires.

L'interaction épithéliale-stromale est importante dans tous les stades de développement mammaire de l'embryogenèse à la lactation de l'adulte. Des expériences ont montré que le mésenchyme pouvait déterminer la morphologie et la fonction de la glande mammaire (Sakakura *et al.*, 1987; Sakakura *et al.*, 1976).

### **1.1. Les cellules stromales sont importantes pour la réponse épithéliale aux œstrogènes.**

Les œstrogènes sont importants pour le développement du cancer du sein. Les cellules cancéreuses répondent aux œstrogènes en produisant des facteurs qui influencent non seulement leur prolifération propre, mais aussi la prolifération de cellules stromales. Ces dernières peuvent également, par voie de conséquence, produire des facteurs pour stimuler la prolifération de cellules cancéreuses (Haslam and Levely, 1985; McGrath, 1983). Les cellules épithéliales répondant à la stimulation des œstrogènes *in vivo* deviennent non-répondantes à la stimulation par les œstrogènes quand ces cellules épithéliales sont isolées du support stromale *in vitro* (Iguchi *et al.*, 1983). Cependant, en coculture, avec des fibroblastes mammaires *in vitro*, cette réponse épithéliale aux œstrogènes est restaurée (Haslam, 1986). Ces résultats suggèrent que dans le cancer du sein certaines actions œstrogéniques sont indirectes et demandent l'interaction avec les cellules stromales.

### **1.2. Les cellules stromales peuvent favoriser la prolifération des cellules cancéreuses épithéliales par effet paracrinien.**

L'effet paracrinien des fibroblastes a été mis en évidence dans le développement de cancer du sein par de nombreux essais de culture *in vitro* avec milieu conditionné de culture de fibroblastes. Dans la plupart des cas, le milieu conditionné de culture de fibroblastes d'origine mammaire favorise la prolifération de cellules épithéliales cancéreuses d'origine mammaire telles que MCF-7 (Cullen *et al.*, 1991; Gache *et al.*, 1998; Lippman *et al.*, 1986; Ryan *et al.*, 1993; van Roozendaal *et al.*, 1992; van Roozendaal *et al.*, 1996; Yee *et al.*, 1988; Yee *et al.*, 1989). De plus, le milieu conditionné de culture de fibroblastes provenant de cancer du sein donne une stimulation plus importante sur la prolifération de cellules cancéreuses (van Roozendaal *et al.*, 1996). Dans certaines expériences, le milieu conditionné de culture de fibroblastes provenant du sein normal ou d'une lésion bénigne (Ryan *et al.*, 1993) ne favorise pas la prolifération des cellules épithéliales, voire inhibe la prolifération épithéliale (Dong-Le *et al.*, 1997).

Le milieu conditionné de culture de fibroblastes humains a un effet mitogène sur des lignées cellulaires cancéreuses mammaires, en augmentant la synthèse d'ADN et la prolifération (Ryan *et al.*, 1993). Ceci supporte la conception que les cellules stromales peuvent participer à

la régulation de la croissance cancéreuse mammaire par la production de facteurs qui modulent la prolifération des cellules cancéreuses (Cullen *et al.*, 1991; Lippman *et al.*, 1986; Yee *et al.*, 1988; Yee *et al.*, 1989). Cet effet sur la prolifération des cellules cancéreuses peut être augmenté par l'addition d'insuline, d'EGF et de 17- $\beta$ -estradiol (Hofland *et al.*, 1995), suggérant que certain(s) facteur(s) soluble(s) soient responsable(s) de cet effet de stimulation.

Cependant, Mukaida (Mukaida *et al.*, 1991) a observé que les fibroblastes de tissu mammaire sont stimulateurs sur la prolifération des cellules MCF-7, tandis que les fibroblastes d'autres sites ne présentent pas cet effet ou ont un effet inhibiteur.

### **1.3. Les cellules stromales peuvent favoriser la formation et la croissance de la tumeur.**

Des études *in vivo* et *in vitro* ont montré un effet des fibroblastes sur la formation tumorale et la croissance de la tumeur (van den Hooff, 1988; Zipori *et al.*, 1987). Quand les fibroblastes sont coinoculés avec les cellules cancéreuses de vessie ou de sein dans des souris nues/nues, la période de latence pour former la tumeur est raccourcie et la croissance de tumeurs est accélérée (Camps *et al.*, 1990). Dans une expérience similaire (Horgan *et al.*, 1987), les fibroblastes mammaires et cutanés étaient coinoculés avec des cellules MCF-7 dans des souris nues/nues, et la formation tumorale a été observée dans 94% de souris contre 49% pour les souris inoculées avec les cellules MCF-7 seules. De plus, la coinoculation des fibroblastes avec MCF-7 favorisait la croissance de la tumeur. Les fibroblastes dérivés de tumeur mammaire ou de tissu normal mammaire aurait le même effet sur la vitesse de développement de tumeur et la taille de la tumeur (Ryan *et al.*, 1993).

### **1.4. Les cellules stromales peuvent favoriser la migration et l'invasion des cellules épithéliales cancéreuses.**

Pour envahir les tissus et former des métastases à distance, les cellules tumorales doivent pénétrer la matrice extracellulaire et atteindre le réseau vasculaire pour être ensuite transportées jusqu'au site d'implantation secondaire. Les cellules tumorales peuvent transmettre des messages par un système de signalisation aux cellules stromales pour qu'elles produisent des facteurs de croissances ou des protéinases et ainsi favoriser la prolifération, la migration et l'invasion des cellules cancéreuses (Dong-Le *et al.*, 1997; Iwazawa *et al.*, 1996;

donc leur effet sur l'épithélium adjacent. Les fibroblastes sont une population de cellules stromales peu caractérisées, ils sont définis en général par leur morphologie fusiforme (spindle-shaped) *in vivo* et des marqueurs non spécifiques tels que vimentine et collagène type 1. On sait que les fibroblastes forment une population hétérogène (Schor *et al.*, 1994; Tamimi and Ahmed, 1987), et dans chaque expérience, une sorte de sous-population de cellules avec des caractéristiques spécifiques pourrait être prédominante sur les autres.

#### **1.6. Les fibroblastes associés à une tumeur mammaire sont différents des fibroblastes normaux.**

En 1969, Nordbye *et al.* (Nordbye, 1969) ont observé que les fibroblastes purifiés de cancer du col utérin humain présentaient une croissance désorganisée comme celle de cellules cancéreuses. Cependant, tous les fibroblastes étaient par ailleurs normaux (Delinassios *et al.*, 1983). Chaudhuri *et al.* ont observé une agglutinabilité différente de ces fibroblastes dérivés du cancer du col utérin (Chaudhuri *et al.*, 1975). Todaro *et al.* ont observé que des lignées cellulaires malignes humaines en culture pouvaient libérer des facteurs comme TGF (transforming growth factor) qui conférait un phénotype malin aux fibroblastes non-transformés (Todaro *et al.*, 1980).

La différence phénotypique entre les fibroblastes de tissu normal et ceux de tissu tumoral a été observée par d'autres chercheurs. Il a été rapporté que les fibroblastes provenant de cancer mammaire, bien que non transformés dans le tissu tumoral, possédaient également un phénotype différent de celui des fibroblastes de sein non-tumoral (Hornby and Cullen, 1995; Hornby and Cullen, 1995). Ces fibroblastes de tissu tumoral ont reçu différentes appellations tels que "activated fibroblasts" (van den Hooff, 1988; van den Hooff, 1991), "abnormal" (Grey *et al.*, 1989), "reactive ou tumor-associated" (Têtu *et al.*, 1993; Têtu *et al.*, 1998). Selon une analyse ultrastructurale, certains auteurs ont classé les cellules stromales de cancer du sein comme "resting fibroblast", "fibroblastes actifs", "myofibroblastes précoces" et "myofibroblastes matures" (Tamimi and Ahmed, 1987). Adams *et al.* (Adams *et al.*, 1988) ont rapporté que les fibroblastes de tumeur mammaire peuvent stimuler la prolifération de MCF-7 *in vitro*, tandis que les fibroblastes de mammoplastie de réduction étaient inhibiteurs. Ryan *et al.* (Ryan *et al.*, 1993) ont rapporté que le niveau de réponse de prolifération dépendait

de la source de fibroblastes, l'effet mitogénique étant plus important avec les fibroblastes de tumeur et de la peau qu'avec les fibroblastes de lésion bénigne mammaire. Cependant, un consensus n'est pas atteint et dans une étude récente, les fibroblastes d'origine cancéreuse ou normale du sein pouvaient stimuler également les cellules cancéreuses d'origine mammaire tel que MCF-7, MDA-MB-231, BT-20 et T47D (Gache *et al.*, 1998).

Un sous-groupe de cellules stromales du cancer du sein montre un élément de différenciation de muscles lisses et désigné myofibroblastes (Ronnov-Jessen *et al.*, 1992). Les myofibroblastes sont des cellules contractiles identifiés 1971 par isolement du tissu de granulation (Gabbiani *et al.*, 1971). Les cellules ont été définies comme des cellules hybridées avec ultrastructure des cellules de muscle lisse et des fibroblastes (Gabbiani *et al.*, 1971). Ces myofibroblastes sont différents des cellules myoépithéliales par la présence de vimentine et l'absence de cytokératine. Bien que ces cellules expriment de l' $\alpha$ -actine, elles sont différentes des cellules musculaires lisses par l'absence de desmine (Ahmed, 1990). Les myofibroblastes ont été impliqués dans certains processus physiologiques et pathologiques, le rôle le plus important des ces cellules étant la contraction lors de la cicatrisation des blessures (Gabbiani *et al.*, 1973; Gabbiani *et al.*, 1971). L'origine des myofibroblastes reste inconnue. Cependant, Oda *et al.* ont observé que les myofibroblastes purifiés de tissu mammaire produisaient les collagènes I et III comme les fibroblastes, et les auteurs ont conclu que les myofibroblastes étaient dérivés de fibroblastes (Oda *et al.*, 1988). Par la coculture des fibroblastes sans expression d' $\alpha$ -actine avec milieu conditionné de MCF-7, l'expression de l' $\alpha$ -actine et le phénotype de myofibroblastes peuvent être induits dans ces fibroblastes d'origine mammaire (Ronnov-Jessen *et al.*, 1992). Des vésicules sécrétoires ont été observées dans les myofibroblastes et non dans les fibroblastes (Oda *et al.*, 1990), impliquant que les myofibroblastes ont la fonction active de sécrétion (Oda *et al.*, 1990). Des expériences ont suggéré que ces myofibroblastes possédaient des fonctions propres comme la déposition de collagène excessif et la contraction donnant la desmoplasie (Tremblay, 1979), une réaction fibrotique associée souvent au cancer mammaire. Le processus d'infiltration des cellules cancéreuses ressemble à une plaie qui ne guérit pas (Tamimi and Ahmed, 1987). On pense que les myofibroblastes peuvent faciliter la motilité des cellules cancéreuses par interaction épithéliale-myofibroblaste via des contacts directs cellules-cellules (Ronnov-Jessen *et al.*,

1992).

La présence des myofibroblastes dans le cancer du sein a été rapportée la première fois dans une étude sur le carcinome tubulaire (Harris and Ahmed, 1977). Une expression de l' $\alpha$ -actine a été souvent observée par analyse d'immunohistochimie dans les cellules stromales dérivées de tissu cancéreux mammaire et rarement dans les cellules stromales dérivées de tissu normal (Brouty-Boye *et al.*, 1994; Sappino *et al.*, 1990). Dans le cancer du sein, 70% (Ronnov-Jessen *et al.*, 1992) à 80% (Sappino *et al.*, 1988) des cellules stromales ont été identifiées comme des myofibroblastes selon l'analyse de l'expression de l' $\alpha$ -actine par immunohistochimie, tandis que les myofibroblastes sont absents dans le tissu normal du sein. Ces résultats ont été confirmés par culture *in vitro* de myofibroblastes à partir de tissu mammaire, dont les myofibroblastes avaient été obtenus à partir de tissus cancéreux et non de tissus normaux (Ronnov-Jessen *et al.*, 1992). La présence de myofibroblastes est également liée à l'histologie du cancer (Schurch *et al.*, 1982). On les observe plus fréquemment dans les carcinomes infiltrants de type tubulaire, lobulaire et canalaire, qui sont fermes et contractiles. Par contre, ils sont peu fréquents dans le carcinome infiltrant de types médullaire et colloïde. Le niveau de myofibroblastes dans les différents types histologiques de cancer mammaire a été associé au taux (Schurch *et al.*, 1982) et au mode (Tamimi and Ahmed, 1987) de croissance tumorale.

Les infiltrats inflammatoires répondent surtout à un phénomène inflammatoire ou à une infection. Ce sont des infiltrats de type commun avec densité cellulaire élevée en macrophages, polynucléaires neutrophiles et éosinophiles, lymphocytes, plasmocytes. Dans le cancer mammaire, on peut voir une infiltration de macrophages qui atteint 80% des cellules inflammatoires (O'Sullivan *et al.*, 1993). Ces macrophages peuvent sécréter le facteur EGF (epidermal growth factor) (O'Sullivan *et al.*, 1993) et sont liés au phénotype invasif du cancer mammaire (Visscher *et al.*, 1995). L'infiltration par ces cellules inflammatoires représente une réaction de l'hôte avec production de protéases putatives dont la cathepsine D (Diment *et al.*, 1988; Roger *et al.*, 1994; Visscher *et al.*, 1995), le MMP-9 (Zakian, 1997) et le MMP-7 (Busiek *et al.*, 1992) par les macrophages associés à l'invasion et les métastases par cellules cancéreuses. Il est démontré que le facteur TGF- $\beta$  produit par les cellules cancéreuses

mammaires pouvaient stimuler les macrophages associés à la tumeur mammaire à produire de l'uPA (Hildenbrand *et al.*, 1998), une protéase importante dans la dégradation de la matrice extracellulaire. Des chercheurs voient même les macrophages associés à la tumeur comme une cible thérapeutique intéressante (Wahl and Kleinman, 1998).

En effet, les cellules stromales du sein sont une population hétérogène avec un phénotype différent (Schor *et al.*, 1994). Il existe des différences fonctionnelles entre certaines populations de fibroblastes cancéreux mammaires et ceux du sein normal (Azzarone *et al.*, 1984)(Maciera-Coelho and Azzarone 1987). Schor *et al.* (Schor *et al.*, 1985) ont observé que les fibroblastes de cancer mammaire migraient plus rapidement dans une matrice de collagène que les fibroblastes de tissu normal. Un facteur soluble, MSF (migration stimulating factor) (Grey *et al.*, 1989) a été purifié à partir de ces fibroblastes associés à la tumeur et non à partir des fibroblastes d'origine normale (Schor *et al.*, 1988). Quand les fibroblastes sont exposés au facteur MSF, il y a une augmentation de capacité de mobilité dans le gel de collagène (Picardo *et al.*, 1991). Bien que la signification de cette découverte ne soit pas bien comprise dans le cancer mammaire *in vivo*, il est possible que ce facteur soluble, MSF, puisse altérer le comportement biologique du cancer du sein en modifiant la mobilité des cellules dans la tumeur, favorisant ainsi l'invasion et la métastase tumorale.

#### **1.7. Le support stromal dans la purification des cellules épithéliales mammaires.**

Les études *in vitro* sur l'interaction épithéliale et stromale dans le cancer du sein sont souvent réalisées par coculture *in vitro* de cellules épithéliales et de fibroblastes de tissu mammaire, ce qui nécessite d'abord une récupération sélective de cellules épithéliales des tissus tumoraux ou normaux mammaires. La purification sélective des cellules épithéliales est aussi la première étape d'une culture à long terme ainsi que l'établissement d'une lignée cellulaire mammaire. Le tissu tumoral mammaire contient une proportion importante de matrice extracellulaire et de cellules stromales, ce qui rend la culture sélective de cellules épithéliales de tumeur mammaire primaire difficile (Sykes J.A. *et al.*, 1970).

Dans une culture primaire à partir d'un tissu mammaire, le tissu tumoral est souvent digéré d'abord par une enzyme en libérant les cellules de la matrice extracellulaire puis l'on obtient

une suspension cellulaire contenant les cellules épithéliales tumorales ou normales aussi que les cellules stromales, qui sont libérées en même temps de la matrice extracellulaire. Cette méthode donne une grande cellularité. Cependant, l'on observe souvent par cette technique une prolifération beaucoup plus importante des cellules stromales, principalement les cellules de type *fibroblast-like* en culture, que les cellules épithéliales. Une autre technique, dite *spilled cell technique*, a été initiée par Lasfargues en 1958 (Lasfargues and Ozzello, 1958). La méthode permet aux cellules épithéliales cancéreuses de migrer sélectivement de cubes tissulaires mammaires découpés et déposés dans un milieu de culture sans effet enzymatique. Bien que les cellules migrées (*spilled*) soient des cellules épithéliales cancéreuses, la cellularité est souvent insuffisante. Par ailleurs, la prolifération de cellules épithéliales a besoin de facteurs de croissance et des interactions cellules-cellules ou cellules-matrice, qui ne sont plus présentes dans la culture *in vitro* après l'isolation de l'organe vivant. D'autre part, bien que les cellules épithéliales aient besoin d'un support stromal, la vitesse de la prolifération *in vitro* est toujours perturbée par la croissance incontrôlable des fibroblastes. Suite à plusieurs passages, les cellules stromales l'emportent généralement et les cellules épithéliales disparaissent progressivement. Donc, le contrôle de la surcroissance des fibroblastes constitue un problème des cultures primaires de cellules épithéliales de cancer mammaire.

Plusieurs modalités ont été étudiées et rapportées pour éviter cette surcroissance de fibroblastes. Sykes (Sykes J.A. *et al.*, 1970) a utilisé un gradient de densité discontinue pour séparer les cellules épithéliales des fibroblastes de tumeur du sein. Cette méthode ne permet pas d'avoir une purification complète. Edwards (Edwards *et al.*, 1980) a utilisé un anticorps spécifiquement contre les fibroblastes. Jones et Haskill ont même employé une sélection sur polyacrylamide (Jones and Haskill, 1973). La méthode utilisée par beaucoup de chercheurs est la culture avec milieu sélectif qui minimise beaucoup l'utilisation de sérum par ajout de substances nutritives, car le sérum favorise plus la croissance des fibroblastes (Ethier *et al.*, 1993). D'autres méthodes ont été rapportées tels que la généticine (Meltzer *et al.*, 1991), la trypsination différentielle (Dairkee *et al.*, 1997), la culture en sandwich créant un microenvironnement avec gradients d'oxygène et des nutriments (Dairkee *et al.*, 1995), un flacon de culture avec un substrat spécial comme Primaria<sup>®</sup> (Falcon) etc.

L'utilisation de cellules 3T3 irradiées comme couche nourricière est une méthode efficace utilisée dans la culture des kératinocytes initiée par Rheinwald et Green (Rheinwald and Green, 1975). Dans leur étude, la prolifération de fibroblastes de tissu cutané ou de tératome pouvaient être inhibée par les cellules 3T3 irradiées en couche nourricière. Cependant, cette méthode a été rarement utilisée dans la culture sélective des cellules cancéreuses à partir des tumeurs mammaires primaires.

La purification de cellules épithéliales constitue la première étape pour l'établissement de cultures à long terme et des lignées cellulaires. Les lignées établies et bien caractérisées sont des outils importants pour étudier l'interaction des cellules stromales et épithéliales *in vitro*. La plupart des lignées disponibles d'origine de cancer mammaire sont, à ce jour, d'origine d'épanchements pleuraux ou abdominaux et donc de métastase. Peu de ces lignées sont d'origine primaire à cause de la difficulté de sélection liée à la contamination des fibroblastes et la prolifération insuffisante des cellules épithéliales *in vitro*. La première lignée mammaire (BT-20) a été établie par Lasfargues en 1958 (Lasfargues and Ozzello, 1958). Des lignées couramment utilisées comme MCF-7 (Soule *et al.*, 1973) et T-47D (Keydar *et al.*, 1979) ont été établies il y a plus de 20 ans. Certaines lignées établies depuis longtemps ont présenté des instabilités génotypiques occasionnelles suite à la culture et des passages trop nombreux *in vitro* (Engel and Young, 1978a). Plusieurs méthodes ont été réalisées et rapportées dans la littérature pour faciliter l'isolement de nouvelles lignées ou favoriser la culture à long terme de cellules cancéreuses mammaires. À part quelques rapports sporadiques, deux grandes séries ont été rapportées. Zoli *et al.* ont étudié 136 tumeurs primaires mammaires (Zoli *et al.*, 1991), seulement une lignée à long terme a été obtenue parmi 136 cas étudiés (Amadori *et al.*, 1993). Dans une autre grande série de McCallum et Lowther (McCallum and Lowther, 1996), 135 tumeurs primaires ont été mises en culture avec un milieu sélectif de MCDB 170 (Gibco). L'étude a réussi à obtenir dix lignées permanentes de ces 135 tumeurs sur une période de 6 ans et demi. Les auteurs ont mis au point une méthode convenable pour un taux de 7% de réussite de culture à long terme.

Tableau 1. Les lignées cellulaires établies à partir des carcinomes primaires du sein humain.

| Nom de lignées | Auteurs         | Histologie | ER/PgR | Dt (h) | Formation                      | P53 mutation | Amplification        |
|----------------|-----------------|------------|--------|--------|--------------------------------|--------------|----------------------|
|                |                 |            |        |        | tumorale dans les souris nu/nu |              | c-myc /ras /c-erbB-2 |
| BT-20          | Lasfargus 1958  | IDC        | -/-    | 30     |                                |              |                      |
| CaMa           | Dobrynin 1963   | Scirrhou   |        |        |                                |              |                      |
| BTM-1          | Martorelli 1969 | Scirrhou   |        | 20     |                                |              |                      |
| BOT-2          | Norquist 1975   | IDC        |        | 16-18  |                                |              |                      |
| BT-410         | Lasfargues 1978 | IDC        | -/+    | 72     | +                              |              |                      |
| BT-474         | Lasfargues 1978 | IDC        | -/+    | 120    | +                              |              |                      |
| Hs578T         | Hackett 1977    | IDC        | -/-    | 24-30  | -                              |              |                      |
| YMB-1          | Yamane 1984     | IDC        | +/+    | 44     |                                |              |                      |
| CAL19A         | Gioanni 1985    | ND         | -/-    | 15     |                                |              |                      |
| CAL18B         | Gioanni 1985    | ND         | -/-    | 30     |                                |              |                      |
| VHB-1          | Vandewalle 1987 | IDC        | +/+    | 30     |                                |              |                      |
| 8701-BC        | Minafra 1989    | IDC        |        | 28.8   |                                |              |                      |
| 21NT           | Band 1990       | IIC        |        | 40     |                                |              |                      |
| 21PT           | Band 1990       | IIC        |        | 40     | +                              |              | -/ND/+               |
| UACC812        | Meltzer 1991    | IDC        | -/-    | 100    |                                |              |                      |
| UACC893        | Meltzer 1991    | IDC        | -/-    | 120    |                                |              |                      |
| BRC-230        | Amadori 1993    | IDC        | -/-    | 30.5   | +                              |              | -/-                  |
| VP185          | McCallum 1996   | IDC        | -/-    |        |                                |              |                      |
| VP207          | McCallum 1996   | IDC        | -/-    |        |                                |              |                      |
| VP223          | McCallum 1996   | IDC        | -/-    |        |                                |              |                      |
| VP229          | McCallum 1996   | IDC        | -/-    |        |                                |              |                      |
| VP267          | McCallum 1996   | IDC        | -/+    |        |                                |              |                      |
| VP298          | McCallum 1996   | IDC        |        |        |                                |              |                      |
| VP303          | McCallum 1996   | IDC        |        |        |                                |              |                      |
| VP137          | McCallum 1996   | IDC        |        |        |                                |              |                      |
| VP331          | McCallum 1996   | IDC        |        |        |                                |              |                      |
| VP342          | McCallum 1996   | IDC        |        |        |                                |              |                      |

IIC: carcinome intracanaulaire et infiltrant, IDC: carcinome canalaire infiltrant,

ER: récepteur d'oestrogènes,

PgR: récepteur de la progestérone,

Dt: temps de doublement,

ND: non déterminé.

## **2. Le matrice extracellulaire dans le cancer mammaire.**

A part l'élément cellulaire, le stroma tumoral comprend une matrice extracellulaire. Il s'agit d'une structure complexe contenant des macromolécules comme des protéines et protéoglycans, qui déterminent l'architecture tissulaire et modulent également la morphologie, la prolifération, l'adhésion ou la migration, ainsi que les capacités biosynthétiques et migratoires des cellules normales et cancéreuses (Foidart *et al.*, 1994). Ce stroma s'insinue entre les structures tumorales et les enserme volontiers de façon très étroite, délimitant les cellules ou les îlots de cellules tumorales. En général, le stroma tumoral des sarcomes est peu abondant contrairement aux carcinomes où il est souvent très abondant et où les cellules tumorales sont parfois diluées.

Les composants de la matrice extracellulaire peuvent être produites par les cellules cancéreuses ou/et par les cellules stromales, tels que fibroblastes et macrophages. Les cellules cancéreuses mammaires modulent la composition de cette matrice extracellulaire en influençant les propriétés des fibroblastes et en interagissant directement avec la matrice extracellulaire.

### **2.1. La composition.**

La matrice extracellulaire peut être aussi divisée en deux catégories selon leur relation structurelle avec le parenchyme: la membrane basale et le tissu conjonctif interstitiel, qui sont différentes par la composition, la localisation et la fonction.

La membrane basale, une structure uniforme et non-cellulaire, est composée principalement par le collagène type IV, la laminine et les protéoglycans. La membrane basale sépare les cellules d'un organe, épithéliales et endothéliales, du tissu interstitiel, fournit un substrat aux cellules et maintient l'architecture nécessaire pour la prolifération, l'orientation et la différenciation des cellules de l'organe, tel que l'épithélialisation (Kefalides *et al.*, 1979).

Le tissu conjonctif interstitiel contient des cellules stromales comme les fibroblastes, macrophages et d'autres types des cellules dépendant du type de tissu (par exemple, ostéoblastes) ainsi que les fibres de collagènes, glycoprotéines et protéoglycans. Le tissu

conjonctif interstitiel peut être divisé en plusieurs sous-groupes qui forment différents types de tissus. Le collagène de type I est la forme principale codistribuée avec le type III dans tous les tissus (Tryggvason *et al.*, 1987). Parmi les autres composants, on trouve la fibronectine, les protéoglycans, l'élastine et d'autres. Le collagène type V est un composant fibrillaire du stroma interstitiel et relie les fibrilles du collagène interstitiel à la matrice de la membrane basale (Broek *et al.*, 1985).

## **2.2. La dégradation de la matrice extracellulaire.**

Pour devenir une tumeur infiltrante et métastatique, les cellules cancéreuses doivent franchir d'abord la membrane basale et ensuite pénétrer la matrice conjonctive pour atteindre le réseau vasculaire, ceci entraîne une perte de continuité des membranes basales sous-épithéliales et une dégradation des composantes de la matrice extracellulaire par plusieurs protéases.

Dans la tumeur, les cellules cancéreuses peuvent stimuler les cellules stromales à produire une quantité importante des protéases impliquée dans la dégradation tissulaire liée à la tumeur (Noël *et al.*, 1994).

## **2.3. Le remodelage tissulaire dans le cancer mammaire.**

Le remodelage de la matrice extracellulaire (la membrane basale et le tissu conjonctif interstitiel) est une étape importante dans le processus d'invasion tumorale, d'angiogenèse et de métastase (Hart *et al.*, 1989; Liotta and Stetler-Stevenson, 1991b). En effet, la dégradation de la matrice extracellulaire ne constitue pas un attribut absolu des tumeurs invasives puisque certaines tumeurs non invasives peuvent également dégrader la matrice. La composition de la matrice extracellulaire dépend d'un équilibre entre la production et la dégradation de ces composantes, telles que collagène, laminine, fibronectine, etc. Les protéases qui participent au remodelage tissulaire sont principalement les métalloprotéinases et les sérines protéinases (Foidart, 1997; Rio *et al.*, 1998). Le cancer mammaire est souvent associé à un dépôt excessif de composants de la matrice extracellulaire, c'est-à-dire la desmoplasie (Martinez-Hernandez, 1988). La desmoplasie contient une quantité importante de collagène interstitiel, fibronectine, élastine et protéoglycans, formant un stroma tumoral fibro-hyperplasique et donnant un aspect de sclérohyalinose. Le stroma désmoplasique constitue une source importante de

myofibroblastes (Barsky *et al.*, 1984). La matrice extracellulaire des cellules cancéreuses mammaires a d'ailleurs un effet mitogénique sur les fibroblastes (Kao *et al.*, 1984). En outre, les cellules cancéreuses mammaires stimulent la prolifération des fibroblastes. L'interaction de ces cellules cancéreuses ou des myofibroblastes activés avec la matrice extracellulaire collagénique provoque cette desmoplasie.

Cependant, le stroma fibreux exubérant peut s'accompagner d'une atrophie importante du tissu cellulo-adipeux dans lequel s'implante le cancer. Il entraîne ainsi une rétraction de la tumeur et de l'environnement péri-tumoral.

#### **2.4. La migration des cellules cancéreuses dans la matrice extracellulaire.**

La migration des cellules cancéreuses est nécessaire pour initier la cascade métastatique, c'est à dire de quitter le site primaire vers le site secondaire où les cellules cancéreuses formeront le foyer métastatique (Liotta *et al.*, 1986). Sans cette capacité à migrer à travers la membrane basale et la matrice extracellulaire, les cellules cancéreuses ne pourraient devenir métastatiques.

L'étude *in vitro* de la migration et de l'invasion des cellules cancéreuses demande de reconstruire *in vitro* un tissu équivalent qui stimule le plus possible la situation tumorale *in vivo*. Le modèle le mieux connu est la chambre de Boyden (Siewerts *et al.*, 1997), composée d'un filtre micropore couvert par des éléments de la matrice extracellulaire comme Matrigel. Cependant, cette approche simule seulement une partie la situation réelle, car la migration des cellules cancéreuses est beaucoup plus complexe *in vivo* où les cellules cancéreuses sont aussi sous l'influence des éléments stromaux tel que les cellules stromales et de l'interaction cellules-cellules et cellules-matrice extracellulaire. Par contre, une reconstruction *in vitro* de type tissu-équivalent contenant non seulement des éléments de la matrice extracellulaire mais aussi des cellules stromales semble préférable pour étudier l'interaction des cellules cancéreuses mammaires et le stroma tumoral. En plus, le modèle tissu-équivalent permet d'observer directement l'invasion des cellules cancéreuses par une analyse histologique.

### **2.5. La matrice extracellulaire favorise la formation tumorale.**

La matrice extracellulaire peut favoriser le développement tumoral *in vivo*, tel que confirmé par des expériences de transplantation de cellules cancéreuses avec Matrigel, un extrait des composantes de la membrane basale (Kleinman H.K. *et al.*, 1986), dans des souris nues/nues. Cet effet de promotion du développement tumoral a été observé pour plusieurs types de cellules cancéreuses tels que cellules du carcinome pulmonaire à petites cellules (Fridman *et al.*, 1990), carcinomes prostatiques (Pretlow *et al.*, 1991), carcinomes mammaires (Noël *et al.*, 1992), cancer du côlon et mélanome. Les cellules MCF-7 ne forment pas de tumeur quand elles sont injectées seules dans les souris nues/nues. Cependant, l'inoculation de cellules MCF-7 et de Matrigel dans les souris nues/nues induit rapidement la formation de nodules tumoraux. (Noël *et al.*, 1992). Le mécanisme impliqué dans cette promotion du développement tumoral n'est pas clair. Il serait cependant lié à l'interaction cellule-cellule et cellules-matrice (Noël *et al.*, 1988; Noël *et al.*, 1993), et à la capacité de liaison des facteurs de croissance (Fridman *et al.*, 1990; Vukicevic *et al.*, 1992).

### **3. Les protéases d'origine stromale dans le cancer mammaire.**

Les enzymes protéolytiques ont été impliquées dans le remodelage des carcinomes et dans la dégradation de différents éléments de la matrice extracellulaire associée à l'invasion tumorale (Liotta *et al.*, 1983; Tryggvason *et al.*, 1987). Toutes les familles de protéases semblent participer à certaines étapes de l'invasion tumorale (Burtin and Durliat, 1990; Duffy, 1992). Dans la famille des sérines protéases, l'uPA est le mieux connu dans la dégradation de la matrice extracellulaire (de Bruin *et al.*, 1987). Dans la famille des cystéines protéases, la cathepsine B a une activité augmentée dans certains cancers (Sloane *et al.*, 1981). Dans la famille de protéases aspartiques, la cathepsine D est étudiée dans le cancer mammaire depuis 1986 (Morisset *et al.*, 1986b; Reid *et al.*, 1986). La famille de métalloprotéases est également étudiée et observée dans plusieurs formes de cancers (McDonnell and Fingleton, 1993).

Cependant, le fait qu'un tissu tumoral contient souvent une activité importante des protéases ne signifie pas toujours que ces protéases sont d'origine des cellules tumorales. Il est vrai que des cellules cancéreuses en culture peuvent produire des protéases et des glycosidases. Cependant, la protéolysis de la matrice extracellulaire est un processus complexe avec

expression anormale de protéases non seulement par les cellules transformées mais également par les cellules non transformées de l'hôte (Tryggvason *et al.*, 1987). Dans la plupart des carcinomes, les cellules stromales, plus particulièrement les fibroblastes, synthétisent également des protéases qui sont sécrétées dans le milieu extracellulaire ou agissent dans les lysosomes, ce sont les protéases extracellulaires d'origine stromale. L'expression des protéases des tumeurs est souvent mesurée par analyse d'extraits tissulaires de tumeurs qui contiennent des cellules de tissu d'hôte, tel que fibroblastes, macrophages, monocytes etc., en plus des cellules cancéreuses. Toutes ces cellules stromales peuvent produire diverses protéases. Il est probable que des cellules stromales de l'hôte jouent un rôle plus ou moins significatif dans le remodelage et la dégradation de la matrice extracellulaire au cours de l'invasion tumorale.

Des études sur l'adénocarcinome du côlon ont bien montré que les cellules stromales et non-malignes étaient fortement impliquées dans la production des molécules qui généraient ou régularisaient la protéolyse extracellulaire au cours de l'invasion tumorale. En effet, l'expression de l'ARNm pour l'uPA (Pyke *et al.*, 1991b), la collagénase interstitielle (Gray *et al.*, 1993), la stromélysine-3 (Rouyer *et al.*, 1994), la gélatinase A (Poulsom *et al.*, 1992; Pyke *et al.*, 1993), la *membrane-type* métalloprotéase de la matrice (MT-MMP) (Okada *et al.*, 1995) et l'inhibiteur tissulaire de MMP-2 (Poulsom *et al.*, 1992) a été observée dans de différentes sous-populations des cellules *fibroblast-like* dans le cancer du côlon. L'ARNm de l'uPAR (urokinase activator receptor) est exprimé dans les cellules cancéreuses et aussi dans les macrophages (tumor infiltrating macrophage) adjacents aux fibroblastes exprimant l'uPA (Pyke *et al.*, 1991b). L'ARNm de PAI-1 (plasminogen activator inhibitor) est également exprimé dans les cellules endothéliales du stroma tumoral (Pyke *et al.*, 1991a) et l'ARNm de gélatinase B dans les macrophages péri-tumoraux (Pyke *et al.*, 1993).

Une situation similaire a été observée dans le cancer mammaire. L'ARNm pour la stromélysine-3 (Basset *et al.*, 1990), la gélatinase A (Poulsom *et al.*, 1993; Wolf *et al.*, 1993) et la *membrane-type* métalloprotéase de la matrice (MT-MMP) (Okada *et al.*, 1995) est exprimée par les cellules stromales *fibroblast-like*, l'ARNm de la gélatinase B par les cellules inflammatoires (macrophages) (Davies *et al.*, 1993; Wolf *et al.*, 1993), et l'ARNm de

l'inhibiteur type 1 de l'activateur de plasminogène (PAI-1) par les cellules endothéliales et d'autres cellules stromales de tissu conjonctif (Dano K *et al.*, 1994).

**Tableau 2. Les cellules stromales exprimant les protéases dans le cancer mammaire.**

| Protéases      | Type de cellules                    | Auteurs                                                       |
|----------------|-------------------------------------|---------------------------------------------------------------|
| uPA            | Myofibroblastes                     | (Nielsen <i>et al.</i> , 1996)                                |
| uPAR           | Macrophages et cellules cancéreuses | (Pyke <i>et al.</i> , 1993)                                   |
| PAI-1          | Cellules endothéliales              | (Bianchi <i>et al.</i> , 1995)                                |
| Gélatinase A   | Fibroblastes                        | (Poulsom <i>et al.</i> , 1993)<br>(Noël <i>et al.</i> , 1994) |
| Gélatinase B   | Macrophages                         | (Nielsen <i>et al.</i> , 1997)                                |
|                | Fibroblastes                        | (Davies <i>et al.</i> , 1993)                                 |
| Stromélysine-3 | Fibroblastes                        | (Basset <i>et al.</i> , 1990)                                 |
| Cathepsine D   | Macrophages et cellules cancéreuses | (Têtu <i>et al.</i> , 1993)                                   |

### **3.1. La stromélysine-3 (MMP-11).**

La stromélysine-3 (ST3) est une nouvelle protéase dans la famille de métalloprotéinases matricielles. Cette protéase a été identifiée en 1990 dans le cancer du sein par analyse soustractive de l'ADNc (Basset *et al.*, 1990). La stromélysine-3 a été classée ensuite comme membre de la famille des MMPs selon la séquence de la protéine déduite de sa séquence d'ADNc, et a été donc nommée MMP-11 (Matrisian, 1992; Nagase *et al.*, 1992). Le gène de ST3 se situe dans le long bras de chromosome 22 (22q11.2) (Levy *et al.*, 1992). Comme d'autres membres de famille des MMPs, la ST3 a également un domaine de pro-région bien conservé, et cette pro-région est clivée quand la ST3 est convertie en forme active. La ST3 contient également un domaine catalytique avec une séquence connue de liaison de zinc<sup>++</sup> et un domaine de l'hémopexine avec une séquence similaire à la protéine de l'hémo-liaison.

La plupart des MMPs, mais non la ST3, sont sécrétées sous forme de zymogènes inactifs. Le pro-domaine de ST3 contient un site additionnel reconnu par la furine, la convertase pro-protéine associée au Golgi (Pei and Weiss, 1995). En conséquence, la forme proenzymatique de ST3, de 60 kDa, est transformée en une forme mature de 45 kDa, à l'intérieur du chemin

sécrétoire puis la ST3 est sécrétée sous forme active (Pei and Weiss, 1995; Santavicca *et al.*, 1996). Une étude récente a montré que cette forme active de 45 kDa était convertie en une séquence interne unique de 35 kDa par l'intermédiaire de bFGF et MMP-dépendant. Il semble que ce fragment de 35 kDa soit spécifique au cancer. Mais cette forme de 35 kDa n'a pas d'activité enzymatique (Mari *et al.*, 1998).

Bien que la forme de 45 kDa soit considérée la forme active majeure de ST3 (Pei and Weiss, 1995), cette protéase possède seulement une faible activité protéolytique et l'activité biologique de cette forme n'est pas claire (Murphy *et al.*, 1993). La ST3 possède la structure de la famille des MMPs, mais son substrat spécifique est complètement différent de celui d'autres membres dans la même famille (Murphy *et al.*, 1993). Un fragment de ST3 recombinant de souris sans le domaine d'hémapexine situé au C-terminal démontrait une activité faible de métalloprotéinase (Murphy *et al.*, 1993). En effet, la ST3 humaine de 45 kDa ne dégrade pas les substrats classiques tels que gélatine, caséine et élastine (Pei *et al.*, 1994). A ce jour, les seuls substrats connus pour la ST3 sont l'inhibiteur de sérine protéase (serpine), l'inhibiteur d' $\alpha$ 1-protéinase ( $\alpha$ 1-PI,  $\alpha$ 1-antitrypsin), et  $\alpha$ 2-antiplasmine (Pei *et al.*, 1994). Comme l' $\alpha$ 1-PI est l'inhibiteur principal circulaire de l'élastase, la dégradation de l' $\alpha$ 1-PI par ST3 pourrait augmenter le dommage tissulaire par l'élastase. Cependant, certaines autres MMPs incluant MMP-1 (collagénase tissulaire) et MMP-3 (stromélysine-1) peuvent hydrolyser également l' $\alpha$ 1-PI (Mast *et al.*, 1991). Comme la ST3 peut dégrader l' $\alpha$ 2-antiplasmine, l'enzyme pourrait aussi augmenter indirectement le niveau local de plasmine. En général, la conversion de pro-MMPs en leurs formes actives dépend de la médiation de la plasmine et la ST3 pourrait donc promouvoir cette conversion et augmenter indirectement la quantité de ces MMPs *in vivo* (Pei *et al.*, 1994). La ST3 humaine hydrolyse également l'IGFBP-1 (l'inhibiteur de l'IGF-1), et donc augmente l'activité biologique de l'IGF-1 et favorise la prolifération et la survie de cellules (Manes *et al.*, 1997).

Bien que la surexpression ait été observée dans des carcinomes primaires ou métastatiques, la ST3 est synthétisée et est exprimée presque uniquement par les cellules stromales réactionnelles de tumeur. Ceci est différent d'autres métalloprotéases matricielles. De plus, l'expression de ST3 a été observée seulement dans les cellules stromales immédiatement

adjacentes aux cellules tumorales (Basset *et al.*, 1990). Dans les foyers primaires ou métastatiques, l'expression de ST3 par les cellules stromales décroît lorsque la distance entre cellules épithéliales malignes et cellules stromales augmente. L'expression la plus forte est située dans les cellules stromales à l'interface épithéliale-stromale. Donc, l'expression de ST3 par les cellules stromales est considérée comme une réponse de l'hôte au remodelage tissulaire associé à la tumeur (Basset *et al.*, 1993).

### 3.1.1. La régularisation de l'expression de ST3.

La régularisation de l'expression de ST3 a été également étudiée. Certains facteurs de croissance ont été recherchés, parmi lesquels, on retrouve des facteurs comme EGF, bFGF/FGF et PDGF. Le bFGF a été le plus étudié à ce jour. Une étude clinique sur le cancer du sein par immunohistochimie a démontré que l'expression de bFGF dans les cellules stromale était associée à l'expression de ST3 par les cellules stromales (Linder *et al.*, 1998). Par administration de FGF (Ahmad *et al.*, 1997; Anderson *et al.*, 1995; Basset *et al.*, 1990; Okada *et al.*, 1995), EGF (Ahmad *et al.*, 1997; Basset *et al.*, 1990) et PDGF (Ahmad *et al.*, 1997; Anderson *et al.*, 1995; Basset *et al.*, 1990) exogènes dans une culture de fibroblastes, la ST3 était activée au niveau de la transcription, mais sans effet au niveau du promoteur (Ahmad *et al.*, 1997). Parmi ces facteurs de croissance, le bFGF donne l'effet d'induction le plus élevé (Basset *et al.*, 1990). Dans une étude avec les ostéoblastes de souris qui produisaient la ST3 en culture, l'administration de FGF-2 a provoqué une diminution de la stabilité de l'ARNm de ST3 sans modifier la transcription; cependant, l'administration prolongée de FGF-2 pourrait augmenter la transcription du gène sans avoir d'effet sur la stabilisation d'ARNm (Delany and Canalis, 1998). Des essais *in vitro* ont suggéré que ces facteurs de croissance, sécrétés par les cellules cancéreuses *in vivo*, pourraient inciter les fibroblastes à produire la ST3 par activation de la transcription du gène. Cette hypothèse a été vérifiée dans une étude récente par coculture *in vitro* des cellules cancéreuses pulmonaires et des fibroblastes d'origine pulmonaire avec l'administration d'anticorps monoclonaux contre le bFGF, le PDGF et EGF. Cette coculture a amené les fibroblastes à produire de la ST3 sous sa forme active de 45 kDa plutôt que sous sa forme majeure de 35 kDa. La neutralisation de l'EGF, le bFGF et le PDGF par ces anticorps n'a pas modifié la production de protéine ST3 sous sa forme active de 45 kDa; mais l'anti-bFGF a significativement inhibé la production de

la forme majeure de 35 kDa. On a conclu que seulement le bFGF, sécrété par les cellules cancéreuses, participait au processus de modification post-traductionnelle de ST3 et non au processus d'induction de l'expression d'ARNm (Mari *et al.*, 1998).

Le promoteur du gène de ST3 contient dans sa région proximale un élément de RARE de type DR1. Cet élément de RARE peut être transactivé par le récepteur de l'acide rétinoïque d'une manière ligand-dépendante et contrôler l'expression du gène de ST3 dans les cellules COS-1 (Anglard *et al.*, 1995). Les effets des rétinoïdes sont réalisés par deux sortes de transactivateurs ligand-dépendants : les récepteurs de l'acide rétinoïque (RARa, RARb, RAR $\gamma$  et leurs isoformes) et les récepteurs X des rétinoïdes (RXRa, RXRb, RXR $\gamma$  et leurs isoformes), ces récepteurs sont des membres de la superfamille de récepteurs nucléaires. Les isomères de l'acide rétinoïque (9C-RA : 9-cis-retinoid acid; et t-RA : all-trans-retinoid acid) à dose physiologique ont montré un pouvoir d'induction sur l'expression de la ST3 au niveau de l'ARNm et de la protéine dans les fibroblastes d'origine mésodermique et supprimait en même temps la collagénase interstitielle. Cet effet d'induction est contrôlé au niveau de la transcription par une *up-regularisation* sur l'élongation de l'ARN nucléaire de ST3. Les hétérodimères de RAR-RXR sont des unités fonctionnelles et nécessaires pour le contrôle du gène de ST3 par l'acide rétinoïque (Guerin *et al.*, 1997). L'acide rétinoïque (9C-RA) semble augmenter l'activité du promoteur du gène de ST3 dans les cellules de rhabdomyosarcomes qui expriment la ST3.

Cependant, l'acide rétinoïque t-RA à dose thérapeutique a montré une suppression de l'effet d'induction sur l'expression de ST3 dans les fibroblastes pulmonaires par bFGF et PDGF (Anderson *et al.*, 1995). Une étude récente a montré le même effet inhibiteur sur la production de ST3 par les cellules stromales de carcinome pancréatique au niveau de la protéine par tous les trans- et 13-cis-acide rétinoïque à la dose thérapeutique (von Marschall *et al.*, 1998).

### **3.1.2. L'expression de stromélysine-3 dans le remodelage tissulaire non-malin.**

La ST3 est exprimée aussi dans l'embryogenèse normale et le remodelage de certains tissus adultes. Dans l'embryon humain, la ST3 est exprimée dans les doigts en développement (Basset *et al.*, 1994). Dans l'embryon de souris, la ST3 se trouve dans la morphogenèse de la

queue, des membranes et du museau (Lefebvre *et al.*, 1995). La ST3 est exprimée dans les cellules stromales en contact avec les cellules épithéliales de l'embryon normal et des tissus adultes. Dans certaines circonstances, l'expression de ST3 dans les cellules stromales est observée en contact avec des cellules épithéliales en voie d'apoptose. Pendant la morphogenèse de la grenouille, la ST3 est spécifiquement exprimée dans le mésenchyme intestinal pendant une période durant laquelle, l'apoptose se trouve dans les cellules épithéliales de l'intestin primaire au moment du remplacement par les cellules épithéliales secondaires (Patterson *et al.*, 1995). Chez l'humain et les rongeurs, la ST3 est exprimée dans les tissus qui sont en période de remodelage intensif tel le placenta, l'utérus en involution et la glande mammaire post-lactation (Lefebvre *et al.*, 1992; Rodgers *et al.*, 1994). Par exemple, les souris femelles, expriment la ST3 dans la glande mammaire en involution après avoir complété le sevrage (Lefebvre *et al.*, 1992). L'expression de ST3 est observée également dans les cellules épithéliales respiratoires au cours de la réparation des blessures, et cette expression est liée à l'acquisition d'un phénotype mésenchymateux dans ces cellules épithéliales au cours de remodelage. (Buisson *et al.*, 1996). Toutes ces données suggèrent que la viabilité, l'activité et le changement des cellules épithéliales normales affectent l'expression de ST3 dans le stroma adjacent.

### **3.1.3. L'expression de stromélysine-3 dans des carcinomes.**

Ces études sur la ST3 ont attiré beaucoup d'attention en cancérologie. En effet, la surexpression de la ST3 a été la première fois observée dans le cancer. La plupart des connaissances sur la ST3 ont été obtenues par ces études cliniques.

Dans le cancer cutané, l'expression de ST3 est associée à différentes histologies : l'expression est très forte et fréquente dans le carcinome basocellulaire (Unden *et al.*, 1996; Wagner *et al.*, 1992), mais absente dans le dermatofibrosarcome (Unden *et al.*, 1996) et le mélanome (Wagner *et al.*, 1992).

Dans le cancer de la sphère ORL, des études ont bien montré une association à l'invasion locale des cellules cancéreuses (Muller *et al.*, 1993; Munck-Wikland *et al.*, 1998; Okada *et al.*, 1995; Polette *et al.*, 1993). Seulement certaines lésions dysplasiques (3/10) présentaient aussi

une expression de ST3 (Munck-Wikland *et al.*, 1998), tandis que 106 des 111 tumeurs invasives présentaient cette expression (Muller *et al.*, 1993).

Dans le cancer colorectal, l'expression de ST3 (Mueller *et al.*, 1997; Okada *et al.*, 1995; Porte *et al.*, 1995; Thewes *et al.*, 1996) était associée à l'invasion locale (Porte *et al.*, 1995) mais sans association à la taille tumorale (Thewes *et al.*, 1996), ni au grade histologique (Thewes *et al.*, 1996).

Dans le carcinome du poumon non à petites cellules, l'expression de ST3 a été observée dans les carcinomes malpighiens et les adénocarcinomes (Anderson *et al.*, 1995). Dans une série d'étude avec des lésions respiratoires (Bolon *et al.*, 1996), la ST3 était absente dans les lésions hyperplasiques et métaplasiques. Par contre, la ST3 était fréquemment exprimée dans les lésions préinvasives tel que dysplasiques et *in situ*. L'expression était observée uniquement dans le carcinome invasif, et cette expression était liée à la progression tumorale (Anderson *et al.*, 1995; Bolon *et al.*, 1997; Urbanski *et al.*, 1992). En effet, l'expression stromale de ST3 était plus fréquente dans le carcinome que dans les lésions atypiques (Karameris *et al.*, 1997).

L'expression a été observée également dans le cancer œsophagien (Porte *et al.*, 1998). Cette expression de ST3 était liée à la progression locale de la tumeur œsophagienne, représentée par l'invasion péri tumorale et la taille tumorale, et un faible taux de survie sans maladie (Porte *et al.*, 1998).

L'expression de ST3 a été aussi observée dans 17 carcinomes pancréatiques parmi 21 cas étudiés (von Marschall *et al.*, 1998).

Parmi tous les carcinomes étudiés à ce jour, la ST3 est absente seulement dans le cancer de la thyroïde (Zedenius *et al.*, 1996) et l'hépatocarcinome, mais plusieurs carcinomes n'ont jamais été étudiés.

La ST3 est aussi absente dans le lymphome malin non hodgkinien (Kossakowska *et al.*, 1996), et le mésothéliome (Urbanski *et al.*, 1992). Ceci suggère que son expression pourrait être

épithélial-spécifique. Cependant, l'expression de ST3 a été observée dans une étude avec 26 sarcomes (Singer *et al.*, 1997).

L'expression de ST3 a été étudiée principalement dans le cancer mammaire. Suite aux études de Basset, l'expression de ST3 a été démontrée dans la plupart des tumeurs invasives, soit dans le foyer primaire ou métastatique (Ahmad *et al.*, 1998; Basset *et al.*, 1994; Chenard *et al.*, 1996; Foidart, 1997; Hahnel *et al.*, 1994; Hahnel *et al.*, 1993; Heppner *et al.*, 1996; Holm *et al.*, 1997; Kawami *et al.*, 1993; Kossakowska *et al.*, 1996; Linder *et al.*, 1997; Pacheco *et al.*, 1998; Têtu *et al.*, 1998), et rarement dans les ganglions métastatiques (Hahnel *et al.*, 1994). Cette expression varie de 60% (Foidart, 1997) à 97.5% (Kawami *et al.*, 1993) dans les tumeurs étudiées selon différents rapports. L'expression de ST3 a été observée également dans des carcinomes *in situ*, mais seulement dans une faible population soit environ de 10% des cas (Basset *et al.*, 1994; Hahnel *et al.*, 1993). La ST3 est absente dans les fibroblastes entourant les cellules épithéliales normales (Basset *et al.*, 1994; Hahnel *et al.*, 1994) et dans les fibroblastes des fibroadénomes (Hahnel *et al.*, 1994). L'expression de ST3 semble associée à l'histologie tumorale : une forte expression a été observée plutôt dans les carcinomes canalaire que dans les carcinomes lobulaire (Ahmad *et al.*, 1998; Chenard *et al.*, 1996). L'expression de ST3 par les fibroblastes est indépendante de la taille tumorale (Chenard *et al.*, 1996), de l'état ganglionnaire (Chenard *et al.*, 1996) et de la distribution microvasculaire (Linder *et al.*, 1997); L'expression de ST3 a été souvent observée chez des patientes pour lesquelles les récepteurs d'œstrogènes sont positifs et l'histologie est le grade 1 (Hahnel *et al.*, 1993). En plus, des études ont observé une bonne corrélation entre l'expression de ST3 par les fibroblastes et une survie globale plus courte (Chenard *et al.*, 1996) ou une faible taux de survie sans maladie (Ahmad *et al.*, 1998; Chenard *et al.*, 1996; Têtu *et al.*, 1998).

Une étude sur l'expression de ST3 dans le cancer du sein incluant 665 patientes avec ou sans envahissement ganglionnaire et un suivi moyen de 10 ans a été effectué par le groupe du Dr Têtu. L'expression de ST3 a été observée dans 86,6% de cas, et était associée avec un âge plus jeune, un envahissement ganglionnaire, un haut grade nucléaire, une histologie canalaire, une aneuploïdie, une expression de HSP-27 et un faible taux de survie sans maladie (Têtu *et al.*, 1998).

On a récemment observé que la ST3 favorisait le développement de tumeur locale dans les souris nu/nu en assurant un support stromal pour l'implantation et la survie des cellules cancéreuses. En effet, la co-injection des cellules de MCF-7 et les cellules de 3T3 transfectées pour le gène de ST3 favorise significativement le développement de tumeurs dans les souris nues/nues (Noël *et al.*, 1996), sans influencer l'invasion ni la prolifération des cellules cancéreuses. Le fait que la ST3 s'exprime au stade précoce de la tumeur implique que cette protéase participe possiblement au développement initial de ces lésions malignes. Cependant, le mécanisme moléculaire par lequel la ST3 favorise le développement tumoral reste inconnu. La production de ST3 dans les cellules stromales semble une conséquence d'une interaction spécifique entre les cellules épithéliales cancéreuses et les cellules stromales adjacentes. Ceci suggère que la ST3 peut participer au stade le plus précoce du développement tumoral par un maintien d'un environnement favorisant la formation tumorale.

De tous ces résultats, il est évident que l'expression de ST3 par les fibroblastes est liée à la malignité tissulaire en cancérologie, à l'invasion locale de la tumeur, et donc pourrait servir à orienter la thérapie expérimentale. La conception actuelle est que l'expression de ST3 dans le cancer est une sorte de réponse par le tissu stromal de l'hôte à la stimulation cancéreuse. La protéase joue un rôle important dans un remodelage tissulaire mais tumoral.

Le mécanisme de stimulation par les cellules épithéliales cancéreuses sur l'expression de ST3 par les fibroblastes reste inconnu. Le fait que l'expression de ST3 soit uniquement présente dans les fibroblastes au contact immédiat des cellules cancéreuses suggère un effet possible de contact direct entre les cellules cancéreuses et les cellules stromales réactionnelles. Cependant, le fait que le bFGF sécrété par les cellules cancéreuses participe seulement à la modification post-traductionnelle implique l'induction de l'expression de ST3 modulée également par des facteurs diffusibles (Mari *et al.*, 1998). Peu d'études dans la littérature ont étudié cette stimulation par des modèles *in vitro*. Une étude récente a montré par une coculture *in vitro* des cellules cancéreuses pulmonaires (NSCLC : non small cell lung cancer) avec les fibroblastes pulmonaires que l'expression des ST3 était inductible dans les fibroblastes pulmonaires spécifiquement par les cellules cancéreuses pulmonaires. La coculture directe et indirecte

donne le même effet de stimulation lors qu'analysée par Northern blot sur la population totale des fibroblastes stimulés (Mari *et al.*, 1998).

Les aspects uniques de la régulation, de la structure et de la fonction de ST3, démontrées par plusieurs études, suggèrent que la ST3 possède certaines autres activités biologiques dans les tissus normaux et tumoraux qui restent inconnues à ce jour (Anglard *et al.*, 1995; Murphy *et al.*, 1993; Noël *et al.*, 1995; Pei *et al.*, 1994; Pei and Weiss, 1995). Le fait que la ST3 soit exprimée dans les cancers dérivés des trois feuillets embryonnaires : l'ectoderme (par exemple, cancer mammaire), le mésoderme (par exemple, sarcome) et l'endoderme (par exemple, côlon), implique que la ST3 est une protéase fonctionnellement nécessaire à la croissance fœtale et que son expression par les fibroblastes dans le cancer est associée à un phénotype primitif typique des tumeurs malignes (Singer *et al.*, 1997).

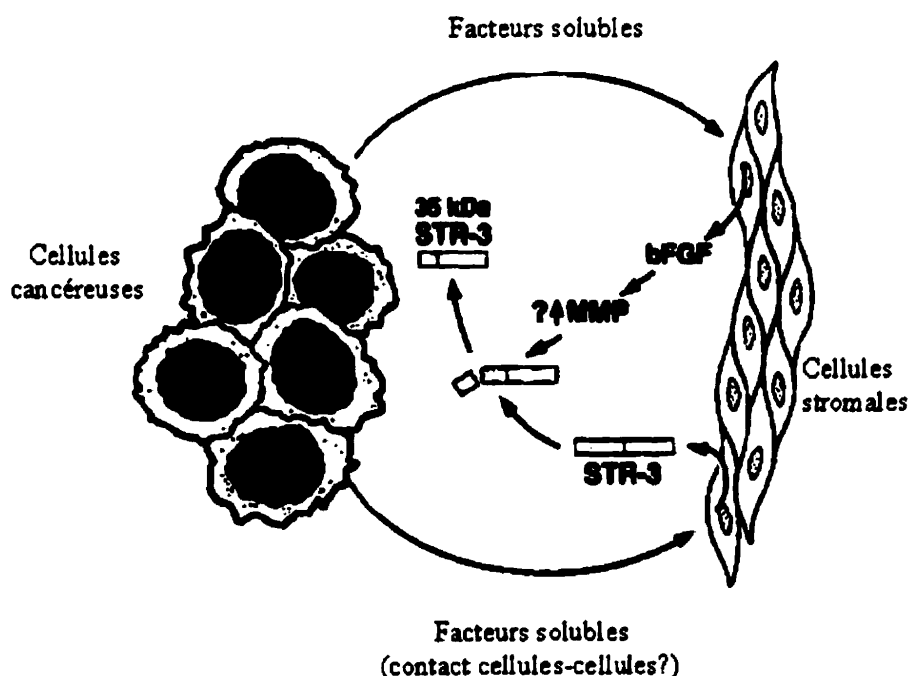


Figure 1. Induction par les cellules cancéreuses de la production de la stromélysine-3 par des cellules stromales (Tiré de Mari, B.P. 1998).

### **3.2. La cathepsine D (CD).**

La cathepsine D est une endoprotéase lysosomiale présente dans toutes les cellules des mammifères. C'est un membre de la famille des protéases aspartiques parmi lesquelles on retrouve les enzymes sécrétoires bien étudiées telles que la rénine et la pepsine (Barrett A.J., 1977). Cependant la cathepsine D est la seule protéase aspartique connue comme étant lysosomiale plutôt que sécrétoire.

#### **3.2.1. La structure et la biosynthèse.**

La cathepsine D humaine consiste en une séquence de 412 acides aminés avec 20 et 44 acides aminés dans les pré- et pro-segments respectivement. (Faust *et al.*, 1985).

La cathepsine D subit un processus protéolytique en multiples étapes au cours de la biosynthèse et de la maturation. La séquence de la partie N-terminale de la précathepsine D (peptide signal) composée de 20 acides aminés module le transport de l'enzyme à travers la membrane du réticulum endoplasmique (ER) (Erickson *et al.*, 1983). Lors de l'entrée de la préprocathepsine D dans le réticulum endoplasmique, le peptide signal est enlevé, et on ne peut donc pas détecter la préproprotéine dans les cellules.

Le propeptide de 44 acides aminés est la partie d'activation qui maintient l'enzyme en état inactif au cours de transport (Erickson *et al.*, 1981). Le clivage de cette proprotéine se tient dans l'endosome ou le lysosome précoce et produit une simple chaîne active (forme intermédiaire) d'environ 48 kDa. Cette molécule est finalement clivée dans les lysosomes en formant une enzyme mature à deux chaînes consistant en un domaine N-terminal de 14 kDa et un domaine C-terminal de 34 kDa, et la forme de 34 kDa est la forme prédominante détectée dans le tissu par Western blot.

La procathepsine D produit deux chaînes (lourde et légère) d'oligosaccharide de mannose (Takahashi *et al.*, 1983). Ces deux oligosaccharides de mannose sont phosphorylés dans le Golgi précoce (cis Golgi network) et la procathepsine D se lie au récepteur de mannose-6-phosphate dans le TGN (trans Golgi network) qui continue vers l'endosome tardif (late endosome: LE) (Kornfeld and Mellman, 1989). La procathepsine D porte ce signal de M6P et

l'enzyme est conduite du Golgi vers le lysosome via l'endosomes par les récepteurs spécifiques de M6P (Kornfeld and Mellman, 1989). Le pH acide dans le lysosome dissocie la combinaison de ces récepteurs et libère la cathepsine D.

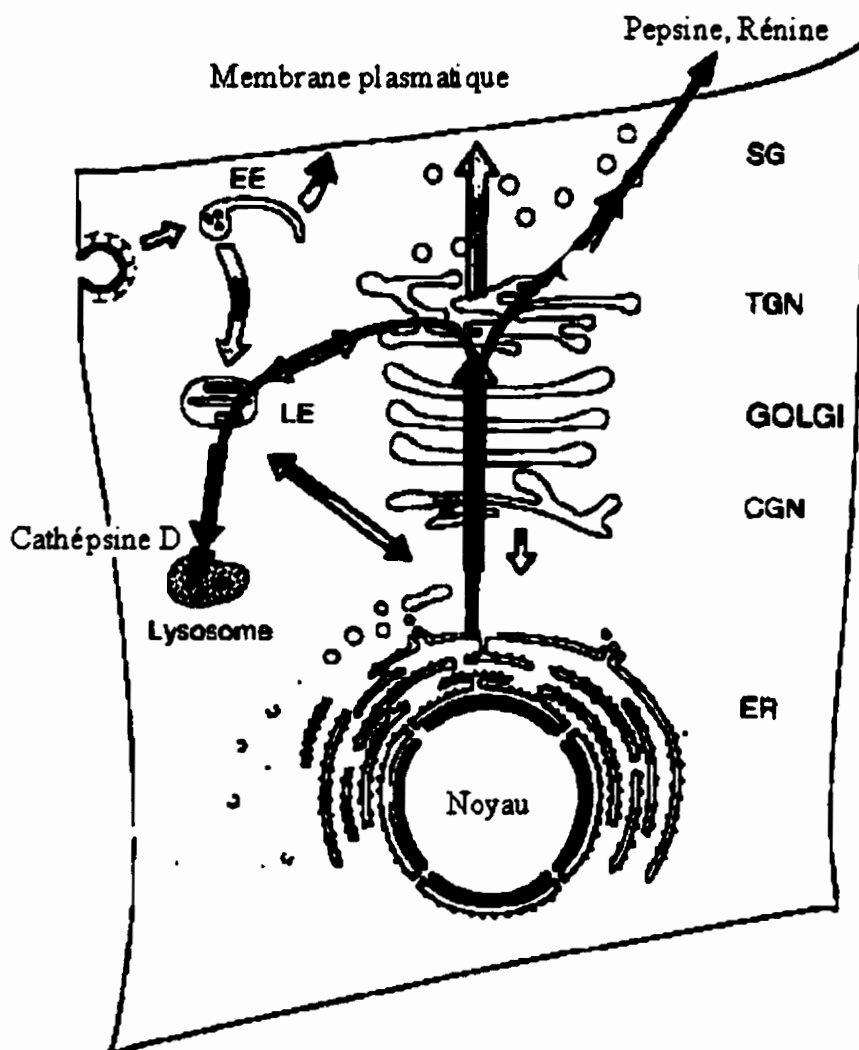


Figure 2. Le processus de biosynthèse et de transport de la cathepsine D.

ER: réticulum endoplasmide; CGN: *cis*-Golgi; TGN: *trans*-Golgi;

SG: granule sécrétoire; LE: *late* endosome; EE: early endosome.

(Tiré de Shewale J.G., Aspartic Proteinases and Their Inhibitors, 1985)

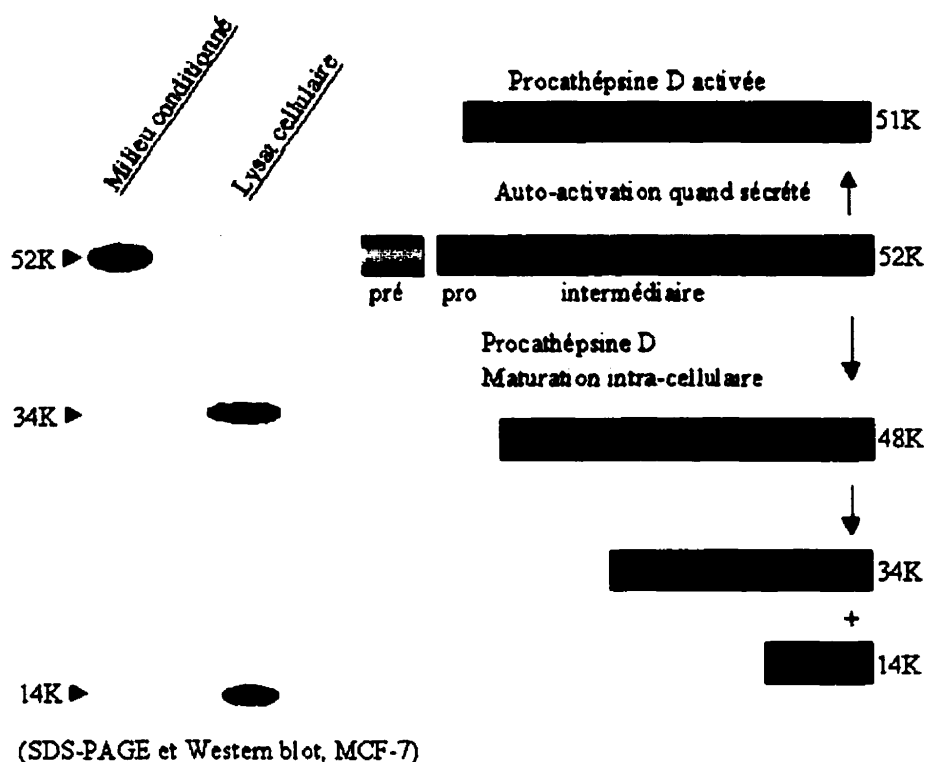


Figure 3. Processus de maturation intracellulaire de la cathepsine D.

(Référé de Henri Rochefort, seminars in Cancer Biology, 1990).

### 3.2.2. Le substrat.

La cathepsine D participe au *turnover* de protéines cellulaires et à la dégradation des protéines par endocytose (Godbold *et al.*, 1998). La cathepsine D possède une activité limitée contre les protéines natives, mais elle possède une activité considérable contre les protéines dénaturées à pH acide (3.5-5.0) (Capony *et al.*, 1986; Morisset *et al.*, 1986a). Les substrats de la cathepsine D sont largement non spécifiques, avec une préférence pour les protéines de haut poids moléculaire. Cette enzyme attaque préférentiellement les liaisons peptidiques situées entre des acides aminés hydrophobes tels que Phe-Phe, Phe-Tyr, Tyr-Leu et Leu-Tyr (Barrett A.J., 1977). L'inhibiteur spécifique de la cathepsine D est la pepstatine.

### 3.2.3. La régulation.

L'expression de cathepsine D est sous le contrôle des œstrogènes dans certaines cellules cancéreuses (Westley and May, 1987). L'expression de l'ARNm de cathepsine D des cellules MCF-7 est 10 fois supérieure en culture avec 17- $\beta$ -estradiol (Cavailles *et al.*, 1988). Cette augmentation de messenger est possiblement due à un effet direct sur la synthèse d'ARNm (Rocheftort, 1990). Les cellules cancéreuses mammaires en culture (Johnson *et al.*, 1993) ou les tumeurs du sein *in vivo* (Maudelonde *et al.*, 1994) ont montré une expression diminuée de cathepsine D dans les cellules cancéreuses sous l'effet du tamoxifène, agent anti-œstrogène. Les ligands de récepteurs d'œstrogènes sont les seuls stéroïdes pouvant induire l'ARNm de cathepsine D, et l'activité de ces agents inducteurs correspondent à leur affinité au récepteur d'œstrogènes et l'activité mitogénique.

Des facteurs de croissance tel que IGF-1, EGF et bFGF peuvent induire l'ARNm de cathepsine D dans les cellules MCF-7 (Cavailles *et al.*, 1988). On croit que ces facteurs de croissance puissent moduler la synthèse d'une protéine qui contrôle indirectement la synthèse d'ARNm de cathepsine D. En plus, ces facteurs de croissance stabilisent l'ARNm de la cathepsine D. Dans les cellules cancéreuses mammaires, les œstrogènes peuvent induire des facteurs de croissance tels que TGF $\alpha$  et IGF-1 qui peuvent augmenter la cathepsine D (Lippman *et al.*, 1986).

### 3.2.4. les activités biologiques de la cathepsine D.

Par digestion intracellulaire, la cathepsine D peut dégrader plusieurs types de protéines, telles que l'ECM pour favoriser la migration des cellules cancéreuses. Elle peut également dégrader ou activer des molécules importantes. On croit que la cathepsine D peut initier la cascade protéolytique de la dégradation de l'ECM par activation de la procathepsine B (van der Stappen *et al.*, 1996) qui active ensuite l'activateur de prourokinase plasminogène (Kobayashi *et al.*, 1991) et par dégradation de la cystatine, un inhibiteur de cystéine protéase (Lenarcic *et al.*, 1988). La cathepsine D peut jouer un rôle dans certaines étapes du processus de métastase, y compris la présentation d'antigènes en présence de MHC de classe II (Deussing *et al.*, 1998). Deux effets biologiques principaux de la cathepsine D sont l'activation de la prolifération cellulaire et de l'activité protéolytique favorisant la métastase.

#### **3.2.4.1. L'effet sur la prolifération des cellules cancéreuses.**

Des études ont démontré que la cathepsine D pouvait favoriser la métastase à distance par stimulation de l'activité mitogénique au lieu de favoriser l'invasion locale par effet protéolytique (Garcia *et al.*, 1990; Liaudet *et al.*, 1994). La cathepsine D peut augmenter la prolifération des cellules cancéreuses, tel qu'observé et rapporté d'abord par Vignon *et al.* en 1986 (Vignon *et al.*, 1986). Dans cette étude, le précurseur de la cathepsine D sécrétée par les cellules MCF-7 a été immunopurifiée et on a observé que ce précurseur accélérât la prolifération des cellules MCF-7 en culture. Cet effet sur la prolifération était indépendant des œstrogènes. Les formes de cathepsine D responsables de cet effet mitogénique peuvent être la forme mature (Vetvicka *et al.*, 1994) et également la forme de précurseur (Liaudet *et al.*, 1994), bien que certains auteurs n'aient pas retrouvé cet effet mitogénique par la forme mature (Karey and Sirbasku, 1988; Stewart *et al.*, 1992), ou par ses précurseur (Stewart *et al.*, 1994).

Le mécanisme responsable de cet effet putatif sur la prolifération cellulaire n'est pas claire. La cathepsine D a été rapportée dans des études comme ayant le même effet que l'IGF-II (insulin-like growth factor) (Vetvicka *et al.*, 1994). Les récepteurs de l'IGF-II fonctionnent comme les récepteurs de mannose-6-phosphate. Il est suggéré que le précurseur de la cathepsine D fonctionne comme un agoniste partiel de l'IGF sur le récepteur de l'IGF-II (Mathieu *et al.*, 1990). Il existe deux autres mécanismes possibles d'activation cellulaire. La cathepsine D peut interagir avec le système de signalisation de l'IGF-I par dégradation de l'IGFBP-3 soluble (IGF binding protein 3), qui est un inhibiteur potentiel de l'IGF (Conover and De Leon, 1994). La cathepsine D peut libérer des facteurs de croissance et des cytokines de la matrice extracellulaire, telles que bFGF. Comme le bFGF est mitogène dans le cancer du sein, la cathepsine D accélère la prolifération des cellules cancéreuses donc indirectement par bFGF (Briozzo *et al.*, 1991).

#### **3.2.4.2. L'activité protéolytique favorisant la métastase.**

Certaines études ont démontré que la cathepsine D pouvait digérer la matrice extracellulaire par ingestion, phagocytose et destruction dans de grandes vésicules acides à l'intérieur des cellules tumorales (Montcourrier *et al.*, 1990; Montcourrier *et al.*, 1994). Le précurseur,

sécrété par les cellules tumorales à l'extérieur des cellules, peut être autoactivé en forme mature dans un environnement de pH acide et dégrader la matrice extracellulaire (Briozzo *et al.*, 1988). L'on retrouve souvent ce milieu acide dans un tissu tumoral (Montcourrier *et al.*, 1997). Par son activité protéolytique, la cathepsine D favorise donc la migration des cellules et le développement de métastases à distance.

On observe de grandes vésicules intracellulaires dans les cellules cancéreuses *in vitro* et le tissu tumoral du sein (Roger *et al.*, 1994). Ces vésicules contiennent un haut niveau de cathepsine D mature (Montcourrier *et al.*, 1990). Les cellules cancéreuses mammaires contenant les grandes vésicules semblent migrer plus facilement dans le Matrigel que celles sans grandes vésicules (Roger *et al.*, 1994). Ces vésicules contiennent des matériaux extracellulaires phagocytosés et peuvent être considérées comme des hétérophagosomes. Les cellules cancéreuses mammaires peuvent rarement phagocyter et digérer des éléments extracellulaires (Spivak, 1973), ce qui semble expliquer une partie de l'activité de cathepsine D. Cependant, cette activité de phagocytose revient principalement aux macrophages, monocytes et polynucléaires neutrophiles.

### **3.2.5. La cathepsine D et le cancer mammaire.**

L'intérêt pour la cathepsine D dans le cancer mammaire est mentionné la première fois vers la fin des années 70 par des études tentant d'identifier des marqueurs protéiques dont leur expression était contrôlée par les œstrogènes dans des lignées cellulaires mammaires et hormono-dépendantes. On a identifié une protéine, de poids moléculaire de 46 à 52 kDa, sécrétée dans le milieu de culture de MCF-7 et cette protéine sécrétée était sous le contrôle des œstrogènes. L'analyse a de plus a montré que cette protéine possédait une activité protéolytique à pH acide (Capony *et al.*, 1986; Morisset *et al.*, 1986b). En 1988, cette protéine a été confirmée comme étant la cathepsine D après l'analyse de la séquence de la protéine et de l'ADNc (Augereau *et al.*, 1988). L'objectif initial était d'identifier un marqueur qui permettrait de prédire la réponse aux œstrogènes, et ainsi aider la planification du traitement clinique comme l'hormonothérapie. Cependant, la relation directe entre la cathepsine D et la réponse de l'hormonothérapie n'a pas été confirmée, la plupart des études subséquentes mettant l'accent sur la signification de l'activité biologique de la cathepsine D tel que son action

protéolytique, ainsi que sa valeur de pronostic dans le cancer mammaire.

La surexpression tumorale de la cathepsine D a été bien étudiée au niveau de l'ARNm et de la protéine (Rochefort *et al.*, 1989), et la plupart des études ont utilisé des cellules MCF-7 comme modèle pour les expériences *in vitro*. L'expression de la cathepsine D dans le cancer mammaire est 2 à 50 fois plus importante que dans le tissu mammaire normal (Capony *et al.*, 1989). La surexpression de la cathepsine D ne se fait pas par amplification du gène et réarrangement chromosomique (Augereau *et al.*, 1988). Dans des cellules malignes comme les cellules MCF-7 en culture, la quantité de précurseur de cathepsine D de 52 kDa augmente significativement et ces précurseurs s'accumulent dans les lysosomes. En conséquence, le processus de la maturation devient plus important avec la production d'une quantité importante de forme mature de la cathepsine D dans les lysosomes ainsi qu'une activité augmentée de la protéase par rapport aux cellules normales. Le précurseur de 52 kDa a été retrouvé également dans le milieu de culture, possiblement par une sécrétion de l'excès de précurseur (52 kDa). La procathepsine D sécrétée à l'extérieur des cellules peut être autoactivée dans un milieu suffisamment acide, que l'on retrouve souvent dans un tissu cancéreux, avec production de la forme mature de la cathepsine D (Briozzo *et al.*, 1988; Erickson, 1989). Le mécanisme de sécrétion n'est pas élucidée à ce jour. On croit que la saturation des récepteurs de M6P peut être responsable de la sécrétion de procathepsine D (Mathieu *et al.*, 1991).

### **3.2.5.1. La valeur pronostique de la cathepsine D dans le cancer du sein.**

La plupart des études sur la cathepsine D sont du domaine de la cancérologie clinique, et portent principalement sur le cancer mammaire. L'intérêt clinique est de savoir si l'expression de la cathepsine D dans un cancer du sein peut prédire le risque de la progression tumorale comme la récurrence et la métastase. L'intérêt clinique est évidente, puisqu'une forte expression de la cathepsine D permettrait d'identifier un sous-groupe de patientes pour lesquelles il existe un haut risque d'évolution de la maladie et justifierait un traitement mieux ciblé.

#### **3.2.5.1.1. Les méthodes analytiques.**

Dans la plupart de ces études, l'expression de cathepsine D a été quantifiée principalement au

niveau de la protéine. Des anticorps monoclonaux (Eng *et al.*, 1994; Garcia *et al.*, 1985) ou polyclonaux (Eng *et al.*, 1994; Reid *et al.*, 1986; Tandon *et al.*, 1990) ont été largement utilisés pour mesurer la cathepsine D dans la tumeur mammaire par trois principales méthodes soit par Western blot (Tandon *et al.*, 1990), ELISA et IRMA (immunoradiometric assay) (Benraad *et al.*, 1992; Freiss *et al.*, 1989; Freiss *et al.*, 1988; Garcia *et al.*, 1985; Rogier *et al.*, 1989), et par immunohistochimie (Reid *et al.*, 1986).

Les méthodes par Western blot, ELISA et IRMA mesurent l'expression de la cathepsine D dans le cytosol du tissu tumoral. La méthode IRMA serait plus sensible (Benraad *et al.*, 1992) que l'ELISA (Maudelonde *et al.*, 1988).

La méthode par immunohistochimie permet de mesurer sémi-quantitativement l'expression de la cathepsine D sur coupes histologiques. Cette méthode permet d'évaluer l'expression au niveau des différentes populations cellulaires.

#### **3.1.5.1.2. L'association avec d'autres facteurs pronostiques.**

Les études initiales voulaient vérifier si la cathepsine D était associée au niveau des récepteurs d'œstrogènes et à la réponse à l'hormonothérapie dans le cancer mammaire, (Garcia *et al.*, 1987; Maudelonde *et al.*, 1988), car l'expression de la cathepsine D était sous la régulation des œstrogènes dans les cellules hormono-dépendantes en culture (Cavaillès *et al.*, 1988; Morisset *et al.*, 1986b; Rochefort *et al.*, 1987; Rochefort *et al.*, 1986; Rochefort *et al.*, 1984; Rochefort *et al.*, 1986; Rochefort *et al.*, 1989; Veith *et al.*, 1983; Westley and Rochefort, 1979). Une seule étude a démontré que l'expression de la cathepsine D était associée à l'état des récepteurs d'œstrogènes chez des patientes préménopausées et périménopausées (Thorpe *et al.*, 1989). En effet, cette corrélation n'a pas été confirmée dans la plupart des études et l'expression de la cathepsine D ne semble pas liée à la réponse hormonale (Garcia *et al.*, 1987; Maudelonde *et al.*, 1988).

Plusieurs études par la suite ont vérifié la relation entre la cathepsine D et d'autres facteurs cliniques : la taille tumorale, l'état ganglionnaire, le grade histologique, l'invasion vasculaire et les récepteurs hormonaux ainsi que certains oncogènes, par exemple, c-erbB-2.

Certaines études ont montré que l'expression de la cathepsine D était associée au grade histologique (Duffy *et al.*, 1991).

Peu d'études ont montré une relation entre l'expression de CD et la taille tumorale (Duffy *et al.*, 1991; Foekens *et al.*, 1993; Gion *et al.*, 1995; Gion *et al.*, 1993; Granata *et al.*, 1991; Kute *et al.*, 1992; Namer *et al.*, 1991; Pelizzola *et al.*, 1996; Pujol *et al.*, 1993; Romain *et al.*, 1990; Seshadri *et al.*, 1994; Spyratos *et al.*, 1992; Spyratos *et al.*, 1989; Stonelake *et al.*, 1994; Thorpe *et al.*, 1989).

Certaines études ont observé une relation entre l'expression forte de la CD et un envahissement ganglionnaire (Foekens *et al.*, 1993; Kute *et al.*, 1992; Namer *et al.*, 1991; Pujol *et al.*, 1993). Cette relation avec l'état ganglionnaire aurait une grande valeur pronostique clinique.

#### **3.2.5.1.3. L'association à la survie de patientes.**

La question la plus importante est si la cathepsine D peut prédire la survie sans maladie et la survie globale de patientes. Plusieurs études cliniques sur ce sujet ont été rapportées dans la littérature.

A ce jour, presque toutes les études publiées sont rétrospectives et les résultats sont variables. La composition des cohortes de patientes, la période de survie, et les méthodes analytiques utilisées sont également différentes.

##### **3.2.5.1.3.1. La mesure cytosolique par ELISA et IRMA.**

La méthode d'ELISA a d'abord été utilisée suivie par la méthode d'IRMA. Les deux méthodes sont commercialisées et couramment utilisées en Europe (Benraad *et al.*, 1992).

La première étude du rôle pronostique de la CD a été réalisée et rapportée par Thorpe *et al.* (Thorpe *et al.*, 1989). L'étude a groupé 396 patientes porteuses d'un cancer mammaire. L'analyse a démontré que la forte concentration de CD était associée significativement à un

faible taux de survie sans maladie, mais sans influence sur la survie totale, dans un groupe de patientes post-ménopausées. Une tendance de cette association a été retrouvée dans le groupe de patientes pré- ou peri-ménopausées. Quand l'expression de cathepsine D était examinée en fonction de l'état ganglionnaire, une forte quantité de cathepsine D était associée significativement à un faible taux de survie sans maladie dans le sous-groupe de patientes pré/peri-ménopausées et sans envahissement ganglionnaire, et dans le sous-groupe de patientes post-ménopausées mais avec envahissement ganglionnaire. De plus, l'expression de la cathepsine D était associée à l'expression des récepteurs d'œstrogènes, un facteur pronostique favorable. Le seuil était établi à 78 pmol/mg et 20 pmol/mg respectivement pour les groupes pré/peri-ménopausés et post-ménopausés.

Une autre étude de la même période a groupé 122 patientes avec cancer du sein (Spyratos *et al.*, 1989) et deux seuils ont été utilisés soit 45 pmol/mg et 70 pmol/mg. Utilisant 70 pmol/mg, toutes les 20 patientes positives pour la cathepsine D ont développé des métastases, tandis que seulement 20 parmi 94 patientes négative pour la cathepsine D ont développé des métastases. L'expression de la cathepsine D était significativement liée à un faible taux de survie sans maladie et un faible taux de survie sans métastase. En analysant avec l'état ganglionnaire, le taux le plus faible de survie sans métastase était observée dans le sous-groupe de patientes avec cathepsine positive et sans envahissement ganglionnaire. Cette étude démontre le rôle important de la cathepsine D comme indicateur efficace de pronostic chez les patientes sans envahissement ganglionnaire pour identification des individus avec haut risque de métastase.

Encouragés par les résultats de ces deux études, plusieurs études ont été effectuées dans le monde pour confirmer la place pronostique de la cathepsine D par la quantification avec la trousse IRMA commercialisée (Duffy *et al.*, 1991; Foekens *et al.*, 1993; Gion *et al.*, 1995; Gion *et al.*, 1993; Granata *et al.*, 1991; Kute *et al.*, 1992; Namer *et al.*, 1991; Pelizzola *et al.*, 1996; Pujol *et al.*, 1993; Romain *et al.*, 1990; Seshadri *et al.*, 1994; Spyratos *et al.*, 1992; Spyratos *et al.*, 1989; Stonelake *et al.*, 1994; Thorpe *et al.*, 1989). Certaines études ont observé une relation entre l'expression forte de la CD et l'envahissement ganglionnaire (Foekens *et al.*, 1993; Kute *et al.*, 1992; Namer *et al.*, 1991; Pujol *et al.*, 1993). La plupart des études ont confirmé le rôle pronostique de la cathepsine D pour tous les sous-groupes de patientes, bien

que différents seuils de positivité aient été utilisés. Certaines études n'ont pas observé d'association entre l'expression de la cathepsine D et le faible taux de la survie, possiblement en raison d'une période trop courte de surveillance (16 mois seuls) (Stonelake *et al.*, 1994). Principalement, une quantité importante de cathepsine D pouvait significativement prédire soit la survie sans récurrence, ou la survie globale ou tous les deux. Ces résultats confirment que la mesure de la cathepsine D par méthode cytosolique a sa valeur pronostique.

Cependant, l'intérêt pronostique de la cathepsine D dans différents sous-groupes semble plus complexe et la plupart de ces études n'ont pas reproduit les résultats de Spyrtos *et al.* (Spyrtos *et al.*, 1989) qui proposait que la valeur pronostique la plus importante de la cathepsine D était chez les patientes sans envahissement ganglionnaire. Deux études qui ont groupé respectivement 162 patientes sans envahissement ganglionnaire (Kute *et al.*, 1992) et 710 patientes sans ou avec envahissement ganglionnaire (Foekens *et al.*, 1993) ont montré que la cathepsine D induisait un mauvais pronostic chez les patientes sans envahissement ganglionnaire, mais une valeur pronostique a été observée dans le sous-groupe de patientes avec envahissement ganglionnaire dans l'étude de Foekens *et al.*. Pour les autres études, la valeur pronostique de cathepsine D a été retrouvée seulement chez les patientes avec ganglions envahis. Les explications possibles de ces résultats différents sont l'emploi de facteurs différents tel que les seuils, les critères finaux comme la survie sans métastase, ou survie sans maladie, la période de surveillance, et le traitement adjuvant administré. Quoi qu'il en soit, il semble que la valeur pronostique de la cathepsine D existe chez les patientes porteuses de cancer du sein avec ou sans envahissement ganglionnaire, mais serait plus grand pour les patientes avec ganglions envahis. L'intérêt clinique d'un marqueur de mauvais pronostic dans les cas dont les ganglions envahis serait discutable, car l'état ganglionnaire est lui-même déjà un fort indicateur de mauvais pronostic et les patientes avec envahissement ganglionnaire seront traitées agressivement en général.

#### **3.2.5.1.3.2. L'analyse par Western blot.**

Certaines études ont utilisé une méthode semi-quantitative par Western blot pour mesurer la cathepsine D dans le cytosol de tumeurs mammaire. Une étude de 397 patientes a montré qu'une forte expression de la cathepsine D était liée à une survie totale plus courte et une

survie sans récurrence plus courte seulement dans le sous-groupe de patientes sans envahissement ganglionnaire. Le risque relatif de décès était 3.9 fois plus important chez les patientes avec forte expression que chez les patientes avec bas niveau de cathepsine D, et cette association n'existait pas dans le sous-groupe avec envahissement ganglionnaire (Tandon *et al.*, 1990). Cependant, une autre étude avec 927 patientes a retrouvé cette corrélation mais seulement dans le sous-groupe de patientes dont les récepteurs d'œstrogènes étaient positifs (Ravdin *et al.*, 1994).

### **3.2.5.1.3.3. L'Analyse par immunohistochimie.**

Plusieurs études cliniques ont utilisé également l'immunohistochimie pour analyser l'expression de la cathepsine D dans le tissu cancéreux. La première étude publiée a rapporté un pronostic amélioré chez les patientes avec expression positive de cathepsine D. Les patientes avec expression de cathepsine D avaient un meilleur taux de survie sans maladie et un meilleur taux de survie globale. La raison éventuelle était que la cathepsine D était sous la régulation des œstrogènes et donc l'expression de cathepsine D représentait l'hormonosensibilité (Henry *et al.*, 1990). C'est le premier rapport que les cellules stromales (macrophages infiltrantes) pouvaient avoir une forte expression de cathepsine D et pouvaient contribuer à la quantité totale de cathepsine D dans le cytosol tumoral. La contribution de la cathepsine D stromale sur le pronostic n'a pas été évaluée dans cette étude.

Les études suivantes par immunohistochimie ont rapporté des résultats différents. Peu d'études ont rapporté une association entre cette expression de cathepsine D par les cellules cancéreuses associées et un mauvais pronostic. Winstanley *et al.* ont analysé l'expression de la cathepsine D par les cellules cancéreuses dans des cas avec ou sans envahissement ganglionnaire et observé que l'expression de la cathepsine D était associée à un mauvais pronostic dans tous les cas étudiés, indépendamment de l'état ganglionnaire (Winstanley *et al.*, 1993). L'association entre l'expression de la cathepsine D et un faible taux de survie a été également retrouvée par Isola *et al.* (Isola *et al.*, 1993) mais seulement dans les cas sans envahissement ganglionnaire et par Kandalaft *et al.* (Kandalaft *et al.*, 1993) mais dans le sous-groupe avec envahissement ganglionnaire.

Cependant, toutes les autres études n'ont pas retrouvé d'association entre l'expression de cathepsine D par les cellules cancéreuses et la survie (Domagala *et al.*, 1992; Joensuu *et al.*, 1995; O'Donoghue *et al.*, 1995; Têtu *et al.*, 1993). L'expression de la cathepsine D par les cellules cancéreuse ne semble donc pas associée à la survie.

En 1993, Têtu *et al.* ont analysé l'expression de la cathepsine D dans une série de 638 patientes porteuses de carcinome mammaire avec envahissement ganglionnaire (Têtu *et al.*, 1993). Les auteurs ont analysé séparément l'expression de cathepsine D dans les cellules cancéreuses et les cellules stromales (macrophages) par immunohistochimie et ont observé que l'expression de cathepsine D produite par cellules stromale et non par cellules tumorale corrélait avec un faible taux de survie sans métastase. Cette corrélation était plus importante dans le sous-groupe de chimiothérapie adjuvante. L'expression de la cathepsine D par les cellules stromales était liée à l'expression de HSP-27, un haut grade de différenciation, l'aneuploïdie et l'absence de récepteurs d'œstrogènes et de la progestérone. Les auteurs suggèrent que l'expression de cathepsine D par les cellules stromales joue un rôle important dans le processus invasif du cancer du sein. Suite à cette étude, deux autre études ont également confirmé cette hypothèse. Dans l'étude de O'Donoghue (O'Donoghue *et al.*, 1995) qui a analysé par immunohistochimie une série de 103 patientes avec carcinome primaire mammaire opérable, l'auteur n'a pas retrouvé non plus de corrélation entre l'expression de cathepsine D par les cellules cancéreuses et la survie, bien que l'expression de cathepsine D ait été observée dans tous les cas. Par contre, la surexpression de cathepsine D par les cellules stromales était liée à un haut grade de différenciation, un taux augmenté de récurrences locales et régionales, un faible taux de survie sans maladie et un faible taux de survie totale. Une autre étude en 1995 rapportée par Joensuu (Joensuu *et al.*, 1995) a analysé 213 carcinomes primaires mammaires par immunohistochimie. Le taux de survie totale chez les patientes sans expression de cathepsine D par les cellules stromales *macrophage-like* était de 75% à 5 ans et de 55% à 30 ans. Chez les patientes avec forte expression de la cathepsine D par les cellules stromales, ce taux de survie était abaissée à 40% à 5 ans et à 20% à 30 ans. La cathepsine D a été observée surtout dans le type histologique canalaire plutôt que lobulaire (80% vs 54%,  $p=0.002$ ). De plus, l'expression de la cathepsine D par les cellules stromales était liée à une prolifération accélérée de cellules tumorales. L'auteur a observé également que la cathepsine

D n'avait pas d'influence indépendante sur la survie dans toutes les catégories de patientes. On a conclu que l'expression de la cathepsine D par les cellules stromales pouvait prédire la survie à long terme. Dans l'étude de Nadji (Nadji *et al.*, 1996) qui a groupé 154 carcinomes canaux et invasifs sans envahissement ganglionnaire, bien qu'il existe une corrélation entre l'expression de cathepsine D par les cellules cancéreuses et la différenciation et le grade nucléaire, c'était la surexpression de cathepsine D par les cellules histiocytaires et fibroblastiques qui corrélait significativement avec la survie totale et la survie sans maladie. L'expression de la cathepsine D par les cellules stromales n'avait pas d'influence sur la survie. L'auteur a conclu que l'analyse de la cathepsine D dans le cytosol n'avait pas de valeur pratique. Une étude récente (Gonzalez-Vela *et al.*, 1999) a analysé 102 échantillons de cancer du sein, et a trouvé que l'expression de cathepsine D dans les cellules stromales était significativement liée à l'envahissement vasculaire, à un haut grade histopronostique, un haut index de mitose et une absence de récepteurs d'œstrogènes.

Le fait que les études par Têtu (Têtu *et al.*, 1993), O'Donoghue (O'Donoghue *et al.*, 1995), Joensuu (Joensuu *et al.*, 1995), Nadji (Nadji *et al.*, 1996), Gonzalez-Vela (Gonzalez-Vela *et al.*, 1999) aient démontré que la cathepsine D par les cellules stromales (macrophages, fibroblastes etc.), et non les cellules cancéreuses, avait une influence pronostique a attiré l'attention des chercheurs cliniques. Les résultats suggèrent que les cellules stromales contribuent une partie de la cathepsine D mesurée dans l'analyse du cytosol tumoral. Cependant deux études ont observé une corrélation significative entre la cathepsine D des cellules cancéreuses par immunohistochimie et le niveau de cathepsine D dans le cytosol mesuré par IRMA (Maudelonde *et al.*, 1992; Roger *et al.*, 1994).

L'analyse différentielle de l'expression de cathepsine D par les cellules cancéreuses et par les cellules stromales donne des informations sur la contribution pronostique individuelle. Ceci suggère que dans l'analyse du cytosol par Western blot, ELISA et IRMA, la quantité totale de cathepsine D contient une partie non négligeable venant des cellules stromales, ce qui pourrait expliquer les divergences de résultats des études par analyse du cytosol. Comme l'indique Westley *et al.* (Westley and May, 1996; Westley and May, 1999), la source de cathepsine D mesurée dans le cytosol tumoral et la valeur pronostique de l'expression de cathepsine D par

différents types de cellules dans la tumeur mammaire reste à établir.

Bien que ce ne soit pas toutes les études qui supportent cette conclusion, il existe une forte évidence que les cellules stromales réactionnelles jouent un rôle dans le cancer mammaire en favorisant par l'activité des protéases y compris la cathepsine D l'invasion et la métastase des cellules cancéreuses.

### **3.3. L'activateur de plasminogène (PA).**

L'activateur de plasminogène est une endopeptidase de la famille des sérines peptidases, caractérisée par la triade catalytique comprenant l'acide aspartique, la sérine et l'histidine. La sérine fonctionne comme un résidu réactif formant un pont covalent avec le substrat. Le groupe de sérines peptidases contient les enzymes associées à la chymotripsine comme les enzymes fibrinolytiques, facteurs de coagulation et celles de la famille de la subtilisine.

Les activateurs du plasminogène sont des sérines protéases normalement actives dans les processus de coagulation, où elles ont pour fonction d'activer le plasminogène en plasmine (Dano *et al.*, 1985; Saksela, 1985). La plasmine est une protéase avec spectre large de substrats. Elle peut catalyser la dégradation de divers substrats dans la matrice extracellulaire, et activer des certaines protéases de la famille des MMPs (Duffy, 1993). Il existe deux types d'activateurs du plasminogène, le type tissulaire (tPA) et le type urokinase (uPA). Ils sont différents en terme de distribution tissulaire et de caractéristiques immunologiques, moléculaire et catalytique. L'uPA est une enzyme de 54 kDa composée de deux sous-unités de 30 kDa et 24 kDa (Dano *et al.*, 1985). La forme tPA native, par contre, est composée d'une seule chaîne polypeptide de 70 kDa (Edlund *et al.*, 1983; Rijken *et al.*, 1982). Analysée par immunohistochimie, l'uPA a été identifiée dans les fibroblastes *in vivo* (Kristensen *et al.*, 1985; Skriver *et al.*, 1984), tandis que le tPA est situé dans l'endothélium vasculaire de plusieurs tissus (Kristensen *et al.*, 1984). Chez l'homme, la fibrinolyse vasculaire est faite principalement par tPA dérivé de l'endothélium. L'uPA est responsable de l'activation du plasminogène qui est présent dans la plupart des tissus extracellulaires. L'uPA peut participer au remodelage normal ou pathologique de la matrice extracellulaire et est impliqué dans le phénomène de progression tumorale (Duffy, 1996). En dehors de son rôle d'activateur du

plasminogène, l'uPA peut également dégrader des composantes de la matrice extracellulaire dont notamment la fibronectine et la laminine (Balian *et al.*, 1979; Liotta *et al.*, 1981), activer d'autres protéases comme la collagénase de type IV, ou encore activer des facteurs de croissance tels que le facteur de croissance hépatocytaire (Duffy, 1996), stimuler la mitogenèse (Binder, 1990) et la migration (Odekon *et al.*, 1992) des cellules. L'uPA se lie à un récepteur membranaire (uPAR) (Duffy, 1993).

En 1988, Duffy a le premier mis en évidence que la présence d'uPA dans les carcinomes mammaires était associée à un pronostic défavorable pour les patientes (Duffy *et al.*, 1988a; Duffy *et al.*, 1988b). En effet, les patientes dont la tumeur contenait un haut niveau d'activité de l'uPA avaient un taux de survie sans maladie (DFS) ou un taux de survie globale significativement plus faible que les patientes dont la tumeur avait un niveau bas d'activité de l'uPA. Ce résultat a été confirmé par plusieurs groupes (Foekens *et al.*, 1992; Grondahl-Hansen *et al.*, 1993), de plus, le haut niveau d'activité de l'uPA est lié à un faible taux de survie globale. Dans le cancer du sein, cette expression stromale est fortement liée à une récurrence de la maladie, et donc l'uPA est considéré comme un facteur pronostique défavorable par plusieurs investigateurs (Duffy *et al.*, 1988a; Duffy *et al.*, 1988b; Evers *et al.*, 1982; Foekens *et al.*, 1992; Grondahl-Hansen *et al.*, 1993; Grondahl-Hansen *et al.*, 1995; Schmitt *et al.*, 1990; Spyrtos *et al.*, 1992).

L'expression de l'uPA a été observée dans les cellules stromales de tissu cancéreux (Wolf *et al.*, 1993). Cette expression de l'uPA par les cellules stromales colorectales est associée au niveau de dysplasie des cellules épithéliales (Sordat *et al.*, 1997). Dans l'étude de Wolf, tous les onze carcinomes canaux mammaires étudiés montraient une expression de l'ARNm de l'uPA par les cellules stromales (Wolf *et al.*, 1993). Dans l'étude de Nielsen, l'ARNm de l'uPA a été observé dans 26 carcinomes canaux parmi 28 carcinomes canaux étudiés et dans tous les 5 carcinomes lobulaires (Nielsen *et al.*, 1996). L'expression de l'uPA a été observée dans les myofibroblastes adjacents des cellules cancéreuses (Nielsen *et al.*, 1996; Romer *et al.*, 1994). Une xénogreffe de cellules MDA-MB-231 dans des souris nues/nues a induit une expression d'ARNm d'uPA et de son récepteur membranaire par les cellules stromales de souris adjacentes aux cellules cancéreuses injectées (Romer *et al.*, 1994),

signifiant que les cellules cancéreuses peuvent induire une production d'uPA et d'uPAR par les cellules stromales. Certains facteurs produits par les cellules cancéreuses, dont TGF- $\beta$ 1 (transforming growth factor - $\beta$ 1), peuvent introduire la production d'uPA par les fibroblastes mammaires (Sieuwerts *et al.*, 1998). En revanche, la plasmine peut activer des proformes de cytokines, de facteurs de croissance et de TGF- $\beta$  (Rifkin *et al.*, 1999).

Dans le cancer du côlon, l'uPA est produite par les fibroblastes, le récepteur de l'uPA (uPAR) par les cellules épithéliales cancéreuses et l'inhibiteur de l'uPA (PAI-1) par les cellules endothéliales (Blasi and Stoppelli, 1999). Il est proposé que l'uPA produite par les fibroblastes se fixe sur l'uPAR des cellules cancéreuses puis favorisent l'invasion tumorale (Grondahl-Hansen *et al.*, 1991; Pyke *et al.*, 1991b; Pyke *et al.*, 1994). Dans le cancer mammaire, l'uPA est produite par les myofibroblastes, et le récepteur de l'uPA est uniquement retrouvé dans les macrophages qui infiltrent le tissu tumoral et non dans les cellules cancéreuses (Bianchi *et al.*, 1994; Blasi and Stoppelli, 1999; Pyke *et al.*, 1993). Il est suggéré que, dans le cancer du sein, les myofibroblastes produisant l'uPA, les macrophages infiltrants produisant l'uPAR et les cellules cancéreuses forment la population cellulaire infiltrante (Nielsen *et al.*, 1996). Dans cette assemblage, l'uPA par les cellules stromales participe non seulement à la dégradation de la matrice extracellulaire mais aussi à la communication intercellulaire par activation des cytokines latentes comme le TGF- $\beta$  (transforming growth factor  $\beta$ ) et HGF (hepatocyte growth factor) (Naldini *et al.*, 1995; Odekon *et al.*, 1994).

Une étude récente a démontré une expression d'uPA non seulement dans les cellules stromales mais aussi dans les cellules cancéreuses. On croit que l'uPA dans les cellules cancéreuses est une forme fixée à l'uPAR (Carriero *et al.*, 1994). Cependant, ce résultat contredit celui où l'uPAR est présente dans les macrophages infiltrants (Bianchi *et al.*, 1994; Blasi and Stoppelli, 1999; Pyke *et al.*, 1993).

### **3.4. Le rôle intégré des protéases stromales sur l'invasion tumorale mammaire : intérêt comme facteurs pronostiques.**

L'établissement du pronostic histologique comprend l'étude de plusieurs paramètres : la structure histologique de la tumeur, l'envahissement vasculaire lymphatique, la

différenciation, l'index mitotique, la gradation histologique, la classification TNM, etc. Les protéases comptent parmi les molécules considérés comme marqueurs tumoraux et impliqués au moins potentiellement dans la métastase tumorale (Duffy, 1996). L'évidence initiale qu'une relation existait entre l'expression des protéases et l'invasion et la métastase cancéreuses était basée sur une corrélation entre le niveau des protéases spécifiques et le potentiel métastatique dans des tumeurs animales (Duffy, 1992).

Il est important de réaliser que les cellules tumorales et les cellules stromales peuvent produire en même temps plusieurs protéases. Les enzymes protéolytiques, qui sont généralement sécrétées sous forme de zymogènes inactifs dans la plupart de cas, appartiennent à différentes familles interdépendantes. Ainsi, des enzymes d'une même famille peuvent s'activer entre elles ou activer celles d'une autre famille (Ramos-DeSimone *et al.*, 1999), ce qui souligne l'extrême complexité des relations existant entre les différentes protéinases, et particulièrement les différents systèmes d'activation. Par exemple, l'activation de la collagénase demande une cascade enzymatique qui implique l'activation hydrolytique de procollagénase par la plasmine, cette dernière devant d'abord être activée par le plasminogène (O'Grady *et al.*, 1981).

Le processus de métastase de tumeur implique une cascade de plusieurs étapes liées et séquentielles lors d'interactions épithéliales/stromales multiples (Fidler and Hart, 1982; Liotta *et al.*, 1983; Schirrmacher, 1985). Toutes ces protéases agissent souvent en synergie, ou successivement (cascade protéolytique), puisque l'invasion tumorale implique de multiples protéinases. (Quigley *et al.*, 1990). Ainsi est-il logique de considérer avec soin l'équilibre entre les protéases et leurs inhibiteurs. C'est seulement lorsque cet équilibre est rompu en leur faveur que les protéases peuvent être activées. En même temps que des protéases, les cellules tumorales et stromales produisent des inhibiteurs de ces protéases.

Si de multiples protéases sont impliquées dans le processus métastatique, l'information pronostique la plus utile et pertinente devrait être obtenue par une mesure combinant de plusieurs protéases dans le cancer mammaire. Il y a donc en fin de compte plusieurs protéases

actives pour attaquer les constituantes de la matrice extracellulaire, et cette synergie peut avoir une grande importance.

#### **4. Objectifs.**

##### **4.1. Général.**

Cette étude vise à tester l'hypothèse selon la quelle les cellules cancéreuses induisent l'expression des protéases par les cellules stromales réactionnelles dans le cancer mammaire et que ces protéases stromales favorisent la progression tumorale.

##### **4.2. Spécifiques.**

- 1) Étudier le rôle de CD, uPA et ST3 d'origine stromale en analysant directement leur expression dans des tissus tumoraux des patientes porteuses d'un cancer mammaire, corrélér l'expression de ces protéases stromales à la survie globale et à la survie sans métastase et effectuer l'analyse avec une population importante de patientes et avec une période de surveillance de long terme;
- 2) Développer une banque des cellules stromales d'origine tumorale afin d'évaluer l'hétérogénéité de réponse des cellules stromales à une même stimulation cancéreuse;
- 3) Démontrer le rôle du support stromal pour la purification des cellules épithéliales et l'établissement de lignée cellulaire à partir des carcinomes mammaires primaires;
- 4) Étudier les interactions entre cellules stromales et cancéreuses par cocultures directes et indirectes afin d'évaluer le rôle des contacts directs et indirects sur la production de protéases;
- 5) Vérifier le rôle de bFGF sur l'induction de MMP-11 par les cellules stromales d'origine tumorale;
- 6) Démontrer que la réponse des cellules stromales d'origine tumorale à une même stimulation est variable d'un individu à l'autre;
- 7) Réaliser la coculture de tissu-équivalent avec les cellules cancéreuses mammaires et les cellules stromales réactionnelles et observer l'interaction épithéliale-stromale principalement pour le remodelage de tissu-équivalent, la migration des cellules cancéreuses et la production des collagénases et gélatinases.

## **CHAPITRE II**

### **CULTURE SÉLECTIVE DE CELLULES ÉPITHÉLIALES MAMMAIRES À PARTIR DE CARCINOMES PRIMAIRES DU SEIN**

## **AVANT-PROPOS.**

**La purification des cellules épithéliales mammaires à partir de carcinomes primaires mammaires est une étape importante et essentielle pour effectuer la recherche *in vitro* dans le domaine de l'interaction stromale-épithéliale du cancer du sein.**

**Ce chapitre porte sur l'utilisation d'une couche nourricière composée de cellules 3T3 irradiées pour isoler de façon sélective des cellules épithéliales de cancers du sein.**

**Le travail de ce chapitre a été principalement effectué dans le Laboratoires d'Organogenèse expérimentale (LOEX). Cet article a été soumis pour fin de publication dans la revue Pathology, Research and Practice.**

## **RÉSUMÉ.**

La recherche *in vitro* sur les interactions stromales-épithéliales dans le cancer du sein requiert souvent l'emploi de cellules épithéliales purifiées de cancer primaire du sein. Cependant, la purification des cellules épithéliales cancéreuses à partir de carcinomes primaires mammaires est un travail laborieux. Deux causes principales expliquent cette difficulté: soit la prolifération lente *in vitro* des cellules épithéliales malignes quand ces cellules sont isolées d'une tumeur humaine; soit une sur-prolifération des fibroblastes réactionnelles qui l'emportent sur la prolifération épithéliale. Plusieurs méthodes ont été proposées dans la littérature et le principe est d'inhiber la surcroissance des fibroblastes. Utilisant une couche nourricière de cellules 3T3 irradiées à 60 Gy, la culture sélective des cellules épithéliales a été réussie dans six carcinomes primaires étudiés. Nous concluons que l'utilisation d'une couche nourricière de 3T3 irradiées comme méthode de culture sélective favorise la purification des cellules épithéliales non seulement par inhibition de la surcroissance des fibroblastes, mais aussi parce que cette couche agit comme support de la prolifération des cellules épithéliales.

**Selective culture of epithelial cells from primary breast carcinomas using  
irradiated 3T3 cells as feeder layer**

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**Running title: selective culture of breast epithelia**

**Summary**

The main drawback to selective culture of human mammary epithelial cell (HMEC) from primary breast cancer is the overgrowth by tumor-associated stromal fibroblasts. This drawback may be overcome by using, in primary culture, lethally irradiated 3T3 cells which act as feeder layer to maintain HMEC proliferation. 3T3 cells, exposed to 60 Gy at confluence, form a specific cellular substrate, which constitutes a barrier to fibroblast attachment. Enzyme-digested breast cells from six primary breast carcinomas were cocultured over lethally irradiated but living 3T3 cells. The method led to the purification of HMEC from all six cancer samples and long-term culture was obtained in one. The epithelial nature of those purified HMEC was demonstrated by their general morphology and by expression of cytokeratins and epithelial membrane antigen. Those results confirm the stimulatory effect of using a stromal feeder layer on breast epithelial cell growth and show that this stromal feeder layer can also control the fibroblast overgrowth. Our results provide an alternative approach in the selective culture of epithelial cells from primary breast carcinoma.

**Keywords:** tumor-derived HMEC, tumor-associated fibroblasts.

## **Introduction**

Breast cancer is a leading cause of cancer-related deaths in North America and in Europe. The availability of cell cultures established directly from patient tumors is an important tool to study breast cancer biology. However, the establishment of such cell lines requires the isolation of malignant cells from breast cancer specimens (Ethier *et al.*, 1993) and selective cultures of human mammary epithelial cells (HMEC) have until now been confronted with a number of technical problems (McCallum and Lowther, 1996). A major problem is the fast overgrowth by tumor-associated stromal fibroblasts in an *in vitro* setting.

To obtain dispersed epithelial cells, most tissue culture techniques require mincing of tissue, followed by an enzymatic digestion of fragments. Although this procedure often leads to an important cellularity, it is rarely satisfactory for primary breast cancer, which contains abundant fibrous stroma (Sykes J.A. *et al.*, 1970). Major improvements in selective culture technology for primary carcinomas have been achieved with the use of selective media (Ethier *et al.*, 1993), partial enzymatic degradation (Dairkee *et al.*, 1997) and other methods such as differential centrifugation (Speirs *et al.*, 1998; Sykes J.A. *et al.*, 1970).

It has been reported that a pure keratinocyte culture, free of fibroblasts, might be obtained with the use of lethally irradiated but living 3T3 cells used as feeder layer (Rheinwald and Green, 1975). This method, however, has rarely been tested in the selective culture of HMEC from primary breast cancers. We hypothesized that cocultures with those living 3T3 cells would produce pure HMEC cultures from primary breast cancers by stimulation of epithelial cell proliferation and inhibition of fibroblast overgrowth. The present report describes this selective method.

## **Materials and methods**

### **Clinical findings**

Six primary breast carcinomas were used in this study and their clinical and histological characteristics are shown on table 1.

### **Specimen dissociation**

Fresh sterile tissue from primary breast tumors was obtained from surgical resection. The diagnosis of those tumors was confirmed by routine histopathological examination. The tissue specimens were then trimmed and placed in Dulbecco modified Eagle's medium (DMEM) with antibiotics at 4°C. Then, the tissue specimen was minced with crossed surgical blades into 1 mm cubes and then digested overnight with collagenase (0.14%) at 37°C under constant shaking. The single-cell suspension was obtained and the cell viability was determined by the trypan blue exclusion technique.

### **Cell culture**

After centrifugation at 250 x g for 10 min, cell pellet was resuspended in culture medium. The final cell suspension was seeded at a concentration of  $2 \times 10^5$  cells/75 cm<sup>2</sup> culture flask (Falcon, Becton Dickinson Inc, Mississauga, Ontario) previously inoculated with lethally irradiated (60 Gy) 3T3 cells (ATCC) at confluence. These 3T3 cells formed a cellular layer acting as substratum in the flasks and were used during one week. The culture medium was as previously reported (Goulet *et al.*, 1996) and consisted of a combination of DMEM with Ham's F12 in a 3:1 proportion (Gibco-BRL, Burlington, Ontario), supplemented with 10 ng/ml human epidermal growth factor (EGF, Chiron Corp., Emeryville, CA), 5 ng/ml 17- $\beta$ -estradiol, 500 ng/ml progesterone, 24.3  $\mu$ g/ml adenine, 5  $\mu$ g/ml crystallized bovine insulin, 5  $\mu$ g/ml human transferrin,  $2 \times 10^{-9}$  M 3,3',5',-triiodo-L-thyronine (Sigma Chemicals, St Louis, MO), 0.4  $\mu$ g/ml hydrocortisone (Calbiochem, La Jolla, CA),  $10^{-10}$  M cholera toxin (Schwarz/Mann, Cleveland, OH), 10% fetal bovine serum (FBS) (Gibco-BRL), 100 IU/ml penicillin G and 25  $\mu$ g/ml gentamicin (Sigma). A few coverslips were placed in the culture flasks on which cells were allowed to grow and on which immunocytochemical assays for the identification of cell characteristics were carried out. All dissociated material was designated as passage 0 upon primary seeding in culture. Flasks were examined daily during the first week. All cultures were kept in a humidified incubator with 5% CO<sub>2</sub> at 37 °C.

All primary cultures were kept in undisturbed conditions, and the culture medium was first changed at the time of cell attachment and spreading was observed. Then, the culture medium

was changed three times a week.

Comparative culture was performed with the same tumor specimens and in the same culture medium, but in which the 3T3 cell layer had been prefixed with methanol at -20°C for 10 minutes followed by washing with sterile phosphate-buffered saline (PBS). Another parallel culture using the same tumor specimens was performed using lethally irradiated 3T3 cells which were recollected by trypsination, admixed with the enzyme-disaggregated cell suspension, and then seeded in normal culture flasks (Falcon).

### **Maintenance of cell culture**

Epithelial cell colonies firmly attached to the flasks but contaminated by fibroblasts were passed again over lethally and freshly irradiated confluent 3T3 cells until the tumor-like epithelial cells were purified. In the absence of fibroblast contamination, if epithelial-like cells were growing slowly due to significant degeneration of 3T3 cells, subcultures were performed in the same conditions. Subcultures were made by exposing the culture to 0.05% trypsin (1:300)-0.02% EDTA solution. Purified epithelial-like cells free of fibroblasts were subcultured regularly in the absence of irradiated 3T3 cell feeder layer. Purified epithelial-like cells were also cultured in Lab-Tek chamber slides (Nunc, Naperville, IL) to perform immunocytochemistry studies.

### **Morphological examination**

All epithelial cells grown in the flasks were directly examined with an inverted microscope and phase-contrast microphotographs were taken periodically.

### **Immunocytochemistry analyses**

Epithelial cells grown on coverslips or chamber slides were washed in PBS for 5 minutes, fixed for 15 min at -20°C with cold methanol then quenched for 5 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. After washing with PBS, slides were covered with normal horse serum during 20 minutes and incubated overnight with each monoclonal antibody at 4°C. Afterwards, slides were rinsed with PBS and exposed for 30 minutes to a secondary biotinylated anti-mouse antibody. After another wash with PBS, slides were

incubated with the avidin-biotin-peroxidase complex (Vector laboratory, Burlingame, CA) and the reaction was revealed with diaminobenzidine (DAB). The coverslips and slides were finally counterstained with Mayer's haematoxylin for 3 minutes and mounted in permount.

The antibodies used were the anti-epithelial membrane antigen (EMA) (mAb, E29, Dako, Mississauga, Ontario), anti-human cytokeratin (mAb, CAM 5.2, Becton Dickinson, Mississauga, Ontario) detecting cytokeratins 8 and 18, anti-vimentin (mAb, V9, Dako) and anti-human  $\alpha$ -smooth muscle actin (mAb, 1A4, Dako).

## **Results:**

### **Settlement and purification of tumor-like cells**

In the flasks containing heterogeneous disaggregated cells (HMEC and stromal cells) seeded over irradiated 3T3 cell layer with supplemented culture medium, epithelial colonies were observed within a few days. However, these HMEC often floated as small spheroid clusters during the first days and adhered later to the 3T3 cell layer. Then the epithelial cell clusters attached to the surface of the culture flask and began to grow as expanding colonies on the flask surface (Fig. 1a & 1b), pushing away the surrounding 3T3 cells and forming an evident interface at the periphery with feeder layer cells (Fig. 1e). This phenomenon was seen in the cultures from all six tumor samples studied. Generally, there was a good cellular proliferation aspect in the interface of 3T3 cells with epithelial colonies. Typical results are shown in figure 1. The first change of culture medium was performed when a few epithelial colonies were clearly growing over irradiated 3T3 cells, this took in general 5 days after seeding. Fibroblast attachment was also observed over irradiated 3T3 cells but the density was minimal and never formed spheroid clusters. This phenomenon occurred generally in the cultures with damaged cellular layer substratum. A completely confluent and intact 3T3 cellular layer was found to be very efficient to obtain epithelial cell purification. However, old feeder layer cells, irradiated long time before, and unconfluent cellular layer gave rise to free spaces facilitating stromal fibroblast settlement.

When dissociated cells from cancer specimen and irradiated 3T3 cells were admixed and simultaneously seeded, we observed also a good proliferation of epithelial colonies in

response to 3T3 cellular support but with more stromal cells proliferating well next to degenerated 3T3 cells. These tumor-associated stromal fibroblasts competed against epithelial cells, enlarged their areas and displaced 3T3 cells. Nevertheless, only rare epithelial colonies were observed in the coculture with methanol-prefixed 3T3 cells. Epithelial colonies failed to develop and they degenerated along with 3T3 cells (Fig. 2a).

Using irradiated 3T3 cells as feeder layer, a pure culture of HMEC, free of fibroblasts, was obtained from six tumor samples from passages two to six (HDQ-P1 at passage 4, HDQ-P2 at passage 3, HDQ-P3 and HDQ-P4 at passage 6, HDQ-P5 and HDQ-P6 at passage 1).

All six purified tumor-derived HMEC proliferated without further support of feeder layers. Serial cultures were obtained in a few passages for each HMEC. Of six purified HMEC lines, long term culture was further obtained only in one cell line (HDQ-P1) which reached passage 115 in DMEM supplemented with 10% of FBS, 5 µg/ml insulin and antibiotics.

### **Culture morphology**

Polygonal and cuboidal epithelial cells formed often cobblestone patches in the culture flasks (Fig. 2b). Domes were frequently observed in these epithelial cell patches, there were often some loose cell colony clusters along the periphery of the epithelial patches (1e). HMEC usually formed adherent patches in the culture flask, became compact but rarely reached complete confluence.

### **Morphological examination**

Microscopic examination of those purified cells revealed a typical epithelial morphology. The majority of those purified tumor-derived HMEC were polygonal and stellate with large nuclei and multiple lobulated nucleoli (Fig. 2c). Aberrant mitotic figures and multinucleate cells were common. Some epithelial cells developed intracytoplasmic vacuoles in their perinuclear cytoplasm.

### **Immunohistochemistry**

All six cell lines showed a strong and diffusely positive immunoreaction for anti-cytokeratin

CAM 5.2 and anti-EMA (Fig. 2d). Immunoreaction with anti- $\alpha$ -actin was negative for all six cell lines. Negative reaction with anti-vimentin was seen in five cell lines, whereas this reaction was weakly and focally positive in the HDQ-P1 cell line.

### **Discussion:**

HMEC isolated from primary breast cancers are important research tools to study cancer cell biology and to develop new treatment strategies. Selective culture and purification of HMEC from primary breast cancer is also the first essential step for establishment of breast cancer cell line. However, most of the time, the isolation of HMEC from a breast cancer by protease digestion is compromised by excessive fibroblast contamination. A successful culture is achieved when epithelial cells proliferate faster than the fibroblasts, which is rarely the case. In fact, the slow proliferation rate of most tumor-derived epithelial cells may relate to either a lack of adequate stromal support in pure epithelial cell culture or the difficulty of single cells to give rise to clonal expansion. Stromal cells are important to stimulate epithelial cell proliferation and this paracrine effect has been well documented *in vivo* and *in vitro* assays (Armstrong and Rosenau, 1978; Brattain *et al.*, 1982; Epstein and Kaplan, 1979). The use of stromal cells as feeder layer to culture human mammary epithelial cells was first reported by Taylor-Papadimitriou *et al.* (Taylor-Papadimitriou *et al.*, 1977). In this study, mitomycin-treated mouse fibroblasts enhanced initial attachment and the growth of mammary epithelial cells in human milk. Later, Smith *et al.* have shown that ultraviolet-irradiated fibroblasts increased higher plating efficiencies in the breast epithelium clone proliferation (Smith *et al.*, 1981). Irradiated mesenchymal cells proved also to provide an adequate stromal support. In fact, Camps *et al.* reported that lethally irradiated transformed fibroblasts could shorten the latency period and increase the rate of human tumor growth *in vivo* (Camps *et al.*, 1990) and Strobel *et al.* showed that irradiated marrow stromal cells can stimulate the growth of human tumor cells, including mammary carcinoma (Strobel *et al.*, 1989). The mechanism responsible for this enhancement is unknown at present, but might involve the production of "growth factors" by stromal cells or increased attachment of epithelial cells due to their interaction with the fibroblasts.

In our experience, coculture of cancer cells with fixed 3T3 cells showed only rare small

epithelial colonies settled and proliferating with difficulty, indicating a loss of stromal support by 3T3 cells. On the contrary, the addition of an irradiated but living 3T3 cell feeder layer to the system provided the best results. In fact, although 3T3 cells were exposed to a radical dose 60 Gy, their biological activities were well retained, while their proliferation capacity was much reduced, which favored a better expansion of epithelial cells. Irradiated 3T3 cells proved also efficient to accelerate keratinocyte proliferation (Green *et al.*, 1979; Rheinwald and Green, 1975).

Rare reports in the literature addressed the influence of using a feeder layer to control fibroblast contamination in the selective culture of HMEC from primary cancers. In 1975, Rheinwald and Green (Rheinwald and Green, 1975) found that irradiated 3T3 cells not only supported the proliferation of keratinocytes isolated from teratomas and human epidermis, but also controlled fibroblast proliferation. Those irradiated 3T3 cells maintained their metabolism and kept some biological activities such as contact inhibition (Rheinwald and Green, 1975). In our assay, irradiated 3T3 cells blocked more significantly fibroblasts than epithelial cell attachment. In fact, we found that fibroblasts are much less adherent than cancer epithelial cells over those cellular layer substrata. Our results suggest that this living 3T3 cellular layer acted as a settlement barrier to tumor-associated fibroblasts and that contact inhibitory effect may be involved.

The use of irradiated 3T3 cells as feeder layer to isolate HMEC from primary breast cancer has been reported in only one recent study (Matouskova E *et al.*, 1998). In this report, which was published while we were completing our study, 3T3 cells were irradiated to 10 Gy and served as feeder layer. Only one tumor sample from a preoperative biopsy specimen was tested. The authors observed epithelial colony expansion in the primary cultures. Only two epithelial colonies were observed after 10 days without changing the medium, and the epithelial cells became stationary in the fourth passage. Nevertheless, our study confirmed the improvement of epithelial colony expansion by the support of irradiated 3T3 cells.

Other approaches aimed at inhibiting the overgrowth of fibroblasts were reported in the literature. Those include the use of selective medium (Ethier *et al.*, 1993), a sandwich system

with nutritional and oxygen gradients (Dairkee *et al.*, 1995), partial enzymatic degradation (Dairkee *et al.*, 1997) and discontinuous density gradients (Speirs *et al.*, 1998; Sykes J.A. *et al.*, 1970). Although the stromal overgrowth was minimized in some reports with selective medium or serum-free medium, contamination of the epithelial fraction with fibroblasts was still apparent in certain cases (Croce *et al.*, 1998; Dairkee *et al.*, 1997), requiring removal of these cells by differential trypsinization (Dairkee *et al.*, 1997). The differential centrifugation was successful at selecting different cell fraction, but was limited by the difficulty to obtain a pure fraction and to optimize centrifugation force and time.

By seeding enzyme-digested cell suspension over lethally irradiated 3T3 cells, we were able to purify HMEC from all six primary breast tumor. These cells were distinct from stromal cells, as confirmed by their morphology. The immunocytochemical analysis also confirmed the epithelial phenotype such as the positive immunocytochemical reaction with anti-human keratin 8 & 18 and with anti-human EMA, as well as the absence of  $\alpha$ -actin. HDQ-P1 cells presented weak and focal vimentin staining. Focal expression of vimentin in breast carcinoma is mostly reported with high grade neoplasms (Domagala *et al.*, 1990).

However, unlike established cell lines in long term culture and our HDQ-P1 cell line, the cultures of HDQ-P2 to HDQ-P6 cells could not be immortalized. Although the short-term culture of HMEC was successful for each of them, the epithelial cells from those five primary carcinomas were unable to keep proliferating in long term culture without further support by 3T3. It is not clear whether clonal growth of certain cell lines obtained with irradiated 3T3 cells is dependent on the long-term support of those stromal cells. Current literature confirms the difficulty to obtain long-term culture and establish new immortalized cell lines from primary breast carcinoma. In a recent report, Amadori *et al.* (Amadori *et al.*, 1993) have established only one cell line from 136 primary breast carcinomas. In another recent study, McCallum & Lowther (McCallum and Lowther, 1996) have obtained only ten cell lines from 135 primary breast carcinomas by using defined medium culture over a 6.5 year period. The advantage of our method is however the greater probability of isolating tumor-derived HMEC for long-term culture since this was achieved in one of 6 cases, which increases the chances of establishing new breast cancer cell lines.

In summary, our results indicate that irradiated 3T3 cell feeder layer may enhance HMEC settlement and proliferation from primary breast carcinomas. Furthermore, intact 3T3 cellular layer constitutes a barrier to tumor-associated stromal fibroblast overgrowth. Those results suggest that this method is relatively simple and less laborious than several others, and the purified HMEC can be studied further for other tests such as karyotyping. Furthermore, this approach may be combined with other selective methods. The ability to easily isolate new cell lines would prove most helpful to support certain research fields such as those related to chemosensitivity and stromal/cancer cell interactions.

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| Cell line | Age of patient | Histology | Grade | Metastase      | ER<br>(fmol/mg) | PgR<br>(fmol/mg) | DNA index | S phase |
|-----------|----------------|-----------|-------|----------------|-----------------|------------------|-----------|---------|
| HDQ-P1    | 50             | Ductal    | 3/3   | N1 (6+/16), M0 | 9               | 3                | 1.58      | 27%     |
| HDQ-P2    | 65             | Ductal    | 3/3   | N1 (3+/7), M0  | 36              | 1                | 1.66      | 22%     |
| HDQ-P3    | 79             | Ductal    | 2/3   | -              | 7               | 1                | -         | -       |
| HDQ-P4    | 68             | Lobular   | -     | N1 (9+/14), M0 | 1               | 1                | 1         | 1%      |
| HDQ-P5    | 51             | Ductal    | 2/3   | N1 (1/10), M0  | 10              | 25               | 1.9       | 7%      |
| HDQ-P6    | 78             | Ductal    | 1/3   | N1 (1+/28), M0 | 5               | 1                | 1.9       | 9%      |

Table 1. Patients and histological characteristics of tumor samples.

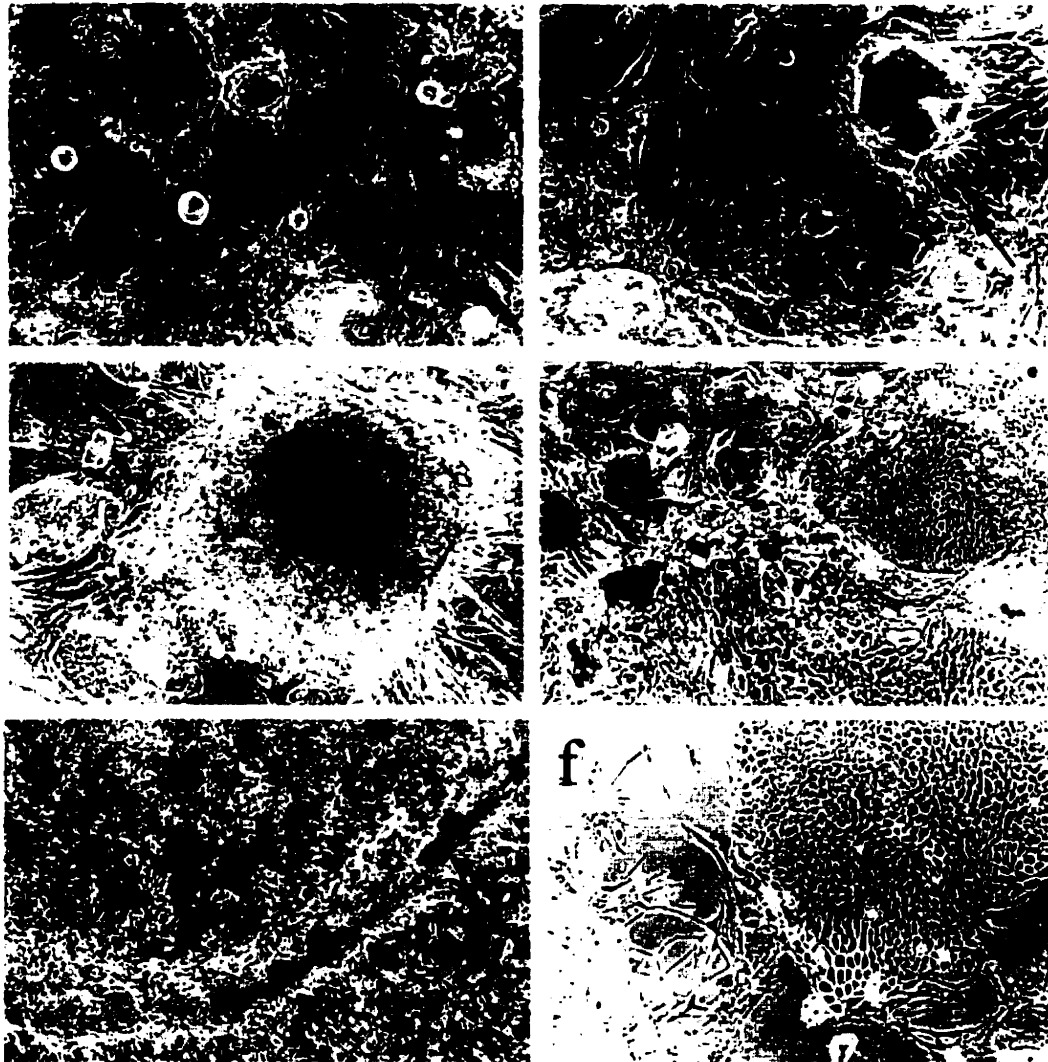


**Figure 1. Selective culture of HMEC.**

**a:** Initial settlement of tumor-like epithelial cell colonies (arrow) over irradiated 3T3 cells (HDQ-P2, P0, day 6, x200), **b:** A tumor-like epithelial colony begins to spread and to grow over the feeder layer (arrow, HDQ-P5, P0, day 18, x200), **c & d:** Examples of morphologies of epithelial colonies over the feeder layer support (HDQ-P1 & HDQ-P3, x100), **e:** Expansion of tumor-like epithelial cells pushing 3T3 cells and forming a distinct border, some cancer epithelial clusters are visible at the interface, showing a good feeder layer support (HDQ-P6, P0, day 26, x40), **f:** Cancer epithelial cell islands with degenerated 3T3 cells (HDQ-P4, P0, day 19, x100).

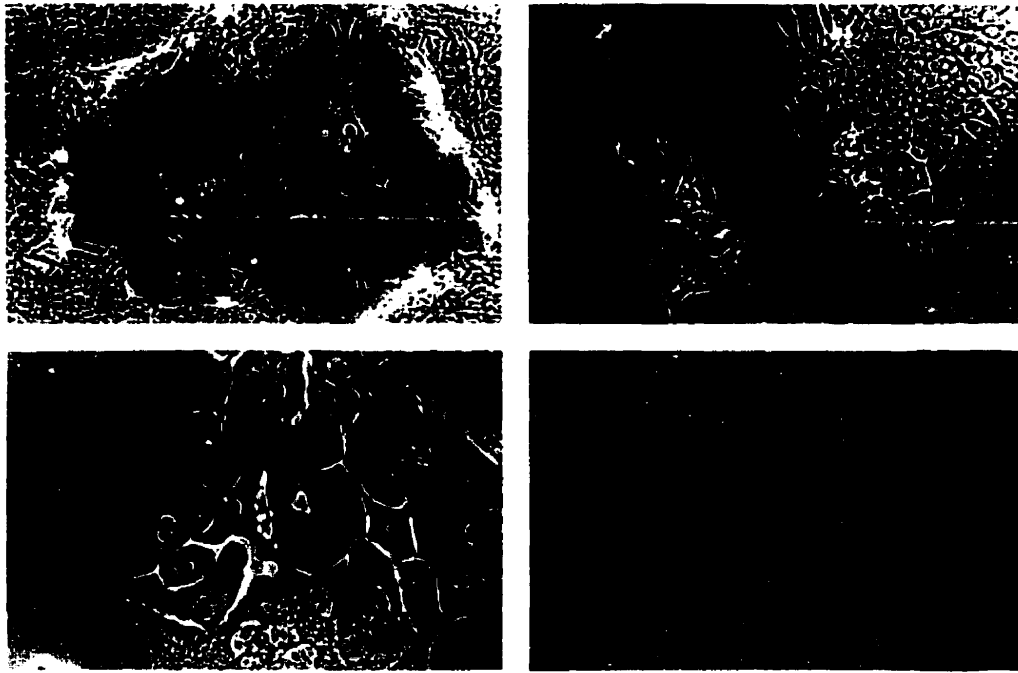


**Figure 2. Selective culture and morphology of HMEC.**

**a: One epithelial cellular colony over prefixed stromal feeder layer cells. No evident active proliferation aspect was observed, b (x200) & c (x400): Cobblestone-like islands composed of polygonal, stellate and cuboidal tumor-derived HMEC, d: Positive expression of EMA in purified HMEC (x200).**

### **CHAPITRE III**

#### **ÉTABLISSEMENT ET CARACTÉRISATION D'UNE NOUVELLE LIGNÉE CELLULAIRE À PARTIR D'UN CARCINOME MAMMAIRE PRIMAIRE**

## **AVANT-PROPOS.**

Ce chapitre présente une nouvelle lignée cancéreuse établie à partir d'un carcinome mammaire primaire. La purification avait été effectuée initialement dans le Laboratoire d'Organogenèse expérimentale, et la culture à long terme et la caractérisation ont été effectuées au Centre de Recherche en Cancérologie. L'analyse karyotypique a été réalisée par une coopération avec l'équipe du Dr. Régen Drouin du Centre de Recherche au Pavillon Saint-François d'Assise. La lignée a été désignée HDQ-P1 signifiant la première lignée établie dans le Service de Pathologie de l'Hôtel-Dieu de Québec. À noter qu'un consentement a été obtenu auprès de la patiente pour utiliser cette tumeur pour fin de recherche. Cet article a été accepté pour publication dans la revue Cancer Genetics and Cytogenetics.

## RÉSUMÉ.

Utilisant la couche nourricière de cellules 3T3 irradiées pour effectuer une culture sélective de cellules épithéliales à partir de carcinomes primaires mammaires, une culture à long terme a été réussie dans un cas parmi six cancers étudiés. La culture à long terme a dépassé 115 passages *in vitro* et la nouvelle lignée est nommée HDQ-P1. La caractérisation incluait une analyse de la morphologie tout au long de la purification, une étude en ultrastructure par microscopie électronique, le karyotype, l'état de mutation de p53 et l'amplification des oncogènes tels que c-myc, c-erbB-2 et c-H-ras. D'autres analyses comprenaient l'expression de la télomérase, le taux des récepteurs hormonaux, la formation de colonies sur culture d'agar et des xénogreffes dans des souris nues/nues. Plus spécifiquement, l'expression de la transcriptase reverse de la télomérase (hTRT : human telomerase reverse transcriptase) confirme que les cellules HDQ-P1 sont immortalisées et le karyotype confirme la malignité de cette lignée cellulaire. De plus, les cellules HDQ-P1 présentent une amplification de c-myc et une mutation de type tronqué de p53 au niveau de l'acide aminé 213 à l'exon 6. Les récepteurs hormonaux sont négatifs pour les œstrogènes et les progestérones. Ces caractères uniques de la lignée HDQ-P1 feront de cette nouvelle lignée mammaire un très bon modèle expérimental pour la recherche sur la biologie du cancer mammaire.

**Establishment and characterization of a new cell line derived from  
human primary breast carcinoma**

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**Running title: new cell line from primary breast cancer cells**

**Summary**

A new cell line, designated HDQ-P1, was successfully established from a primary ductal infiltrating mammary carcinoma by using a 3T3 feeder layer lethally irradiated to 60 Gy. The HDQ-P1 cells have been grown in culture for over 115 passages and have a doubling time of 60 hrs. Characterization of the cell line was performed. This included morphology by light and transmission electron microscopy, karyotype, growth rate, telomerase expression, tumor antigen expression, xenograft implantation into nude mice, colony formation in soft agar, p53 sequencing and gene copy number of c-myc, c-erbB-2 and c-H-ras oncogenes.

The epithelial nature of this cell line was confirmed by ultrastructural analysis, expression of cytokeratins and epithelial membrane antigen. The HDQ-P1 cells possess an extensively rearranged and polyploid karyotype with an average of 20 recurrent marker chromosomes. Scatchard analysis demonstrated that both primary tumor and HDQ-P1 cells were estrogen and progesterone receptor negative. The HDQ-P1 cells have the same expression of human telomerase reverse transcriptase as other established cell lines such as MDA-MB-231, SK-BR-3 and MCF-7. Direct DNA sequencing showed a point mutation that yielded to a stop codon at the amino acid 213 in exon 6 of p53 gene. A 5-fold amplification of c-myc was observed in HDQ-P1 cells. No amplification of c-erbB-2 and c-H-ras genes was observed. This cell line presents unique characteristics and may prove to be a good experimental model to investigate breast cancer biology.

**Keywords:** primary breast cancer, epithelial cancer cell culture

## **Introduction**

Breast cancer is a leading cause of the cancer-related deaths in North America and in Europe. Cell culture established directly from patient tumors is an important model for examining and manipulating potentially relevant molecular and cellular processes underlying malignant breast disease. Hence, well-characterized cancer cell lines are powerful research resources for studying cancer cell biology and for the development of new strategies against cancer. However, selective culture of neoplastic cells and then successful establishment of new cell lines has rarely been reported from primary breast carcinoma (McCallum and Lowther, 1996). A major obstacle may be the slow proliferation rate of cancer cells (von Fournier *et al.*, 1980) as opposed to the fast overgrowth by tumor-associated stromal fibroblasts (Cailleau *et al.*, 1974; Engel *et al.*, 1978b; Soule *et al.*, 1973; Speirs *et al.*, 1998), as well as normal epithelial cells (Dairkee *et al.*, 1995). Although many efforts have been focused on the purification of epithelial cancer cells in selective culture from primary breast carcinomas (Dairkee *et al.*, 1995; Ethier *et al.*, 1993), the majority of breast cancer-derived cell lines have been of metastatic origin (Cailleau *et al.*, 1974; Kurebayashi *et al.*, 1995; Siwek *et al.*, 1998; Smith *et al.*, 1987; Zoli *et al.*, 1997).

By using lethally irradiated but metabolically active 3T3 cells cocultured as feeder layer in the cancer cell selective culture with primary breast cancer, we have recently established a new cell line, designated HDQ-P1, from a primary ductal infiltrating breast carcinoma. The present report describes the establishment and characterization of this new breast cancer cell line.

## **Materials and methods**

### **Clinical findings**

HDQ-P1 cell line was obtained from a primary breast carcinoma of a 50-year-old woman who underwent radical mastectomy in November 1995 for a G3, estrogen (ER) and progesterone (PgR) receptor negative, infiltrating ductal carcinoma of the breast. Metastatic involvement was present in 6 of 16 examined axillary lymph nodes. No distant metastase was detected. The patient was still alive without recurrence 3 years after the diagnosis.

### **Specimen dissociation**

Fresh sterile tissue of the primary breast tumor was obtained immediately after surgical resection (tumorectomy). Tumor diagnosis was confirmed by routine histopathological examination. The tissue specimen was then trimmed and placed in Dulbecco modified Eagle's medium (DMEM) (Gibco-BRL, Burlington, Canada), with antibiotics at 4°C. Then, the tissue specimen was minced with crossed surgical blades into about 1 mm cubes and then digested with collagenase (0.14%) at 37°C overnight with shaking. The single-cell suspension was obtained and the cell viability was determined by the trypan blue exclusion technique.

### **Cell culture**

After centrifugation at 250 x g for 10 min, the cell pellet was resuspended in culture medium. The final cell suspension was seeded at a concentration of  $2 \times 10^5$  cells/75 cm<sup>2</sup> culture flask (Falcon, Becton Dickinson Inc, Franklin Lakes, NJ) previously covered with confluent and lethally irradiated (60 Gy) 3T3 cells (ATCC) at confluence. These 3T3 cells formed a cellular layer as substrata in the flasks and were used no more than one week after the irradiation. The culture medium consisted of a combination of DMEM with Ham's F12 in a 3:1 proportion (Gibco-BRL), supplemented with 10 ng/ml human epidermal growth factor (EGF, Chiron Corp., Emeryville, CA), 5 ng/ml 17- $\beta$ -estradiol, 500 ng/ml progesterone, 24.3  $\mu$ g/ml adenine, 5  $\mu$ g/ml crystallized bovine insulin, 5  $\mu$ g/ml human transferrin,  $2 \times 10^{-9}$  M 3,3',5',-triiodo-L-thyronine (Sigma Chemicals, St Louis, MO, USA), 0.4  $\mu$ g/ml hydrocortisone (Calbiochem, La Jolla, CA, USA),  $10^{-10}$  M cholera toxin (Schwarz/Mann, Cleveland, OH, USA), 10% fetal calf serum (FCS), 100 IU/ml penicillin G and 25  $\mu$ g/ml gentamicin. A few coverslips were placed in the culture flasks in order to carry out immunocytochemical assays for the identification of cell characteristics. All dissociated material was designated as passage 0 upon primary seeding in culture. Flasks were examined daily during the first week. All cultures were kept in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

Culture medium was first changed at the time of cell attachment and spreading was observed. Then, the culture medium was changed three times a week.

**Maintenance of cell culture**

Tumor-like cell colonies firmly attached to the flasks but contaminated with fibroblasts were passed again over lethally and freshly irradiated confluent 3T3 cells until the tumor-like epithelial cells were purified. In absence of fibroblast contamination, if epithelial-like cells were growing slowly due to significant degeneration of 3T3 cells, subcultures were performed under the same conditions. Subcultures were made by exposing the culture to a 0.05% trypsin (1:300)-0.02% EDTA solution. Purified epithelial-like cells free of fibroblasts were subcultured regularly in absence of irradiated 3T3 cell feeder layer.

In order to test the dependence of cancer cells on hormones and some growth factors, cells were cultured not only with the above highly supplied growth medium, but also simply with DMEM plus 10% FCS, 100 IU/ml penicillin G and 25 µg/ml gentamicin.

**Source of other cells**

The normal skin fibroblasts (NSF) were obtained from reduction mammoplasties. The fibroblast line F9846 was purified from primary breast cancers. Some other malignant cells used in the study were MDA-MB-231, SK-BR-3, MCF-7 and BT-20 (ATCC). All cells were grown in DMEM with penicillin, streptomycin and 10% FCS in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

**Morphological examination**

All tumor-like epithelial cells grown in the flasks were directly examined with an inverted microscope and phase-contrast microphotographs were taken periodically.

Ultrastructure of cells was performed by transmission electron microscopy. The cell pellets were fixed in 3% glutaraldehyde and postfixed in osmium tetroxide. Thin sections were counterstained with uranyl acetate and lead citrate. The sections were examined with a JEM 1200EX electron microscope (JEOL Industries, Tokyo, Japan).

**Immunocytochemistry analyses**

Tumor-like cells grown on coverslips were washed in phosphate-buffered saline (PBS) for 5

minutes, fixed for 15 min at -20°C with cold methanol, then quenched for 5 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. After washing with PBS, slides were covered with normal horse serum during 20 minutes and incubated overnight with each monoclonal antibody at 4°C. Afterwards, slides were rinsed with PBS and exposed for 30 minutes to a secondary biotinylated anti-mouse antibody. After another wash with PBS, slides were incubated with the avidin-biotin-peroxidase complex (Vector laboratory, Burlingame, CA) and the reaction was revealed with diaminobenzidine (DAB). The coverslips and slides were finally counterstained with Mayer's haematoxylin for 3 minutes and mounted in permount.

Monoclonal antibodies used for epithelial markers were the anti-epithelial membrane antigen (EMA) (Dako, Mississauga, Ontario, Canada) and anti-human cytokeratins including CAM 5.2 (Becton Dickinson, Mississauga, Ontario) detecting cytokeratins 8 and 18; 34βE12 (Dako) detecting cytokeratins 1, 5, 10 and 14; anti-cytokeratin 7 (Dako) and anti-cytokeratin 20 (Dako). Other antibodies used were anti-vimentin (Dako), anti-human α-smooth muscle actin (Dako). Besides immunoreaction with anti-EMA and anti-human cytokeratin monoclonal antibodies, the cell line HDQ-P1 has been tested with anti-cathepsin D (Ab-1, Oncogene Research Products, Cambridge, MA), anti-p53 (PAb 1801, Oncogene), anti-HER-2 (CD11, Signet Labs, Dedham, Mass) and anti-human carcinoembryonic antigen (CEA) (Dako).

### **Growth kinetics**

The doubling time of the HDQ-P1 cell population at passage 9 and passage 88 *in vitro* was determined by counting the cell number at regular intervals. On day 0, 10<sup>5</sup> viable tumor-like cells were seeded in triplicate in each of 6-well plates (Nunc, Naperville, IL) with different culture medium. Cells were counted in triplicate with a haemocytometer. The growth curve was plotted and the doubling time was calculated from regression equation of the curve.

### **Estrogen and progesterone receptors**

A cytosolic extract was obtained from cell pellet ( $3 \times 10^7$ ) of HDQ-P1 cells at passage 11 cultured with the medium containing estradiol and progesterone and also at passage 75 cultured without neither 17-β-estradiol nor progesterone. Cytosolic levels of estrogen and

progesterone receptors (ER, PgR) were measured by column chromatography on hydroxylapatite as reported (Bélanger *et al.*, 1985). Results were reported in femtomoles per mg of protein and were defined as positive if there was  $> 10$  femtomoles  $\text{mg}^{-1}$  of protein. Positive and negative cytosolic materials were analyzed at the same time for quality control. The results were compared with those obtained with original biopsy specimens.

#### **MUC1 analysis by Western blot**

The cells in culture were washed twice with ice-cold PBS, and the proteins were extracted with lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecylsulfate and 50 mM Tris pH 8.0) for 1 h at 4 °C. The supernatants were centrifuged at  $10,000 \times g$  for 30 min at 4 °C and the quantity of the protein was measured using DC Protein Assay kit (Bio-Rad, Mississauga, Ontario). Twenty-five  $\mu\text{g}$  of each protein extract were heated with  $\beta$ -mercaptoethanol, separated by electrophoresis in a 7.5% polyacrylamide gel and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec). The membrane was blocked with 2% BSA for 1 h, incubated at room temperature for 2 hrs in Tris-buffered saline (TBS) containing anti-EMA mAb (2  $\mu\text{g}/\text{ml}$ , Dako), washed and finally incubated with peroxidase-conjugated goat anti-mouse polyclonal antibody (Jackson Immunoresearch, West Grove, Pennsylvania). After washes, antibody binding was detected by chemiluminescence (Renaissance<sup>R</sup>, NEN Life Science Products, Boston, MA). After that, the membrane was deprobed and reblocked as above. The membrane was then treated overnight at 37 °C with 10 mU/ml V. cholerae sialidase (Boehringer Mannheim, Laval, Quebec) in 50 mM sodium acetate pH 5.5, 4 mM calcium chloride and 1 mg/ml BSA, and probed with anti-EMA as previously.

#### **Conventional cytogenetics**

Cells from passage 8, 24, 32 and 60 at the exponential phase of growth were arrested by adding 0.04  $\mu\text{g}/\text{ml}$  colcemid for 2 to 4 hours and incubated at 37°C. The cells were detached with trypsin (0.25%) and pelleted by centrifugation at 2000 rpm for 7 minutes. Hypotonic treatment was performed in 0.075 M KCl at 37°C for 15 min. The cells were then fixed three times in freshly prepared ice-cold fixative (3 part methanol/1 part glacial acetic acid) for 15

min each (Drouin *et al.*, 1988). A few drops of the cells re-suspended in fresh fixative were dropped onto glass slides in a humidity and temperature controlled chamber (Thermotron Industries, Holland, Michigan) at 36% humidity and temperature of 25°C. The slides were allowed to air dry for about 2 to 3 days before performing GTG-banding (G-band using trypsin and Giemsa staining) (Drouin *et al.*, 1988). GTG banding was produced by treating the slides with 1% fresh trypsin in NaCl 0.9% for 20 to 60 seconds and staining with 4% Giemsa (Harleco) for 5 minutes (Drouin *et al.*, 1988). Chromosomes of a total of 289 mitoses were counted after GTG-banding and 6 metaphases were karyotyped.

### **Molecular cytogenetics**

The following probes were used: Digoxigenin-labeled coatasome 1, 6, 7, 16, 18 and 19 total chromosome probes (Oncor, Gaithersburg, MD) and biotinylated p53 DNA probe (Oncor). The slides were soaked in 2 x SSC (0.15 M NaCl, 0.015 M sodium citrate) pH 7 at 37°C for 1 hour and dehydrated in a 70%/80%/100% ethanol series. Just prior to hybridization the chromosome preparation was denatured in 70% (vol/vol) formamide/2 x SSC pH 7, for 90 sec at 70°C and then dehydrated at 4°C. The hybridization mixture (total volume of 10 µl containing the specific probe) was then denatured at 70°C for 10 min. Following denaturation, the coatasome probes were incubated for 2 hours at 37°C and then added on the slides. A cover slip was then placed and the slides incubated overnight (16 hours) in a humidified chamber at 37°C. They were then washed at 43°C as follows: once using 50% formamide in 2 x SSC for 10 min and once in 2 x SSC for 10 min. Finally, they were washed twice in PBD (Oncor, Cat.#S1370-7) for 5 min at room temperature.

Sites of hybridization were visualized by indirect immunofluorescence. First, 100 µl of a 1:50 dilution of mouse monoclonal antidigoxigenin antibody (Boehringer Mannheim) for coatasome detection and of rabbit antibiotin antibody (Enzo diagnostic Inc., New York, NY) for p53 detection were added onto the slides, followed by incubation in a humidified chamber at 37°C for 45 min; slides were washed twice for 5 min in PBD at room temperature. A second incubation was carried out for 30 min at 37°C with 100 µl of a 1:50 dilution of digoxigeninylated sheep antimouse IgG (Boehringer Mannheim) and of biotinylated goat antirabbit IgG (Vector Laboratories), followed by two washes. Slides were then incubated for

30 min at 37°C with 100 µl of a 1:100 dilution of sheep anti-digoxigenin-rhodamine Fab fragments (Boehringer Mannheim) and of streptavidine-FITC (Fluorescein Isothiocyanate conjugate, Gibco-BRL), followed by two washes. The slides were soaked in PBS for 1 min and then stained with DAPI II (Vysis, 125 ng/ml) for 5 min at room temperature. All the pictures were taken from an Olympus BX60 with an image analysis system using a black & white digital camera (IMAC-CCD 930) coupled with the Metasystems *in situ* imaging system (ISIS 2) software version 2.5. Separate filter sets were used to view hybridization signals or chromosomes, and the images were merged using the image analysis system.

### **Analysis of DNA ploidy and cell cycle**

Cells were obtained from cell suspensions. They were pelleted by centrifugation, resuspended in 1 ml of 0.1% solution of sodium citrate containing 0.02 mg/ml RNase, 0.3% NP-40, 0.05 mg/ml propidium iodide and incubated for 30 min in the dark at 4°C. Human tonsil tissue stained under the same conditions was used as an external diploid standard. DNA analysis was performed on an EPICS ELITE ESP flow cytometer (Coulter). Up to 10,000 HDQ-P1 cells were measured for their DNA content by their relative PI fluorescence. The emitted fluorescence was processed with multiparameter data acquisition and display system to give a single-parameter histogram. In a normal sample, a diploid peak appears on the histogram at the same channel as with the standard tonsil peak. Any aneuploid cell population would appear as displaced either to the right (hyperdiploid) or to the left (hypodiploid) of the standard diploid peak (G0/G1 cells) channel. The DNA index (DI) is determined by dividing the G0/G1 peak channel of the sample by the G0/G1 peak channel of the tonsil diploid cells.

### **Anchorage independent growth**

The ability of anchorage independent growth of the purified cancer epithelial cells was determined by the formation of colonies in soft agar at passage 10 and at passage 20. Single cell suspension containing  $10^5$  cells/35-mm petri dish was dispersed in a solution containing 0.3% bacto-agar (Difco Laboratories, Detroit, Michigan) in epithelial culture medium described above. This was layered over a 0.6% bacto-agar solution in DMEM-Ham's F12 (3:1) supplemented with 10% FCS and antibiotics. Dishes were incubated at 37°C and fed biweekly with epithelial cell culture medium. Formation of colonies was determined by

inverted microscopy after 2 and 4 weeks post-seeding.

### **Tumorigenicity in nude mice**

Tumor-like cells were injected with  $3.5 \times 10^6$  and  $1 \times 10^7$  cells in 0.2 ml culture medium into 6 to 7 week-old CD1-nu/nu female nude mice (Charles River Canada Inc., St-Constant, Quebec) at passages 11, 16, 50 and 100. Each nude mouse was subcutaneously injected in flanks. Mice were observed weekly and were kept for two months.

### **RT-PCR analysis of human telomerase reverse transcriptase (hTERT)**

The isolation of RNA was performed according to Chomczynski and Sacchi (1987). RT-PCR was performed as described previously with some modification (Wang *et al.*, 1997). Briefly, 1 µg of total RNA purified from HDQ-P1 cell lysates was used in the reaction of RT. PCR amplification was carried out in 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTP, 1.5 µM of each primer containing 1 unit of Taq DNA polymerase (Gibco-BRL) and ¼ of the reverse transcription mix in a final volume of 25 µl. hTERT mRNA was amplified using the primer pair 5'-CGGAAGAGTGTCTGGAGCAA-3' (sense strand) and 5'-GGATGAAGCGGAGTCTGGA-3' (antisense strand) which yields a 125-bp product. Following an initial denaturation at 94°C for 5 minutes, the reaction conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, with a final extension time of 10 minutes at 72°C. PCR was carried out in a GeneAmp PCR system 9600 (Perkin-Elmer/Cetus, Norwalk, USA). PCR products were separated on 6% polyacrylamide gels and stained with ethidium bromide. Concomitant amplification, but in the separate tubes, of β-actin was used as internal control with the primers 5'-CGTGGACATCCGCAAAGAC-3' and 5'-CTCGGCCACATTGTGAACT-3' (Johnson *et al.*, 1996). PCR using these primers yields a 484-bp product. The RNA from cell lines MDA-MB-231, SK-BR-3 and MCF-7 was used as positive control and the RNA from normal human mammary fibroblasts as negative control.

### **DNA sequencing for analysis of mutation in p53**

High molecular genomic DNA from HDQ-P1 cells was obtained as cited above. The genomic DNA was amplified by two consecutive rounds of PCR using the p53 specific primers. First

round of amplification was carried out with 100 ng genomic DNA in a 100 µl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 50 pmol of each primer and 2.5 units of Taq DNA polymerase (Gibco-BRL). After 94°C for 5 min, the reaction mixtures were subjected to 40 cycles of amplification in GeneAmp PCR system 9600 (Perkin-Elmer Cetus), where each cycle consisted of 94°C for 30s, 55°C for 30s and 72°C for 60s. The last cycle was followed by a final elongation at 72°C for 10 min. Negative controls containing no template DNA were included. For the second round of amplification, 1 µl of the first PCR reaction was used and amplification carried out using 10 pmol of primers. Reaction conditions were same as above. After verification by electrophoresis in the 6% polyacrylamide gel, the amplified DNA was then purified with Qiaquick PCR purification kit (Qiagen, Mississauga, Ontario) and sequenced using specific primers on an automatic sequencer (ABI 373A Stretch). A duplicate preparation of genomic DNA from HDQ-P1 cells was used for duplicate PCR and sequencing analysis.

Following primer sets were chosen based on the gene sequence (Buchman *et al.*, 1988) and used for the amplification of p53 gene: Set I for the first round amplification of exon 5-6: 5'-AGGAGGTGCTTACACATGTTTGT-3' (sense: 12951-12973) and 5'-GGTTATAGGGAGGTCAAATAAGC-3' (antisense: 13546-13524) resulting a fragment of 596 bp; Set II for the second round amplification of exon 5-6: 5'-TCACTTGTGCCCTGACTTTCAACT-3' (sense: 13008-13031) and 5'-AGGTCAAATAAGCAGCAGGAGAA-3' (antisense: 13536-13514) resulting a fragment of 592 bp; Set III: 5'-GCGCACTGGCCTCATCTTGG-3' (sense: 13692-13981) and 5'-GTGCTAGGAAAGAGGCAAGGAA-3' (antisense: 14683-14662) for first and also second round amplifications of exon 7-8 resulting a fragment of 722 bp. Primers used for the sequencing of exon 5-6 were 5'-TCACTTGTGCCCTGACTTTCAACT-3' (sense: 13008-13031) and AGGTCAAATAAGCAGCAGGAGAA (antisense: 13536-13514). Primers used for sequencing of exon 7-8 were respectively 5'-GTCCTGCTTGCTTACCTCGCTT-3' (antisense: 14603-14582), 5'-GTGCTAGGAAAGAGGCAAGGAA-3' (antisense: 14683-14662) and 5'-GCGCACTGGCCTCATCTTGG-3' (sense: 13962-13981).

### **Gene copy number**

High molecular weight DNA was extracted from cellular pellet using digestion buffer (0.1 mg/ml proteinase K, 100mM NaCl, 10 mM Tris-HCl pH 8, 25 mM EDTA pH 8 and 0.5% sodium dodecylsulfate). Genomic DNA was digested respectively with EcoRI for c-erbB-2, SacI for c-myc and BamHI for c-H-ras. 10 µg DNA of the digested preparations were fractionated by electrophoresis in a 0.8% agarose gel, transferred to nylon Hybon-N membranes (Amersham Pharmacia Biotech), and cross-linked by UV irradiation. Prehybridization and hybridization were carried out at 68 °C, 5x SSC, 5x Denhardt' solution and 100 µg/ml of sheared salmon sperm DNA. Hybridization was done with the probes labeled by random priming to a specific activity of at least  $10^8$  cpm/µg. After washing to a final stringency of 0.1x SSC at 68 °C, the membranes were placed on a phosphor screen, and then analyzed by ImageQuANT (Molecular Dynamics, Sunnyvale, CA). The membranes hybridized with c-erbB-2, c-H-ras and c-myc probes were then deprobed and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The oncogene amplification of each sample was represented by the ratio of the oncogene signals over the GAPDH signal. The degree of oncogene amplification in HDQ-P1 cells was calculated by comparison with the ratio in NSF.

The DNA probes used were: c-erbB-2, a fragment of 2055 bp corresponding to human gene c-erbB-2 subcloned into SmaI pBSKS, and c-myc, a fragment of 207 bp subcloned into SmaI pBSRS corresponding to 4957-5163 human c-myc gene sequence (Saito *et al.*, 1983) (gift of Dr. Claude Labrie, CHUL, Laval University, Quebec, Canada). The DNA probe for c-H-ras was a 6 kb fragment homologous to the human c-H-ras oncogene (Pulciani *et al.*, 1982) (gift of Anna Gasperi-Campani, Morgagni-Pierantoni Hospital, Italy). Hybridization with a probe corresponding to human GAPDH (697 bp corresponding to 4205-4623) (Tokunaga *et al.*, 1987) was used as an internal standard to determine the amount of DNA in each sample.

### **Results**

#### **Settlement and purification of tumor-like cells**

In the flasks containing heterogeneous disaggregated cells (cancer and stromal cells) seeded over irradiated 3T3 cell layer with highly supplemented culture medium, epithelial cells

floated as small spheroid clusters during the first days and adhered later to the 3T3 cell layer. Tumor-like epithelial colonies were then observed, pushing away the surrounding 3T3 cells and then began to grow as expanding colonies on the flask surface (Fig. 1a). The first change of culture medium was performed when a few epithelial colonies were clearly growing between irradiated 3T3 cells, this took about 5 days after seeding. Fibroblast attachment was also observed over irradiated 3T3 cells but the density was minimal and never formed spheroid clusters, neither colonies. Using irradiated 3T3 cells as feeder layer, a pure culture of cancer epithelial cells free of fibroblasts was obtained in passage 5 for HDQ-P1 cells (Fig. 1b).

### **Morphological examination**

Morphological examination demonstrated a typical epithelial morphology for those purified tumor-like epithelial cells. The tumor-like epithelial colonies were made of polygonal cells (Fig. 1b, 1c & 1e). The nuclei were large, pleomorphic and lobulated with prominent nucleoli. Abnormal mitotic figures and multinucleate cells were common. The nuclear-cytoplasmic ratio was high. At the ultrastructure level, HDQ-P1 cells were polygonal and attached to each other with tight junctions and desmosomes. Abundant microvilli were present at the apical border. Moreover, intercellular lumina with protruding microvilli and bundles of tonofilaments were also observed (Fig. 2).

### **Immunocytochemistry**

HDQ-P1 cells showed a strong and diffusely positive immunoreaction with anti-EMA, anti-cytokeratin CAM 5.2 (Fig. 1f), anti-cytokeratin 7 and anti-keratin 34 $\beta$ E12. However, the immunoreaction was negative with anticytokeratin 20 and anti- $\alpha$ -actin. Anti-vimentin reaction was weakly and focally positive. A few cells expressed cathepsin D. There was a lack of p53 accumulation and of c-erbB-2 overexpression. Similar findings were observed on the tissue section of the tumor.

### **Cell growth *in vitro* and Growth kinetics**

In the earlier stage of culture, the cells proliferated as adherent patches in the culture flask (Fig. 1b). In long term culture, HDQ-P1 cells proliferate in monolayer (Fig. 1c) and can reach complete confluence. The effect of different nutrients and growth factors added to the culture

medium was particularly evident in the early stage cultures of HDQ-P1 cells. HDQ-P1 cells did not proliferate well in DMEM supplemented with 10% FCS only. With highly growth factor-enriched medium, HDQ-P1 cell proliferation was improved. The growth curves are showed in Figure 3. The doubling time of HDQ-P1 cells *in vitro* at passage 9 was 121 hours (~5 days) for the culture with enriched medium. Recent cultures showed that HDQ-P1 cells grow well with DMEM supplemented only by 10% FCS and insulin (5 µg/ml), without further need of the highly enriched medium described above. Figure 3b shows the growth rate of HDQ-P1 at passage 88, the doubling time reached 60 hrs.

### **Estrogen and progesterone receptors**

Analysis by column chromatography on hydroxylapatite showed cytosolic level of estrogen and progesterone receptors of 45 fm/mg and 18 fm/mg respectively for HDQ-P1 cells at passage 11 when the cells were cultured with 17-β-estradiol and progesterone. Those receptor levels fell to 1 fm/mg for estrogen receptor and 7 fm/mg for progesterone receptor level without further 17-β-estradiol and progesterone in culture medium. The same method was used to analyze the estrogen and progesterone receptor level in the original tumor and the estrogen receptor level was 9 fm/mg and progesterone receptor level was 3 fm/mg.

### **Western blot Analysis**

Western analysis indicates the presence of MUC1 in the HDQ-P1 cells, as detected with the anti-EMA mAb. Treating the membrane with *Vibrio cholerae* sialidase resulted in an increase in intensity of the anti-EMA signal of HDQ-P1 and BT-20, whereas the signal was barely affected in the other samples (Fig. 4).

### **Cytogenetics**

Chromosome counting of 289 metaphases from passage 8, 24, 32 and 60 revealed that 100% of epithelial cancer cells had a polyploid karyotype. The distribution of chromosome number illustrated in Figure 5 shows, for each passage, a peak in the hypotriploid range and a second peak in the hyperpentaploid range.

Cytogenetic analysis of HDQ-P1 cells showed an extensively rearranged karyotype. In

particular, on the average 20 (varying from 14 to 29) recurrent marker chromosomes (unidentified origin) were observed in different metaphases. Figure 6 illustrates a representative hyperpentaploid karyotype of an HDQ-P1 cell from passage 60. In general, chromosome 6 and chromosome 10 were found in three copies in hypotriploid cells and in five copies in hyperpentaploid cells. Also, chromosomes X, 13, 16, 18 and 20 were the most frequently underrepresented chromosomes in hypotriploid cells (0 to 1 copy of these chromosomes), as well as in hyperpentaploid cells (1 to 2 copies of these chromosomes). The chromosomes most frequently involved in structural rearrangements were chromosomes 1, 7 and 19. Translocations involving chromosome 1 in breast cancer cell line have been observed by others (Meltzer *et al.*, 1991).

These results were further investigated by FISH (fluorescence *in situ* hybridization). Chromosome specific composite DNA probes (coatasome probes) were used to specifically paint chromosomes. Parts of metaphases showing chromosome painting using different coatasome probes are illustrated in Figure 7. Interestingly, we found that chromosomes 16 and 18, frequently underrepresented in their normal copies, were involved in multiple chromosomal rearrangements (insertion, translocation, etc...) leading to marker chromosomes. This can be visualized in figure 7d and 7e where hybridization signals partially paint several chromosomes. Also, we confirmed the highly rearranged states of chromosomes 1, 7 and 19 (Fig. 7a, c and f). Hybridization of coatasome 6 shows that chromosome 6 is present in three or five normal copies (Fig. 7b). Finally, FISH studies revealed the presence of 6 to 9 copies of p53 gene in most of the HDQ-P1 cells (green spots in Figure 7a).

### **DNA analysis**

DNA analysis of the HDQ-P1 cells revealed a complete aneuploid cell population (DNA index=1,39). DNA ploidy performed on the original tumor showed a DNA index of 1,58. This is consistent with the cytogenetic findings.

### **Anchorage independence growth**

HDQ-P1 cells were capable of growing in soft agar culture with more than 50 cells per colony. Representative results are shown in the Figure 1d.

### **Tumorigenicity in nude mice**

Sub-cutaneous injections of up to  $1 \times 10^7$  viable HDQ-P1 cells of four different passages into nude mice have not resulted in tumor development after 8 weeks. Although tumor-like formations were observed, these pseudo-tumoral formations disappeared with time.

### **hTERT mRNA expression**

RT-PCR revealed that HDQ-P1 cells expressed hTERT mRNA, as other established breast cancer cell lines such as MDA-MB-231, SK-BR-3 and MCF-7 (Fig. 8), while no expression of hTERT was found in normal skin and F9846 fibroblasts.

### **P53 sequencing**

DNA sequencing of p53 exons 5-8 was carried out and one point mutation was identified in exon 6 at codon 213 as a stop codon (CGA → TGA). The result was confirmed by the sequencing from both sense and antisense and by duplicate sequencing with a duplicate genomic DNA preparation.

### **Gene copy number**

Genomic DNAs extracted from HDQ-P1 cells was digested with either EcoRI, SacI or BamHI, and hybridized by the Southern blotting technique with different probes homologous to cellular protooncogenes c-myc, c-erbB-2 and c-H-ras. The copy number of c-myc in HDQ-P1 cells was increased ~5 fold compared to that in NSF. No significant increase in gene number was observed for c-erbB-2 and c-H-ras (Fig. 9). After the digestion with BamHI, a rearrangement of c-H-ras was observed in HDQ-P1 cells (Fig. 9).

### **Discussion**

Cell lines from primary breast carcinomas are among the most difficult to establish in tumor tissue culture (McCallum and Lowther, 1996). Several methods have been designed to obtain long term culture. The use of stromal cells as feeder layers to culture human mammary epithelial cells was first reported by Taylor-Papadimitriou *et al.* (Taylor-Papadimitriou *et al.*, 1977). In their study, mitomycin-treated mouse fibroblasts have been shown to enhance initial

attachment and the growth of mammary epithelial cells in human milk. Later, Smith *et al.* have shown that ultraviolet-irradiated fibroblasts yielded higher plating efficiency for breast epithelial clones (Smith *et al.*, 1981). Irradiated fibroblasts were also found to provide good stromal support. Lethally irradiated transformed fibroblasts could shorten the latency period and increase the rate of human tumor growth *in vivo* (Camps *et al.*, 1990) and irradiated marrow stromal cells could stimulate the growth of human tumor cells, including mammary carcinoma (Strobel *et al.*, 1989). In 1975, Rheinwald and Green (Rheinwald and Green, 1975) found that lethally irradiated 3T3 cells could not only support serial proliferation of keratinocyte lines of teratomatous origin and human epidermal cells, but could also control fibroblast proliferation. The use of irradiated 3T3 cells as feeder layer for cell lines established from primary breast cancer has never been reported. By seeding an enzyme-digested cell suspension over lethally irradiated 3T3 cells, we have isolated epithelial cancer cells from six primary breast cancers (data not shown) and obtained long term culture of one of them designated as HDQ-P1. Although lethally irradiated to 60 Gy, these 3T3 cells retained their metabolism, and maintained some biological activities such as contact inhibitory effect over tumor-derived fibroblast overgrowth (Rheinwald and Green, 1975) and the potential to accelerate human tumor cell growth, as observed with keratinocyte cultures (Green *et al.*, 1979; Rheinwald and Green, 1975).

The cell line, HDQ-P1, has been cultured up to passage 115 and stored frozen. The epithelial phenotype of HDQ-P1 cells was checked regularly and has been confirmed because of its morphological appearance in monolayer culture, its ultrastructural features and the expression of epithelial markers. The ultrastructural features generally accepted as specific traits of epithelial cells (Buehring and Hackett, 1974) were found in HDQ-P1 cells, such as tight junctions, desmosomes and abundant microvilli on the cell border. The immunocytochemical analysis confirmed also the epithelial phenotype such as the positive immunocytochemical reaction with anti-human keratins 8 and 18, and with anti-human EMA, as well as the absence of  $\alpha$ -actin. High expression of type II neutral-to-basic human cytokeratins 7 and 8 in HDQ-P1 cells is typical for cells of ductal epithelial origin. Indeed, the expression of cytokeratin 7 is restricted to ductal and certain glandular epithelia (Moll, 1994). We found that HDQ-P1 cells present weak and focal vimentin staining, a factor associated with higher grade malignancy

(Domagala *et al.*, 1990).

The elements confirming the neoplastic nature of HDQ-P1 cell line were its karyotypic abnormalities, positivity for cytokeratins 8 and 18 and gene abnormalities. The clonal growth in semisolid agar media and cancer cell morphology support also the neoplastic aspect. However, no tumor could grow in nude mice. It is well known that breast cancer cell lines subcutaneously injected into the flank in nude mice tend to be less tumorigenic and less metastatic than other cell lines derived from pulmonary, renal and colon carcinomas (Price *et al.*, 1990). In fact, a lack of tumorigenicity in nude mice has been reported in a few other cell lines, such as the Hs578T line (Hackett *et al.*, 1977). Certain factors such as a need for high level of hormonal stimulation influence the tumor formation in nude mice. Indeed, HDQ-P1 cells were cultured with 17- $\beta$ -estradiol and progesterone. Another possibility is that the tumorigenic potential of the cell line HDQ-P1 is marginal, which could be tested by inoculation *in vivo* with fibroblasts (Camps *et al.*, 1990; Noel *et al.*, 1998) or with other support material such as Matrigel (Noel *et al.*, 1992).

The HDQ-P1 cells displayed an aneuploid karyotype and a high modal chromosome number. This confirms the neoplastic nature of HDQ-P1 cell line. In fact, aneuploidy is frequently observed in breast cancers, but not in normal or reactive mesothelial cells, and has been reported to be a marker of poor prognosis (Toikkanen *et al.*, 1989). Little is known about the cytogenetic features of breast cancer. No characteristic abnormalities have been reported, but other investigators showed that the chromosome 6 was the most frequently gained chromosome (McCallum and Lowther, 1996) and that chromosome 1 was the most frequently involved in structural rearrangements (Amadori *et al.*, 1993; McCallum and Lowther, 1996). Among the possible reasons for such limited data are technical difficulties and frequent evidence of a large number of rearrangements. The cytogenetic analysis of HDQ-P1 cells showed a stable but extensively rearranged hyperploid karyotype.

The chromosome abnormalities, not only confirm the malignant state of the cell line, but also are likely to be similar to the aberrations found in the breast carcinoma from which the cell line HDQ-P1 was derived. Indeed, the same karyotype abnormalities remained from passage 8

to 60. Since the initial chromosome aberrations are likely conserved during the culture of the epithelial cancer cells, this procedure to produce cell lines from breast carcinoma might be very helpful to identify chromosome abnormalities in breast carcinoma.

Activation and stabilization of telomerases are required for cellular immortality and oncogenesis (Greider, 1998). Of the three subunits forming the telomerase complex (hTR, TP1 and hTERT), the expression of hTERT but not that of hTR or that of TP1 was significantly correlated with telomerase activity (Takakura *et al.*, 1998). Expression of hTERT is observed at higher levels in cancer cell lines and malignant tumor tissues (Nakayama *et al.*, 1998; Takakura *et al.*, 1998). The level of expression of hTERT mRNA in HDQ-P1 cells was comparable to other established breast cancer cell lines such as MDA-MB-231, SK-BR-3 and MCF-7, confirming that HDQ-P1 cells are immortalized. This contrasts with normal and tumor-associated fibroblasts, which do not express hTERT mRNA.

At passage 9, the doubling time of HDQ-P1 cells with rich medium was approximately 5 days. This is in keeping with the observation of Petersen and Deurs (Petersen and Van Deurs, 1987) that the breast cancer cells are among the most slowly growing populations even in highly growth factor-enriched medium. However, in a later culture (passage 88), HDQ-P1 cells had an accelerated proliferation rate with a doubling time of 60 hours in DMEM only supplemented with insulin and FCS. This is typical of an established cancer cell line that becomes independent from most other nutrients and growth factors. This is in agreement with other reports on cancer cell culture from primary breast carcinomas (Cailleau *et al.*, 1974; Soule *et al.*, 1973) which showed that cancer cells grow slowly in early passages and acquire a fast growth rate in later passages.

Mutations of the p53 tumor suppressor gene are present in 15-50% of breast carcinomas (Elledge and Allred, 1994). Expression of high levels of wild type p53 arrests the cell cycle in G1 phase in response to DNA damage, in order to allow DNA repair. Loss of this gene function allows the propagation of cells with genetic damage. Point mutation is a common mechanism of p53 gene alteration in human cancers. Up to 87% (Greenblatt *et al.*, 1994) of mutations occur in exons 5-8 covering amino acids 126-306, and the site of the mutations

observed may have important consequences on the biological activity of the protein (Halevy *et al.*, 1990; Kern *et al.*, 1991). The p53 protein and gene have been analyzed in HDQ-P1 cells respectively by immunocytochemistry and by DNA sequencing. One point mutation of the p53 gene has been found in exon 6, at amino acid position 213, resulting in a stop codon (CGA→TGA). In breast cancer, mutation of p53 in the codon 213 results either truncation (stop codon) or polymorphism, but the truncation (CGA-TGA) is more common in this site (Hainaut *et al.*, 1998). The p53 point mutations have been reported in about 50% of established breast cancer cell lines, and the mutation sites covered exons 5-8, at amino acids 132, 134, 157, 175, 194, 234, 249, 273, 280, 285 (Bartek *et al.*, 1990; Runnebaum *et al.*, 1994). Among the cell lines studied (Bartek *et al.*, 1990; Runnebaum *et al.*, 1994), a missense mutation was the most frequent occurrence. Point mutation with stop codon was found only in the MW1C 6.3 cell line (TAC→TAA, codon 234/exon 7), and in that cell line, no p53 protein accumulation was found with the polyclonal antibody 1801 (Runnebaum *et al.*, 1994). A nonsense mutation (stop codon) has not been reported in amino acid 213 of breast cancer cell lines (Hainaut *et al.*, 1998). No p53 protein accumulation was found in HDQ-P1 cells by IHC using the anti-p53 (PAb 1801) antibody. This antibody targets codon 46-55 product as epitope and can react with both mutant and wild-type human p53 protein (Banks *et al.*, 1986).

Alteration of oncogenes by point mutation, amplification, or rearrangement may be important in the etiology of human cancers. c-myc, c-erbB-2 and c-H-ras are most frequently involved in breast cancer. Increased c-erbB-2 (Scorilas *et al.*, 1993; Seshadri *et al.*, 1994) and c-myc gene (Varley *et al.*, 1987; Yokota *et al.*, 1986) copies have been associated with a poor prognosis in breast cancer. Amplification of the c-myc gene has been reported in up to 32% primary breast carcinomas (Escot *et al.*, 1986). However, the c-myc amplification was found in only a few established breast cancer cell lines (Watanabe *et al.*, 1992). In the report of Kozbor *et al.*, among five cell lines studied (BT-20, SKBR-3, MCF-7, CAMA-1 and SKBR-5), only SKBR-3 exhibited a 10-fold amplification of the gene (Kozbor and Croce, 1984). HDQ-P1 cells may therefore be helpful to understand the role of c-myc gene alteration on breast cancer progression. However, no c-erbB-2 amplified gene was found in HDQ-P1 cells, neither protein overexpression by IHC. The c-erbB-2 amplification has been reported in up to 30% of breast cancers (Slamon *et al.*, 1987). Concomitant c-erbB-2 and c-myc amplification has

rarely been reported. Garcia *et al.* reported only one case with c-erbB2 and c-myc amplification among 125 primary breast cancers, suggesting that alterations of either c-myc or c-erbB2 genes may occur in two different groups of breast carcinomas (Garcia *et al.*, 1989). A significant relationship between tumor proliferation and c-myc but not c-erbB-2 amplification has been reported (Kreipe *et al.*, 1993). No c-H-ras amplification was observed in HDQ-P1 cells. In fact, c-H-ras amplification is rare in breast cancer. Garcia *et al.* found no amplification of this gene among 129 primary breast cancers (Garcia *et al.*, 1989). A restriction fragment length polymorphism was found in HDQ-P1 cells after digestion with BamHI. The protooncogene c-H-ras occurred as fragments of sizes ranging from 6 to 9 kb (Goldfarb *et al.*, 1982) because of allelic polymorphism in the flanking regions (Capon *et al.*, 1983). More heterogeneity of c-H-ras locus was reported in cancer than in normal genomes (Krontiris *et al.*, 1985).

The MUC1 mucin, also called episialin, polymorphic epithelial antigen, and epithelial membrane antigen, is a highly glycosylated normal component of epithelial cells (Patton *et al.*, 1995). It has been suggested that MUC1 expression may influence immune recognition and cellular adhesion processes, and hence play a role in tumor growth and metastasis (Hilkens *et al.*, 1995). MUC1 expression as well as its glycosylation status are often altered in breast cancer (Lloyd *et al.*, 1996), which has led to its use as a tumor marker for that pathology (Tondini *et al.*, 1988). The differences in the band patterns on probing with anti-EMA likely reflect the high degree of polymorphism of the protein (Patton *et al.*, 1995) and/or variations in mucin glycosylation among samples. The latter possibility is suggested by the enhanced reactivity of HDQ-P1 and BT-20 with anti-EMA, following sialic acids cleavage. Such unmasking effect has already been described for other MUC1-specific antibodies (Ho *et al.*, 1995). Although the biological significance of alterations in MUC1 biosynthesis is still unclear, the recent efforts toward the development of MUC1-based breast cancer immunotherapy confirm the clinical interest of the mucin (Graham *et al.*, 1996).

A majority of breast cancer cell lines are ER negative and hormone-unresponsive, and the MCF-7, T-47D and ZR-75-1 cells are only three major hormone-dependent cell lines available for *in vitro* studies in breast cancer. The estrogen and progesterone receptors in HDQ-P1 cells

were not significantly modified by those hormones. In fact, the estrogen receptor level was increased from 9 fm/mg to 45 fm/mg and the progesterone receptor level changed from 3 fm/mg to 18 fm/mg when the cells were cultured in the presence of 17- $\beta$ -estradiol and progesterone. After hormone withdrawal, the estrogen receptor levels fell from 45 fm/mg to 1 fm/mg and progesterone from 18 fm/mg to 7 fm/mg. Overall, estrogen and progesterone receptors in the current culture should be considered as negative and comparable to the original tumor.

In summary, by using a feeder layer of lethally irradiated 3T3 cells, we have established a new cell line from one primary mammary carcinoma. This method may be helpful as an alternative approach in the selective long-term culture of cancer cells from primary breast cancer. The cell line is typical of breast cancer cells with distinct ultrastructural, immunological and genetic characteristics. Furthermore, compared to other cell lines, HDQ-P1 cells reveal two distinct features, namely an amplification of c-myc and one point mutation of p53. The cell line may represent a new model for further *in vitro* studies on breast cancer.

### **Acknowledgment**

The authors wish to thank Danielle Murray, Marie-Anne Quesnel, Nathalie Tremblay and Marc Bronsard for excellent technical assistance. J.L. holds a studentship from the Division of Pathology and Faculty of Medicine, Laval University, and R.D. is a research scholar of the Cancer Research Society Inc./Medical Research Council of Canada Program and B.T. is a research clinician scholar of the Fonds de la Recherche en Santé du Québec (FRSQ).

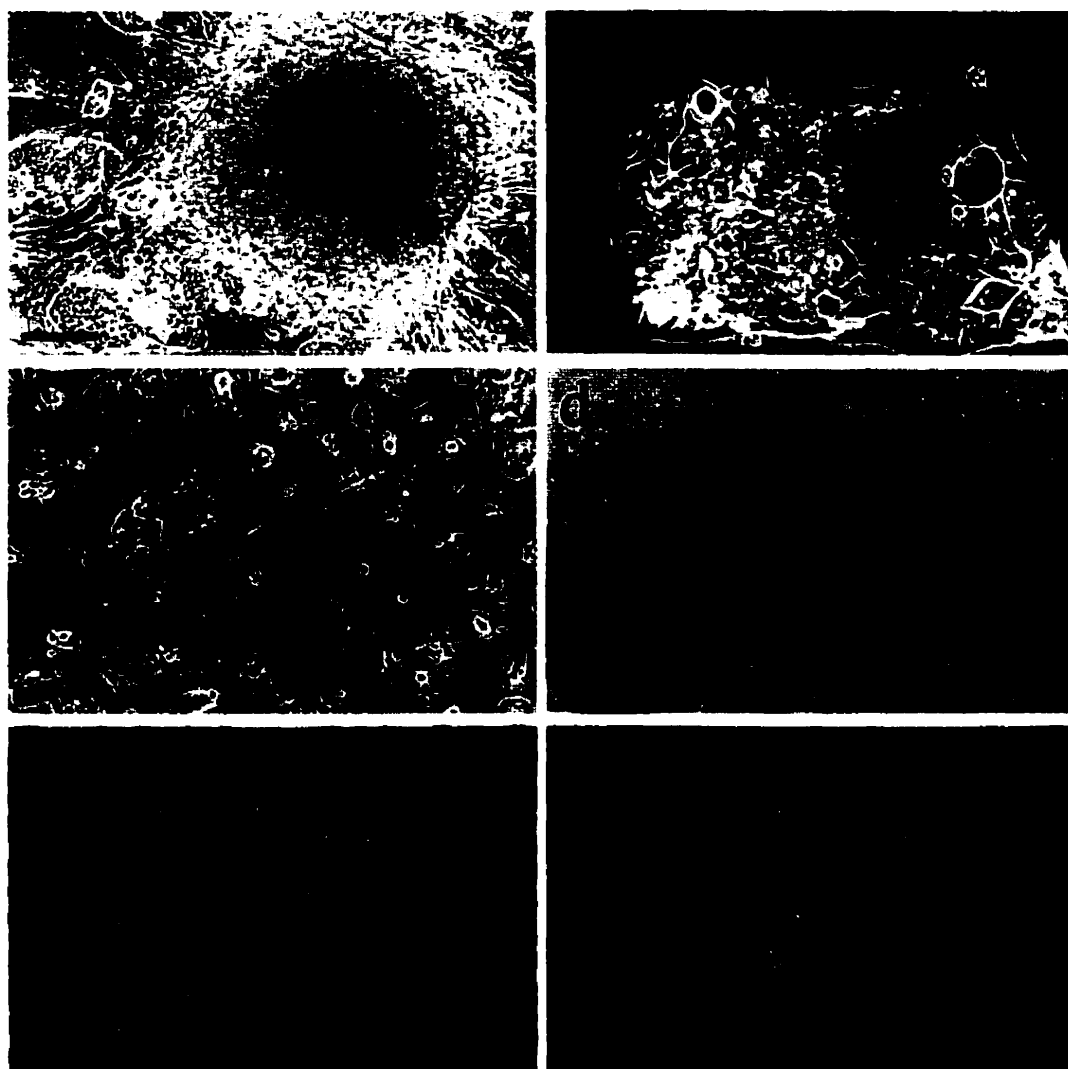


**Figure 1. Selective culture and morphological observation of HDQ-P1 cells**

**a.** Proliferation morphology of tumor-like epithelial cell colonies in the culture with irradiated 3T3 cells (scale bar: 200  $\mu\text{m}$ ); **b.** purified epithelial-like cells growing as cellular patch in the early cultures (scale bar: 400  $\mu\text{m}$ ); **c.** HDQ-P1 cells in the 21st passage of culture (scale bar: 200  $\mu\text{m}$ ), **d:** formation of tumor-like colonies by HDQ-P1 cells in agar culture (scale bar: 100  $\mu\text{m}$ ); **e.** morphology of HDQ-P1 cells (Haematoxylin & Eosin) showing polygonal cells with large, pleomorphic and lobulated nucleoli (scale bar: 100  $\mu\text{m}$ ); **f.** positive immunoreaction with anti-cytokeratin CAM 5.2 (scale bar: 50  $\mu\text{m}$ ).

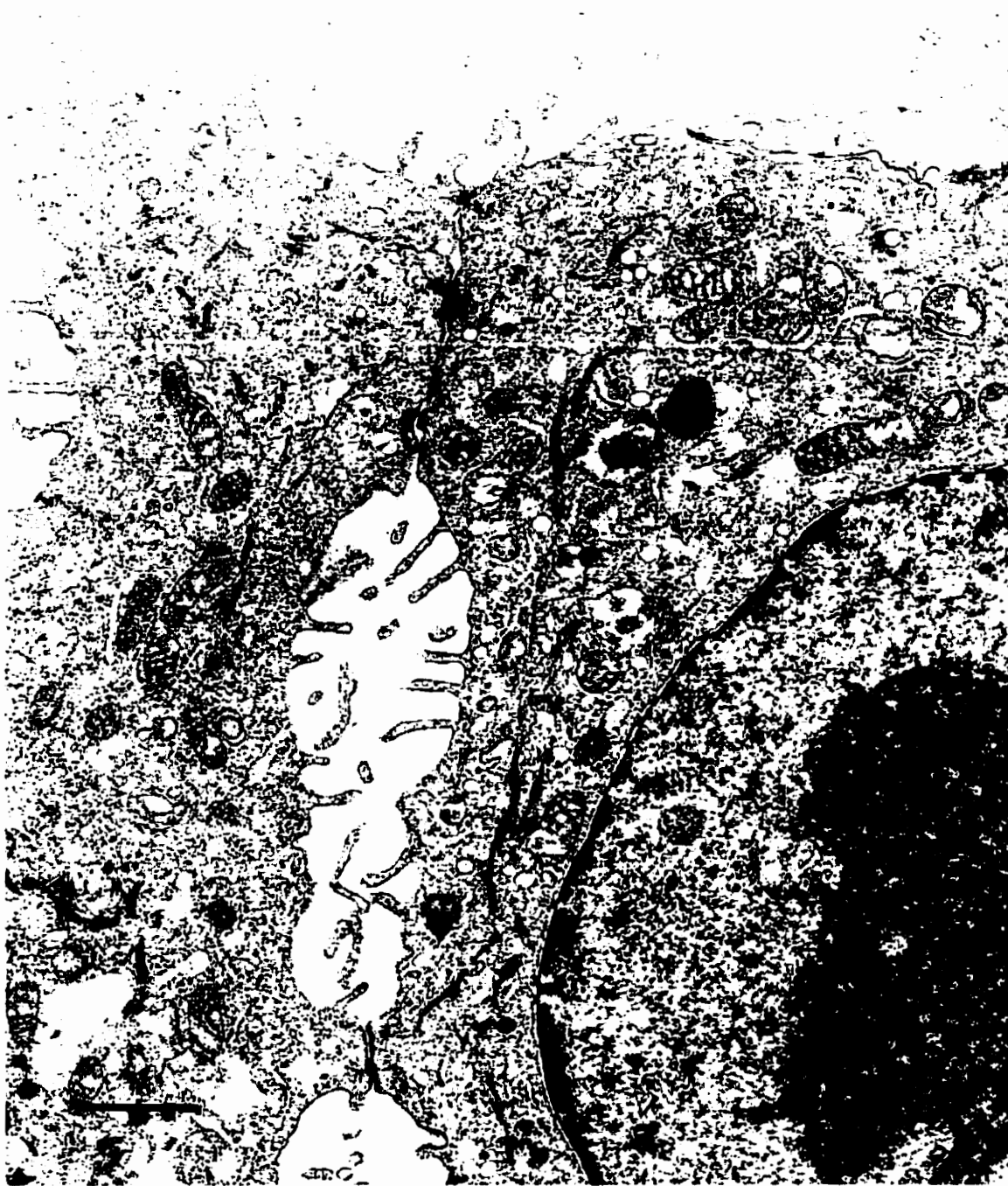


**Figure 2.** Ultrastructure of HDQ-P1 cells. The cells contain bundles of tonofilaments and are attached by tight junctions and desmosomes which delineate intercellular lumina with protruding microvilli (scale bar: 1  $\mu$ m).

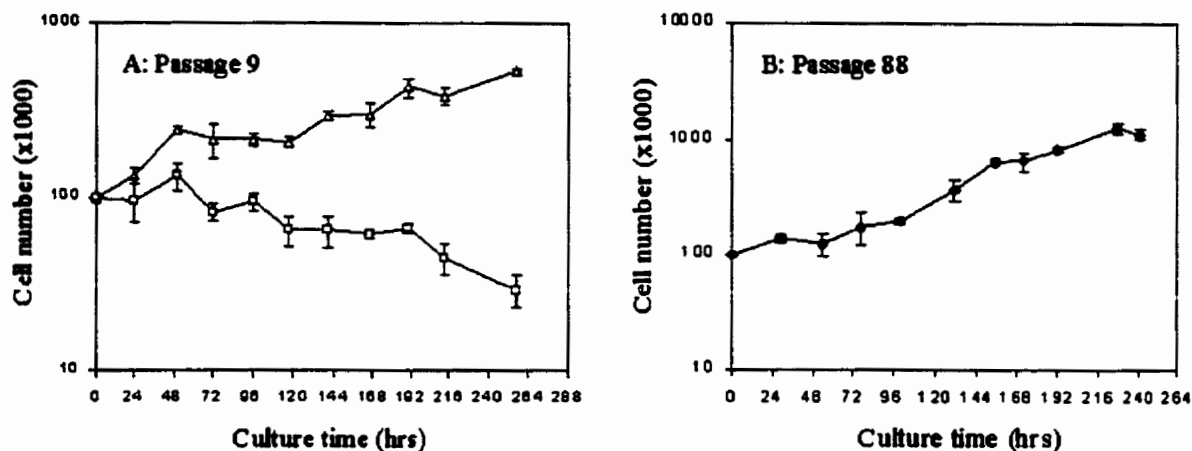


Figure 3. Growth curves of HDQ-P1 cells

a. The growth rate of HDQ-P1 cells at the 9th passage with highly enriched medium ( $\Delta$ ) and with DMEM only supplemented by 10% FCS ( $\square$ ). The figure shows a strong dependence of cells on nutrients and growth factors and hormones. With highly enriched medium, the doubling time (DT) of HDQ-P1 cells was 121 hrs ( $R^2=0.8837$ ); b. growth rate of HDQ-P1 cells at the 88th passage in the culture with DMEM supplemented by 10% FCS and 5  $\mu\text{g/ml}$  crystallized bovine insulin (DT=60 hrs,  $R^2=0.9634$ ).

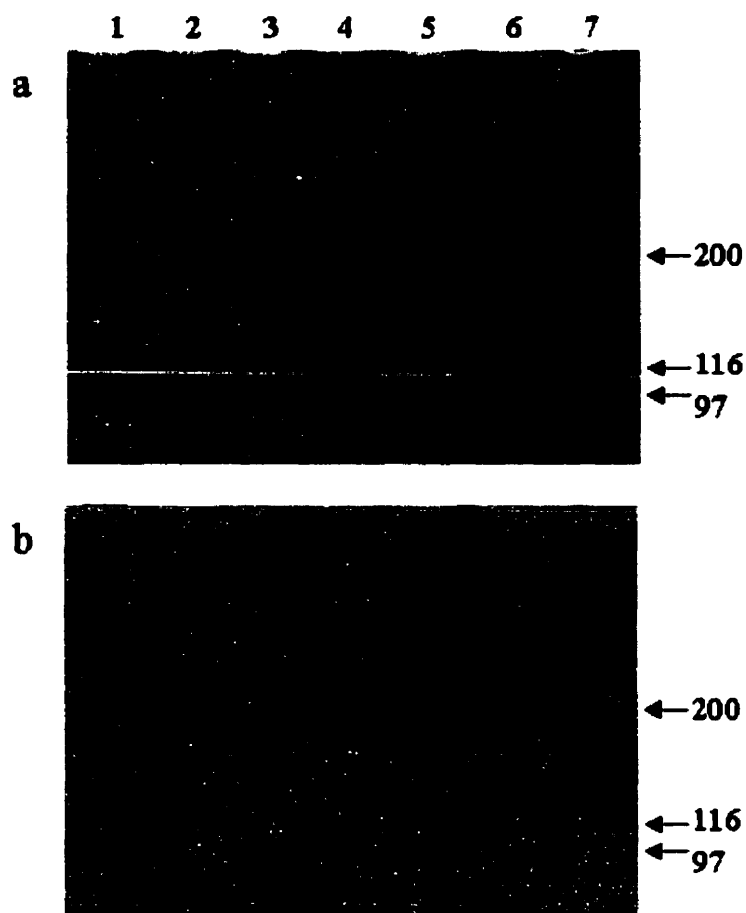


**Figure 4. Western blot analysis of MUC1 in cell lines in culture**

**a: intact membrane; b: membrane treated with *V. cholerae* sialidase. Samples are identified as: 1. Normal skin fibroblasts; 2. Tumor-associated fibroblasts (F9846); 3. SK-BR-3; 4. MDA-MB-231; 5. HDQ-P1; 6. BT-20; 7. MCF-7. Arrows indicate molecular weight standards (kDa).**

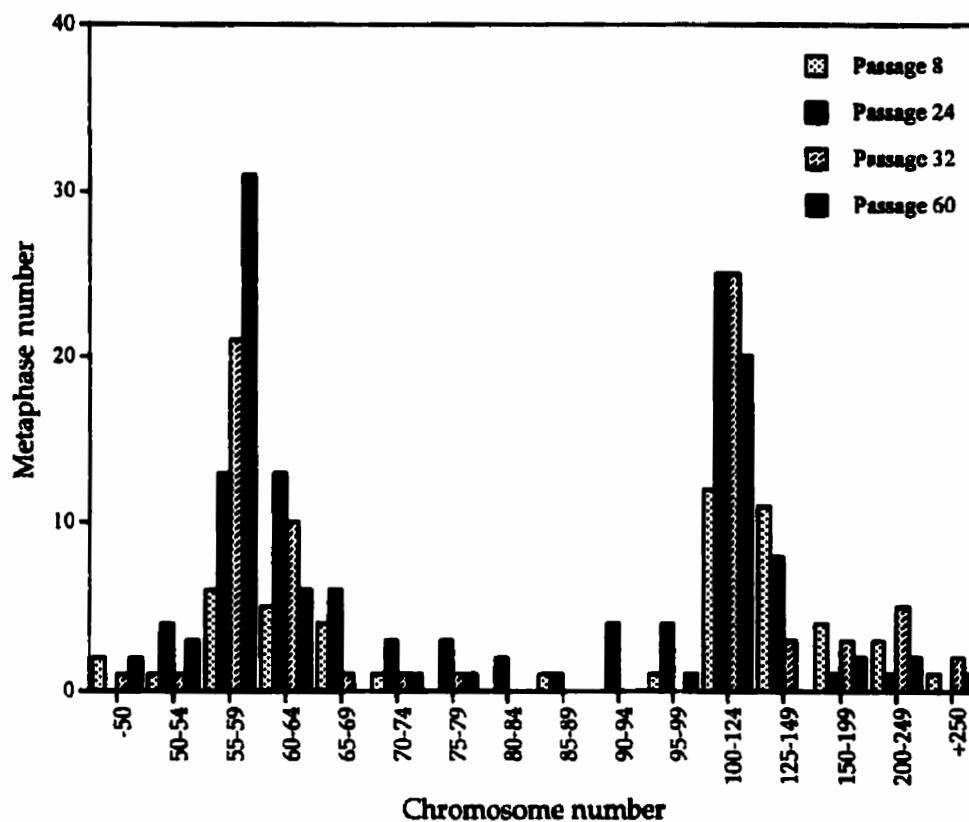


Figure 5. Chromosome number distribution of HDQ-P1 epithelial cancer cell line at passages 8, 24, 32 and 60.

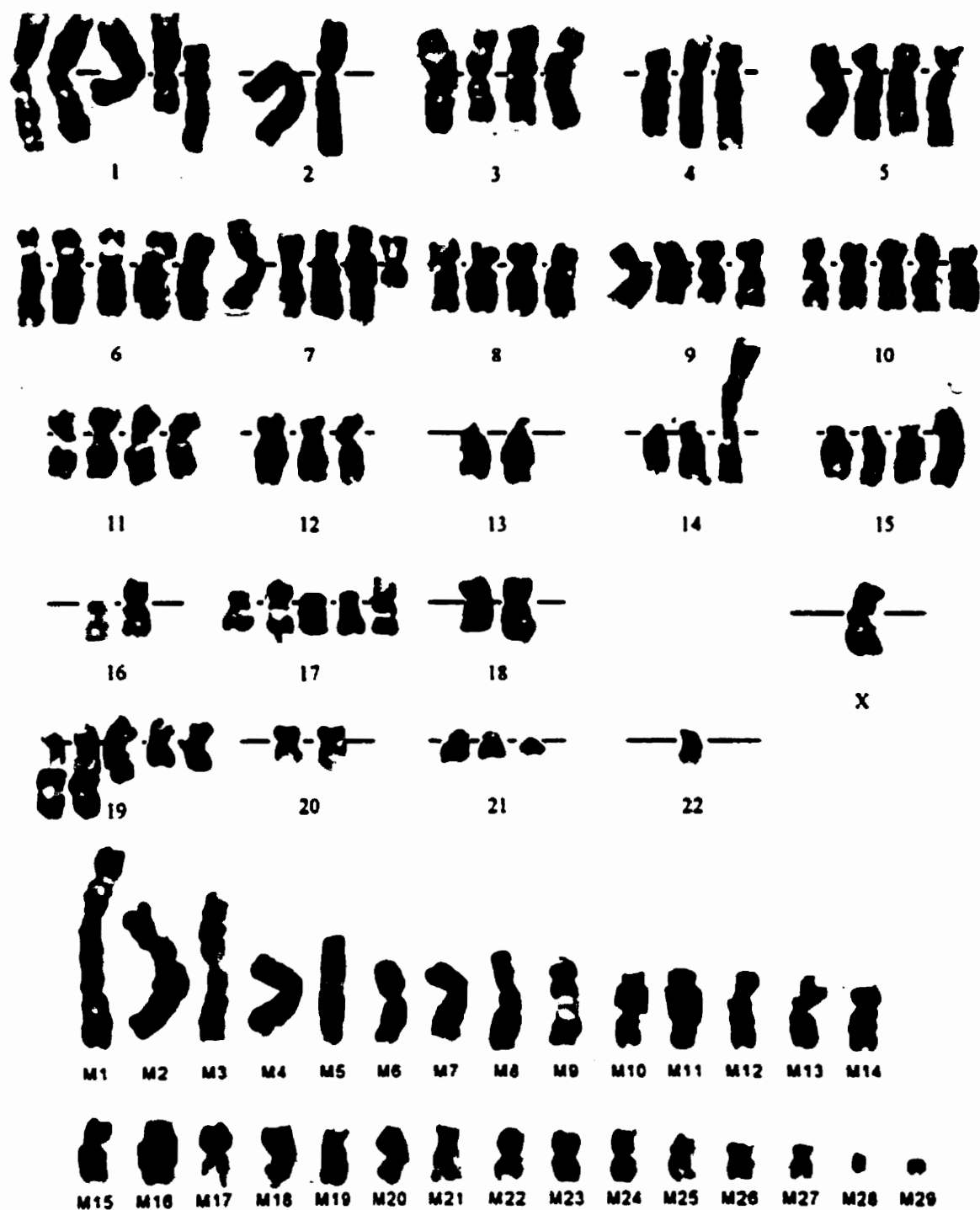


Figure 6. Representative GTG-banded karyotype of an epithelial cancer cell from passage 60 showing hyperpentaploidy. Twenty nine marker chromosomes are identified as M1 to M29.

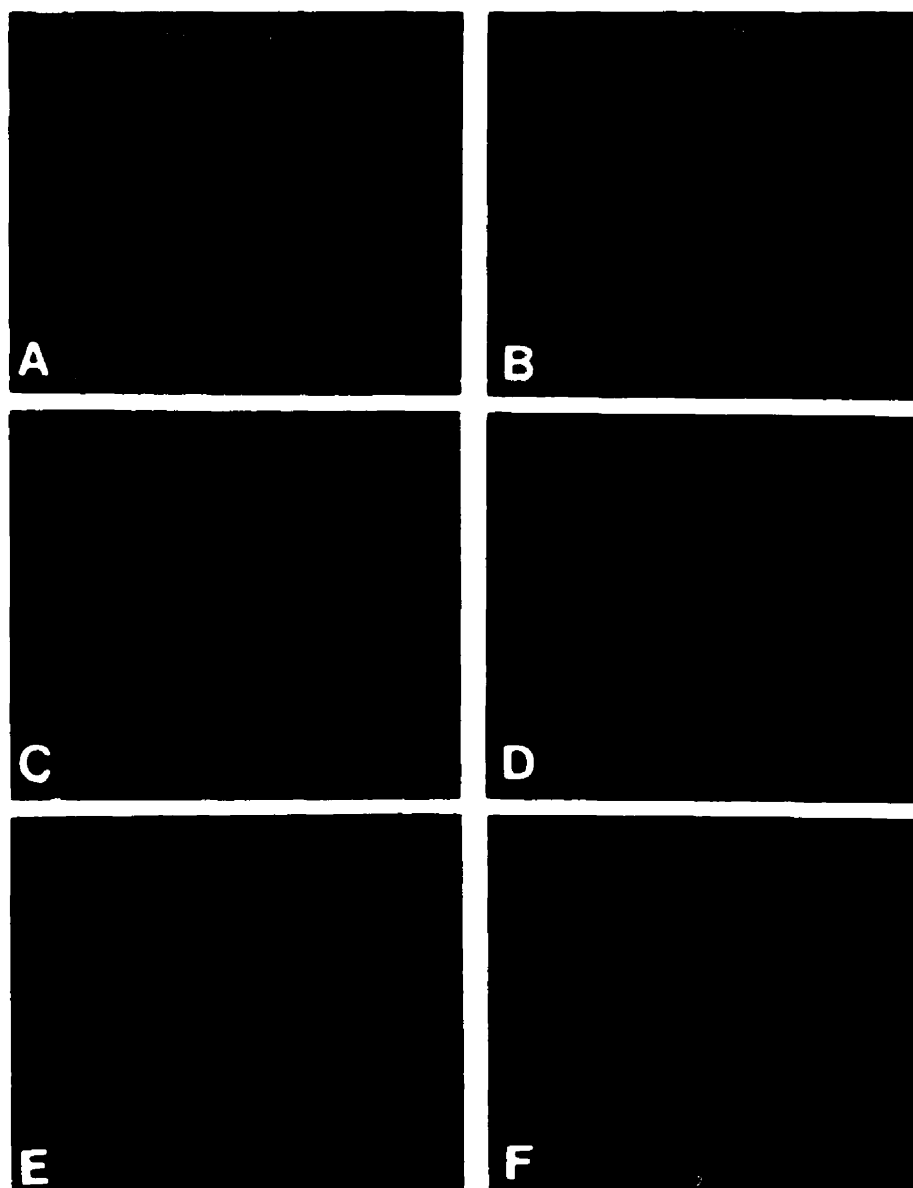


**Figure 7. Chromosome aberrations in epithelial cancer cells studied by FISH.**

a. Simultaneous hybridization of coatasome 1 total chromosome digoxigenin-labeled probe (red) and p53 specific DNA biotinylated probe (green); b. Hybridization of coatasome 6 total chromosome digoxigenin-labeled probe; c. Hybridization of coatasome 7 total chromosome digoxigenin-labeled probe; d. Hybridization of coatasome 16 total chromosome digoxigenin-labeled probe; e. Hybridization of coatasome 18 total chromosome digoxigenin-labeled probe; and f. Hybridization of coatasome 19 total chromosome digoxigenin-labeled probe. Chromosomes were counterstained using DAPI II.

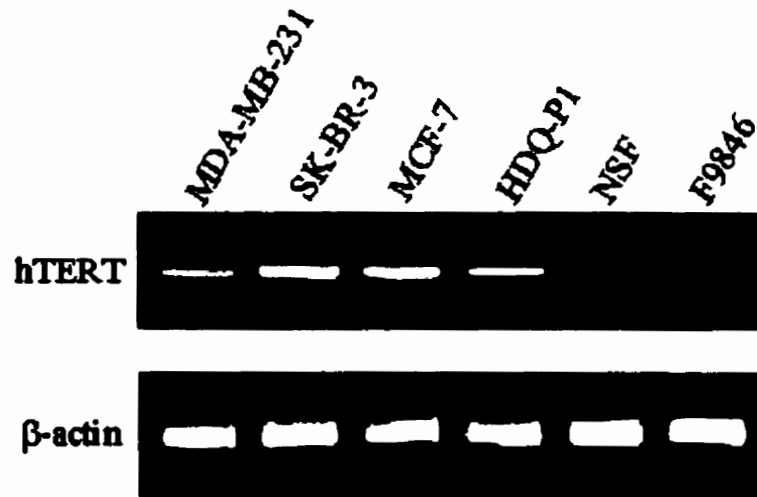


Figure 8. hTERT mRNA expression in breast cancer cell lines

HDQ-P1 cells expressed hTERT mRNA to levels comparable to other breast cancer cell lines: MDA-MB-231, SK-BR-3 and MCF-7, while non malignant fibroblasts failed to express hTERT.

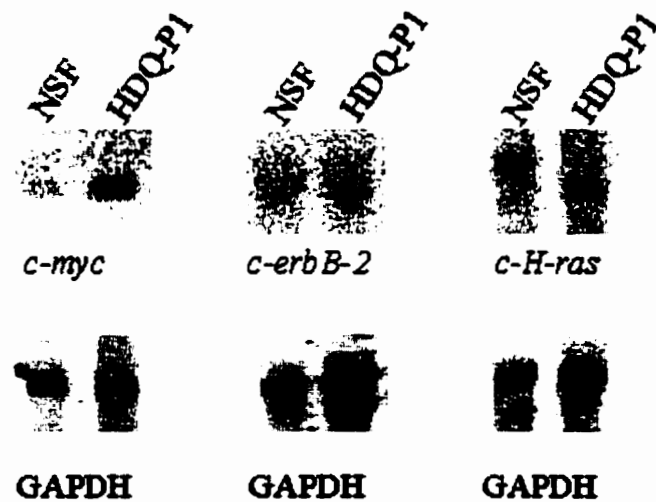


Figure 9. c-myc oncogene amplification in HDQ-P1 cells

The HDQ-P1 cells reveal c-myc oncogene amplification compared to normal skin fibroblasts, whereas no significant c-erbB-2 and c-H-ras amplification was observed. A BamHI RFLP was observed in the HDQ-P1 cells.

## **CHAPITRE IV**

### **EXPRESSION DE LA STROMÉLYSINE-3 PAR LES CELLULES STROMALES RÉACTIONNELLES ASSOCIÉES À LA TUMEUR MAMMAIRE**

## **AVANT-PROPOS.**

La stromélysine-3 (ST3) est une métalloprotéase matricielle identifiée en 1990. C'est une protéase pratiquement limitée aux cellules stromales. Des analyses cliniques ont démontré que son expression stromale associée à une progression tumorale dans certaines formes de cancers dont le cancer mammaire. Cependant, il existe peu d'information sur le rôle des interactions entre cellules stromales et cellules cancéreuses mammaires sur la production de ST3. L'objectif de cette étude est d'élucider à l'aide d'un modèle *in vitro*, l'expression de stromélysine-3 par des cellules stromales mammaires sous l'influence de cellules épithéliales cancéreuses. Cet article a été soumis pour publication à la revue American Journal of Pathology.

## RÉSUMÉ.

La stromélysine-3 (MMP-11) est une métalloprotéase matricielle (MMP) identifiée en 1990 par hybridation par soustraction d'un cancer du sein. L'expression de la MMP-11 a été observée à ce jour dans de nombreux cancers mais uniquement dans les cellules stromales réactionnelles situées à proximité des cellules cancéreuses. De rares analyses cliniques suggèrent que l'expression de la MMP-11 est associée à un pronostic défavorable. Le rôle présumé de la MMP-11 est d'assurer un remodelage de la matrice extracellulaire et, ainsi, de favoriser l'invasion et l'implantation tumorale. La MMP-11 est une cible thérapeutique intéressante puisqu'elle est produite par des cellules bénignes et donc moins susceptibles de développer des mutations et résistances en cours de traitement. Cette étude visait donc à démontrer que la surexpression de MMP-11 par des cellules stromales était une réaction spécifique à une stimulation par des cellules cancéreuses. Nous avons étudié l'expression de MMP-11 par hybridation *in situ* à l'aide de sondes d'ARN et par immunohistochimie avec un anticorps monoclonal dans un modèle mammaire *in vitro*. Dans ce modèle, nous avons placé des fibroblastes purifiés de plusieurs cancers du sein en coculture directe ou indirecte avec des cellules de trois lignées cancéreuses mammaires (MDA-MB-231, SK-BR-3 et MCF-7) et des cellules épithéliales normales mammaires. L'expression de MMP-11 était observée uniquement par les fibroblastes situés à l'interface avec les cellules épithéliales cancéreuses. Cette étude confirme que l'expression de la MMP-11 est sous l'influence des cellules cancéreuses et qu'un effet de contact direct entre ces deux types cellulaires était principalement responsable de cette stimulation. Elle démontre de plus, pour la première fois, que les fibroblastes de différentes patientes répondent de façon variable à une même stimulation.

**Stromelysin-3 overexpression by mammary tumor-associated fibroblasts  
under *in vitro* breast cancer cell induction**

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**Running title: stromelysin-3 expression in breast cancer associated fibroblasts**

## **Abstract**

**Stromelysin-3 (ST3) is a matrix metalloproteinase (MMP) overexpressed by reactive stromal cells of most carcinomas. Recent clinical studies demonstrated a correlation between ST3 expression by tumor stromal cells and local invasiveness and poor survival in breast cancer patients, suggesting that ST3 plays a critical role in cancer progression and that this protease might be a potential target for new therapeutic strategies. To date, only rare studies used an *in vitro* model to investigate the effect of cancer induction over ST3 expression by stromal cells. In order to better understand host response pattern in stromal expression of stromelysin-3 in breast cancer, we have investigated stromelysin-3 expression by stromal cells isolated from primary breast carcinomas. Those tumor-associated fibroblasts were co-cultivated with benign and cancer epithelial cells of mammary origin, and stromelysin-3 expression was analyzed with the use of both *in situ* hybridization and immunocytochemistry. The results showed that ST3 expression by stromal cells was cancer-specific. In fact, three malignant cancer cell lines (MDA-MB-231, SK-BR-3 and MCF-7) have equally induced ST3 overexpression by tumor-associated stromal cells, while normal mammary epithelial cells and an immortalized normal mammary epithelial cell line (184A1) failed to induce ST3 expression. The tumor induction effect was much important in direct cell-cell contact coculture. The induced protease expression was only on stromal cells located close to epithelial cells and not influenced by the addition of basic fibroblast growth factor (bFGF) and anti-bFGF to the culture medium. Moreover, tumor-associated fibroblasts from different primary tumors gave different responsiveness to a similar putative cancer stimulation, suggesting a different host reaction in tumor invasion. We therefore hypothesize that the induction effect is more likely via a cell-cell contact mechanism. Our results provide further evidence that ST3 expression is limited to stromal cells located close to cancer cells and that cancer cells and not benign epithelial cells can induce MMP expression by stromal cells. Those data suggest that efforts should be aimed at identifying cancer cell factors responsible for ST3 expression by stromal cells.**

**Key words: Stromelysin-3, breast cancer, matrix metalloproteinases, stromal cells**

## Introduction

Stromelysin-3 (ST3) is a relatively new member of the matrix metalloproteinase (MMP) family. Identified in 1990 by subtractive cDNA library screening of a human breast carcinoma and a fibro-adenoma, ST3 has been found to be overexpressed by breast carcinomas (Basset *et al.*, 1990). The protein sequence, as deduced from its cDNA, indicated an homology between ST3 and other MMPs and has been designated as MMP-11 (Murphy *et al.*, 1991). Many recent clinical studies have found that ST3 was overexpressed by reactive stromal cells of several human malignancies such as breast (Ahmad *et al.*, 1998; Basset *et al.*, 1990; Basset *et al.*, 1994; Chenard *et al.*, 1996; Hahnel *et al.*, 1994; Hahnel *et al.*, 1993; Heppner *et al.*, 1996; Holm *et al.*, 1997; Kawami *et al.*, 1993; Kossakowska *et al.*, 1996; Linder *et al.*, 1997; Pacheco *et al.*, 1998; Têtu *et al.*, 1998), lung (Anderson *et al.*, 1995; Bolon *et al.*, 1996; Bolon *et al.*, 1997; Karameris *et al.*, 1997; Urbanski *et al.*, 1992), head and neck (Muller *et al.*, 1993; Munck-Wikland *et al.*, 1998; Polette *et al.*, 1993), colorectum (Newell *et al.*, 1994) (Kossakowska *et al.*, 1996; Mueller *et al.*, 1997; Porte *et al.*, 1995; Thewes *et al.*, 1996), esophagus (Porte *et al.*, 1998), pancreas (von Marschall *et al.*, 1998) and skin cancers (Unden *et al.*, 1996; Wagner *et al.*, 1992). Some of those studies have shown that overexpression of ST3 transcripts or proteins correlated with clinically malignant local invasiveness (Bolon *et al.*, 1996; Bolon *et al.*, 1997; Karameris *et al.*, 1997; Muller *et al.*, 1993; Munck-Wikland *et al.*, 1998; Porte *et al.*, 1995; Porte *et al.*, 1998). ST3 was absent in hyperplastic and metaplastic lesions whereas the enzyme was frequently expressed in preinvasive (dysplastic and *in situ*) lesions and uniformly present in invasive carcinomas (Bolon *et al.*, 1996). ST3 overexpression was associated with a poor overall or disease-free survival in a few studies on breast (Ahmad *et al.*, 1998; Chenard *et al.*, 1996; Têtu *et al.*, 1998) and esophageal carcinomas (Porte *et al.*, 1998). ST3 has been proposed as a potential prognostic factor (Ahmad *et al.*, 1998) and also a potential target for tumor control management (Anderson *et al.*, 1995).

In all those clinical studies, ST3 expression was expressed by stromal cells adjacent to cancer epithelial cells. Although the mechanism by which the tumor cells induce surrounding stromal cells to produce ST3 remains unknown, those data suggest that ST3 production by stromal cells in tumor tissues may be a response by host stromal cells to putative stimulation messages derived from cancer cells. A recent report showed that human breast cancer cells could

directly activate the ST3 promoter in normal breast fibroblasts and hence increase gene expression at the transcriptional level (Ahmad *et al.*, 1997). In another recent *in vitro* study using a coculture system with Northern and Western blot analyses, non small cell lung cancer (NSCLC) cells were found to induce ST3 expression by normal pulmonary fibroblasts (Mari *et al.*, 1998). It has been hypothesized that tumor-derived growth factors might induce enzyme production by stromal cells and that ST3 may be implicated in the initial process of cancer cell migration through extracellular matrix degradation (Basset *et al.*, 1990; Basset *et al.*, 1993; Wagner *et al.*, 1992). One recent animal study has shown that ST3 expression by stromal cells promote the tumor take in nude mice rather than the activation of tumor proliferation and invasion (Noel *et al.*, 1996).

The fact that only stromal cells in close contact with cancer cells respond to tumor induction stimulation suggests that the effect is more likely via a cell to cell contact. However, certain data suggest that a paracrine activation is also involved in ST3 production. Certain growth factors such as bFGF (Ahmad *et al.*, 1997; Anderson *et al.*, 1995; Basset *et al.*, 1990; Okada *et al.*, 1995), EGF (Ahmad *et al.*, 1997; Basset *et al.*, 1990) and PDGF (Ahmad *et al.*, 1997; Anderson *et al.*, 1995; Basset *et al.*, 1990) were reported in some studies to stimulate ST3 production at the transcriptional level (Anderson *et al.*, 1995; Basset *et al.*, 1990; Okada *et al.*, 1995). However, a recent *in vitro* study found that bFGF does not increase the induction but only the post-transductional processing at a unique internal sequence by producing a tumor-specific 35 kDa ST3 protein which lacks enzymatic activity (Mari *et al.*, 1998). The biological activities of this protease in cancer progression is unclear.

To date, a majority of clinical studies on ST3 mRNA expression has been performed on tumor tissue sections. However, in order to better understand the role of cancer cell/stromal cell interactions and the influence of the host fibroblast phenotype on ST3 expression, the use of only rare *in vitro* assays were performed to confirm the influence of cancer-stromal cell interactions on ST3 expression (Ahmad *et al.*, 1997; Mari *et al.*, 1998). Moreover, in these *in vitro* assays, only fibroblasts from normal tissues were used and ST3 expression was tested by Northern and Western blot analyses. This prompted us to investigate the patterns of expression of ST3 in primary breast carcinomas by an *in vitro* model using not only normal skin

fibroblasts (NFS) but also those purified from primary breast carcinomas (tumor-associated fibroblasts). All those fibroblasts were co-cultivated with benign and malignant breast cell lines and ST3 expression was documented by *in situ* hybridization and immunocytochemistry.

## **Materials and methods**

### **Cancer tissues**

Nine primary breast carcinomas were used in this study. There were seven invasive ductal and two invasive lobular carcinomas. In each case, a fresh sterile tissue specimen of the tumor was obtained immediately after surgical resection. The diagnosis of those tumors was confirmed by routine histologic examination. The tissue specimens were trimmed and placed in Dulbecco modified Eagle's medium (DMEM) with antibiotics at 4°C. Tumor tissues were then minced with crossed surgical blades into about 1 mm<sup>3</sup> fragments and digested overnight with collagenase (0.14%) at 37°C under gentle constant shaking. A single-cell suspension was obtained and the cell viability was determined by the trypan blue exclusion technique. Tumor tissues have been obtained from our bank of breast cancer that has been approved by the hospital ethical committee. Each patient whose tissue is recorded in our tumor bank is asked to sign an informed consent form.

### **Stromal cells**

Tumor-derived fibroblasts were obtained from primary breast cancers by serial passages. Only cells obtained between passages 5 and 10 were used for this study. Normal skin fibroblasts (NSF) were obtained from normal skin tissue of mammary origin. Cells were maintained in DMEM with 10% fetal bovine serum (FBS). Cell lines were incubated at 37°C with 5% CO<sub>2</sub>. The fibroblastic nature of those cells was confirmed by their morphology, the expression of vimentin, and the absence of cytokeratins.

### **Epithelial cell lines**

The epithelial cells used in the study were human breast cancer lines MDA-MB-231, SK-BR-3, MCF-7 and an immortalized normal human mammary epithelial cell line 184A1 (ATCC). The MGH-U4 Cell line corresponding to dysplastic urothelial cells was kindly provided by Dr. Y. Fradet (Université Laval, Québec, Canada). Normal mammary epithelial (NME) cells and

keratinocytes were kindly provided by Dr. M. Rouhabia (Université Laval, Québec, Canada).

### **Direct coculture**

$2 \times 10^5$  fibroblasts were seeded on chamber slides (Nunc, Naperville, IL) treated previously by poly-L-lysine and  $1 \times 10^5$  epithelial cells were added when those fibroblasts reached 90% confluence. The cells were cultured with DMEM supplemented with 10% FBS during 24 hrs, and then washed twice with DMEM. Coculture was continued with DMEM during two to four days. At the end of this period, the chamber slides with cells were washed twice by phosphate-buffered saline (PBS) and frozen at  $-80^\circ\text{C}$  until use for immunohistochemistry. For *in situ* hybridization, the cells in chamber slides, after washing with PBS, were fixed with 4% paraformaldehyde at room temperature for 20 minutes. The fixation was stopped with the use of 3x PBS and the fixed cells in chamber slides were dehydrated and stored at  $-80^\circ\text{C}$  until use. In the selective coculture, anti-human bFGF mAb (5  $\mu\text{g/ml}$ , clone FB-8, Sigma, Oakville, Ontario) and bFGF (10 ng/ml, Sigma) were added to the coculture medium. Purified fibroblasts without epithelial cells in the coculture served as negative control. The coculture was repeated in duplicate for each cell line.

Another type of direct coculture was performed by using cell suspension after enzymatic digestion of primary breast tumors and seeding of  $2 \times 10^5$  mixed stromal and cancers cells in suspension on chamber slides without prior cell selection. Those slides had also been treated previously by poly-L-lysine and the same process cited above has been followed.

### **Indirect coculture**

Indirect coculture was performed using the Transwell culture system (0.4  $\mu\text{m}$  pore size, Corning Costar, Cambridge, MA).  $1 \times 10^5$  fibroblasts were first seeded in the lower chamber and 24 hrs after,  $1 \times 10^5$  epithelial cell lines in the upper chamber of the Transwell systems. The coculture was maintained for the first 24 h with DMEM and 10% FBS and then replaced by DMEM after two washings with DMEM, for two to four days in 5%  $\text{CO}_2$  incubator. At the end of the coculture, the fibroblasts in the lower chamber were used for the analysis. The fibroblasts cultured in the same series without epithelial cells in the upper chamber served as negative control.

The fibroblasts were also cultivated with daily changed conditioned medium obtained from cancer cell culture. The conditioned medium was obtained by culturing cancer cell lines in DMEM without FBS during 24 hrs. The culture medium was collected and centrifuged at 250 x g for 10 min. The supernatant was then stored at 4°C until use. The culture was also repeated in duplicate for each cell line.

### ***In situ* hybridization**

*In situ* hybridization was performed as reported previously (Têtu *et al.*, 1998) but with a few modifications. Fixed cells in chamber slides were rehydrated in graded alcohol and then treated with 0.2 N HCl for 20 min at room temperature to denature proteins. The cells were then deproteinated in 40 µg/ml pronase at 37°C for 15 min, acetylated for 10 min in 0.5% (v/v) acetic anhydride/0.1 M triethanolamine pH 8 and prehybridized in 2x SSC /50% formamide. Hybridization was performed overnight with <sup>35</sup>S-labeled (>1x10<sup>5</sup> cpm/µl) antisense RNA probe at 55°C in a sealed moisturized chamber. After hybridization, the samples were treated with RNase A (Sigma, 20 µg/ml, 30 min at 37°C), stringently washed twice (2x SSC/formamide 50%, 60°C 1 hr) to remove unhybridized probe and dipped into Kodak autoradiography emulsion type NBT2. Slides were kept in light-tight box for 2-4 weeks, were developed and counter-stained with hematoxylin and eosin. Dark-field microscopy was used to identify areas with high grain density. The negative controls were performed under the same condition using sense RNA probes. Plasmids for ST3 were kindly provided by Pr. P Basset, (IGBMC, Illkirch, France). The 1759 base pairs stromelysin-3 cDNA was subcloned in the EcoRI site and extended from nucleotides (nt) 346 to 2105. Reduction of the probe length was achieved by partial alkaline hydrolysis. Optimal *in situ* hybridization was obtained with 100 bp probes.

### **Immunocytochemistry**

The cells were first fixed in acetone for 10 minutes and quenched for 5 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity in the tumor samples. After washing with PBS, slides were covered with normal horse serum during 20 minutes and incubated overnight with each monoclonal antibody at 4°C. Afterwards, slides were rinsed with PBS and exposed for 30

minutes to a secondary biotinylated anti-mouse antibody. After another wash with PBS, slides were incubated with the avidin-biotin-peroxidase complex (Vector laboratory, Burlingame, CA) and the binding of the antibodies was revealed with diaminobenzidine (DAB). The slides were finally counterstained with Mayer's haematoxylin for 3 minutes and mounted in permount. Monoclonal antibodies used for epithelial markers were anti-epithelial membrane antigen (Dako, Mississauga, Ontario, Canada) and anti-human cytokeratins (CAM 5.2, Becton Dickinson, Mississauga, Ontario) detecting cytokeratins 8 and 18. Other antibodies used were anti-vimentin (Dako), anti-human  $\alpha$ -smooth muscle actin (Dako) and anti-CD68 (KP1, Dako). The anti-human ST3 antibody 5ST-4A9 was kindly provided by Pr. P Basset (IGBMC, Illkirch, France).

### **RNA preparation and RT-PCR analysis**

The isolation of total RNA was performed according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described (Wang *et al.*, 1997) with a few modifications. Briefly, 1  $\mu$ g of total RNA was used in the reaction of RT. PCR amplification was carried out in a final volume of 25  $\mu$ l containing 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 100 nM of each primer, 1 unit of Taq DNA polymerase (Gibco-BRL, Burlington, Canada) and 5  $\mu$ l of reverse transcription mix for ST3 amplification. Concomitant amplification, but in the separate tube, of human  $\beta$ -actin was performed in the same condition but with pre-optimized diluted reverse transcription mix for quality and semi-quantitative evaluation of mRNA of each sample. For selective semi-quantitative analysis, 2  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P] dCTP was added to each reaction mixture.

Primers used for amplification human  $\beta$ -actin specific sequences were 5'CGTGGACATCCGCAAAGAC3' and 5'CTCGGCCACATTGTGAAC3' (Johnson *et al.*, 1996). PCR using these primers yielded a 484-base-pair (bp) product. The ST3-specific sequences were amplified by using primer 5'TGGGTGTACGACGGTGAAAA3' and primer 5'CATGGGTCTCTAGCCTGATA3', which yielded a 449-bp product (Johnson *et al.*, 1996).

Following an initial denaturation at 94°C for 5 minutes, samples were amplified by 25 cycles

of denaturation at 94°C for 30s, annealing at 60°C for 30s, extension at 72°C for 45s, and ended by extension at 72°C for 10 min in a DNA thermal cycler (Perkin-Elmer/GeneAmp system 9600).

PCR products were separated on 6% polyacrylamide gels in TBE (50 mM Tris, 40 mM boric acid, 0.5 mM EDTA), stained with ethidium bromide and the PCR product expression level was documented with an AlphaImager 2000 (Alpha Innotech Corporation). The radioactive gels were exposed to phosphor screen, scanned with a Storm 860 scanner (Molecular Dynamics Inc, Sunnyvale, CA) and analyzed using ImageQuaNT program (Molecular Dynamics Inc).

Each amplification was performed in duplicate. Negative controls were included in each experiment: Reverse transcription was carried out in the absence of RNA (RT-control). PCR was carried out from the RT-control or substituting reverse transcription buffer to the cDNA template. The RNA of tumor tissue T12499 was used in RT-PCR in every experiment batch as a positive control.

## **Results**

Most of all purified tumor-derived fibroblasts were fibroblast-like cells and some of them were myofibroblast-like in culture. The mesenchymal origin of fibroblast-like cells purified from primary breast carcinomas and that of fibroblasts purified from the normal breast tissues were confirmed by the lack of human cytokeratin expression (8 & 18), presence of vimentin and their spindle-cell morphology. Unexpectedly, immunoreaction was positive for CD68 (KP1) in all cases.

### ***In vitro* direct coculture model**

In the direct coculture assay, cell proliferation resulted in a reconstituted structure reminiscent of the tumor seen on the original section. In general, cancer epithelial cells formed colonies, surrounded by a monolayer of fibroblasts. There was a well-defined stromal/epithelial interface. This phenomenon was more evident in the coculture with MCF-7 and in the coculture assay with stromal cells and epithelial cancer cells originated from the same primary breast tumors (Fig 1a, 1b, 1c and 1n).

### ***In situ* analysis for ST3**

ST3 mRNA expression was documented in the tissue sections of all tumor tissues from which tumor-associated fibroblasts have been purified. All tumors expressed ST3 by *in situ* hybridization but to varying levels, and the expression was limited to fibroblasts located close to cancer epithelial cells. Representative results are shown in Figures 1d, 1g, 1j, and 1m.

ST3 mRNA expression was weak or even absent in *in vitro* unstimulated tumor-derived fibroblasts purified and isolated from each of the nine primary tumors (Fig. 1e, 1h and 1k). After direct coculture with cancer cells, overexpression of ST3 transcripts reappeared in those tumor-derived fibroblasts purified from each original primary breast cancer. In each case, ST3 mRNA expression was observed only in fibroblasts located in close contact with tumor cells and colonies. Under similar coculture conditions and same cancer cell line stimulation, ST3 mRNA expression was however much variable among those nine cases (Fig. 1f, 1i, 1l and 1o). No clear difference on ST3 mRNA expression was observed in each single case under the stimulation of any of the three breast cancer cell lines: MDA-MB-231, SK-BR-3 and MCF-7 (Fig. 2a, 2b and 2c). The coculture with MCF-7 yielded larger epithelial colonies surrounded by fibroblasts expressing ST3 mRNA. In the coculture with MDA-MB-231, epithelial cancer cells were isolated among the stromal cell proliferation and the positive signal was more focal. In the direct coculture with NME and 184A1 cells, as well as keratinocytes, no expression of ST3 was observed (Fig. 2h, 2i and 2j). Coculture with MGH-U4 cells induced weak but focally positive signals in the fibroblasts (Fig. 2g).

In the coculture of NSF with breast cancer cell lines, a positive but weak signal of ST3 mRNA was also observed in the NSF located close to the cancer cells (Fig. 2l).

In the coculture involving stromal and cancer cells from the same tumor, expression of ST3 was also found in stromal cells relocated close to spheroid epithelial cell colonies (Fig. 1o).

Comparable induction effect on fibroblasts was observed with the use of an anti-human ST3 antibody (Fig. 2d). As for ST3 mRNA, 184A1, NME cells and keratinocytes did not induce

### **ST3 protein expression.**

The addition of anti-bFGF (Fig. 2e) and bFGF (Fig. 2f) did not influence ST3 mRNA and protein expression by tumor-derived fibroblasts and NSF.

### **Indirect coculture**

Fibroblasts co-cultivated indirectly with epithelial cancer cells seeded in the Transwell system were analyzed by RT-PCR. The PCR conditions were initially optimized to avoid ST3 amplification saturation compared to the positive control T12499. A standard curve was first obtained from RT-PCR reactions performed for human  $\beta$ -actin by using serial dilutions of cDNA obtained by RT with RNA from primary breast tumor T12499. As illustrated in Figure 3a, the experimental graph showed a linear region of amplification. The initial assays have shown that dilutions of cDNA volume ranging from 1/64 to 1/128 used for stromelysin-3 amplification was suitable for  $\beta$ -actin amplification.

Indirect coculture with MDA-MB-231, SK-BR-3 and MCF-7 resulted in ST3 mRNA overexpression in normal fibroblasts and tumor-derived fibroblasts from some but not all breast cancers. F4059 showed a positive response to cancer cell induction (Fig. 3b), while some others as F9277 and F11151 responded merely to the induction by indirect coculture (Fig. 3d). ST3 mRNA level by normal skin fibroblasts was not significantly changed after the stimulation with breast cancer cell lines (data not shown). The stimulation of those fibroblasts with the conditioned medium from a cancer cell culture gave the same results as obtained by the coculture with the Transwell system (Fig. 3c). No evident difference of induction was found with either MDA-MB-231, SK-BR-3 or MCF-7 breast cancer cell lines (Fig. 3b).

### **Discussion**

The fact that stromal expression of stromelysin-3 is associated with tumor invasiveness and poor survival indicates that this matrix metalloprotease participates in some cancer progression (Têtu *et al.*, 1998). Many studies reveal also that proteases of stromal origin play a crucial role in mammary carcinoma development and metastasis process (See review (Johnsen *et al.*, 1998). The production of stromal proteases in cancer tissues is, in fact, one host reactive

pattern in process of tumor formation and invasion. Stromal-cancerous epithelial interaction results in tumor-associated tissue remodeling. However, few is known about stromal response patterns for stromelysin-3 production in breast cancer, as many clinical analyses of stromelysin-3 were performed with tumor tissue. To date, only rare studies confirmed in an *in vitro* model the induction of ST3 expression by stromal cells under cancer cell stimulation (Ahmad *et al.*, 1997; Mari *et al.*, 1998). One study combined non-small cell lung cancer cells (NSCLC) and normal pulmonary fibroblasts (Mari *et al.*, 1998). Using direct and indirect coculture methods, this study showed that ST3 was overexpressed by normal human pulmonary fibroblasts under NSCLC cell stimulation and that this induction was tumor-specific in lung cancer. In another assay (Ahmad *et al.*, 1997), the authors have found that MCF-7, MDA-MB-231 and ZR75 induced ST3 expression by human forefront skin fibroblasts. However, the expression was weak compared to the expression of the primary tumors and the authors concluded that the *ex vivo* condition of the study might only partially mimic the *in vivo* situation. In both *in vitro* assays, ST3 mRNA expression by fibroblasts was analyzed by Northern blot and fibroblasts from normal tissues were used. We have adopted a different approach and developed a tumor/stromal coculture assay aimed at reconstituting a structure reminiscent of a primary tumor on a slide. Moreover, the study used not only normal fibroblasts but also mammary tumor-associated stromal cells, as well as cancer cells from primary tumors or established mammary cancer lines. The induction of ST3 expression was assessed by immunocytochemistry and *in situ* hybridization, which allowed us to define the location of cells responding to cancer cell stimulation.

With those reconstituted breast tissue models, cell morphology and distribution of the positive signal of stromelysin-3 expression differed according to the epithelial cell lines used. Coculture of stromal cells with MCF-7 and cancer cells purified from primary tumors produced tumor islands or spheroid with well defined stromal/epithelial interface, while the coculture with the MDA-MB-231 or MGH-U4 was made of isolated cells within this spindle cell population and the coculture with SK-BR-3 was somewhat intermediate and formed small irregular islands and single cells, reflecting the different behavior and proliferation mode of those different cancer cell lines. The co-cultivated cells on the slide reproduced well the tumor tissue section from the primary breast tumor.

Tumor-associated fibroblasts purified from each breast cancer had a typical morphology of fibroblast-like cells in culture and expressed vimentin by immunocytochemistry. Some cells were myofibroblast-like with  $\alpha$ -actin expression. However, we have found that all these fibroblast-like cells from mammary origin consistently showed a positive immunoreaction with anti-CD68 (KP1). CD 68 is a marker of macrophage or myeloid lineage (Micklem *et al.*, 1989). Essentially, macrophages but not fibroblasts or myofibroblasts express CD68. (Tsang and Chan, 1992). It has been reported that fibroblast-like cells *in vitro* might transdifferentiate from monocytes *in vivo* and might keep this pan-macrophage antigen (Reuter *et al.*, 1995). However, other studies have reported CD 68 expression in non macrophage cells, such as in tumor cells of sarcomas, lymphomas, malignant melanomas and carcinomas (Cassidy *et al.*, 1994; Tsang and Chan, 1992). One report concluded that CD 68 was basically a lysosomal marker. Those data suggest that fibroblast-like cells in breast cancer are possible multipotential cells with the capability to modulate from fibroblast-like cells to macrophages under specific conditions.

In the direct coculture using a mixture of stromal and cancer cells released from the same whole digested primary breast tumor, all stromal cells, located close or remote to malignant cells in the original tumor, were mixed and redistributed into culture chambers. The fact that only the fibroblasts relocated at the stromal/epithelial interface overexpressed ST3 mRNA provides further evidence that only stromal cells immediately surrounding the neoplastic cells were reactive to putative inductive signals from cancer cells. The production of ST3 by so-called reactive stromal cells therefore seems to be a response to some messages from epithelial cancer cells. When purified and thus isolated from their cancer environment, tumor-associated fibroblasts with strong ST3 expression in original primary tumor (F9846 in Fig. 1d) stopped expressing ST3 (Fig. 1e). When the fibroblasts were admixed in the new *in vitro* environment with epithelial cancer cells, ST3 overexpression resumed and was limited to fibroblasts situated close to the epithelial front (Fig. 1f and Fig. 2a-c). The induction was shown as tumor-specific and, in fact, no ST3 expression was found in the fibroblasts cocultivated with normal epithelial cells such as NME, 184A1 and keratinocytes. The presence of weak but focally positive expression of ST3 in the fibroblasts cocultivated with MGH-U4 cells is interesting.

The MGH-U4 cell line was obtained from a bladder epithelium with focal severe urothelial atypia. Severe atypia or dysplasia in bladder epithelium is considered as a premalignant abnormality (Murphy and Soloway, 1982). The fact that the MGH-U4 cells could induce ST3 expression supports *in vivo* findings in which ST3 was frequently expressed in preinvasive (dysplastic and carcinoma *in situ*) lesions (Bolon *et al.*, 1996). Those results confirm that cancer cells may induce ST3 overexpression by their neighboring stromal cells and vice versa that those neighboring stromal cells can respond to this stimulation.

In contrast to other *in vitro* models aimed at investigating cancer cell/stromal cell interactions, we have studied interaction of cancer cells, not only with normal breast skin fibroblasts, but also with fibroblasts purified from primary breast carcinomas. It has been well established that tumor-associated fibroblasts are phenotypically different from normal fibroblasts (Hornby and Cullen, 1995). Those tumor-associated fibroblasts have been designated as "activated" (van den Hooff, 1988; van den Hooff, 1991), "abnormal" (Grey *et al.*, 1989) or "reactive" (Têtu *et al.*, 1993; Têtu *et al.*, 1998) in malignant breast tumors. Furthermore, under similar conditions, using cancer cell stimulation and an *in vitro* direct coculture model, the level of ST3 expression differed among the fibroblasts from nine individual tumors. Indeed, marked expression was observed in the coculture with F9846; while no evident ST3 expression was found with F14700. Therefore, the capacity to respond to the induction of the same breast cancer cells differed not only between fibroblasts obtained from normal skin and tumors but also among those obtained from different breast tumors. Although the small number of cases does not allow us to draw strong conclusion, our data suggest that the host phenotype may influence the capacity of its stromal cells to express ST3. Therefore, since recent literature trends to link ST3 overexpression with a poorer prognosis (Ahmad *et al.*, 1998; Chenard *et al.*, 1996; Têtu *et al.*, 1998), our data suggest that not only cancer cell characteristics but also certain host stromal cell features may influence cancer aggressiveness.

The fact that an indirect coculture can induce ST3 expression suggests that the induction was caused at least in part by diffusible/soluble factor(s). In the indirect Transwell coculture system, the epithelial and stromal elements shared the same growth medium and were separated by an one mm space, a very long distance at the cellular level. In order to

compensate for the low sensitivity of Northern blot analyses, we have applied the RT-PCR technology to analyze the stimulation effect in the indirect coculture and ST3 overexpression was detected in part of our cases. In the direct coculture, the distance between stromal and epithelial elements merely reached a few  $\mu\text{m}$  and the signal was stronger in closer fibroblasts. Those data suggest that the induction of ST3 expression by cancer cells was largely through direct cell-cell contact or through secretion of locally concentrated soluble factors. Our data show that the distance between epithelial cancer cells and stromal cells may be critical to the induction of ST3 production. Although we cannot rule out the influence of some diffusible factors, the high level of ST3 mRNA expression at the stromal/epithelial interface strongly suggests the predominance of direct communication of messages from tumor to stromal cells.

In the literature, bFGF has been regarded as a potential activator of ST3 expression. However, Basset *et al.* failed to relate bFGF to ST3 promoter activation (Ahmad *et al.*, 1997). In the study of Mari *et al.* (Mari *et al.*, 1998), bFGF effect has been shown to be regulated only at the post-translational level. bFGF induced active zymogen cleavage to a 35 kDa fragment the function of which remains unknown. Our results indirectly support those reports. Anti-bFGF was used to neutralize endogenous bFGF and exogenous bFGF was added to increase the putative cytokine effect. However, no evident influence was observed at either transcription or transduction level in comparison with the simple coculture with cancer cells.

In conclusion, our study confirms that breast cancer cells can induce ST3 expression by normal and tumor-associated fibroblasts and that this induction is cancer-specific. The ST3 mRNA expression and protein production were only present in the fibroblasts located close to cancer cells, suggesting that the distance is a limiting factor to tumor stimulation. The tumor-associated fibroblasts from different tumors, however, respond differently to this tumor induction, resulting in a variable expression of ST3 and suggesting that different phenotypes exist among those host but tumor-associated fibroblasts and among patients. Furthermore, the induction effect was stronger on fibroblasts purified from breast tumors than on normal skin fibroblasts, suggesting that tumor fibroblasts are more sensitive to cancer cell activation than normal fibroblasts. The induction effect by breast cancer cells may be partly mediated by some diffusible factors. However, our data suggest that cell-cell direct contact plays the most

important role on ST3 induction. Our data provide further evidence that stromal/cancer interaction as well as stromal and cancer cell phenotypes are important in the regulation of ST3 production implicated in the breast cancer progression.

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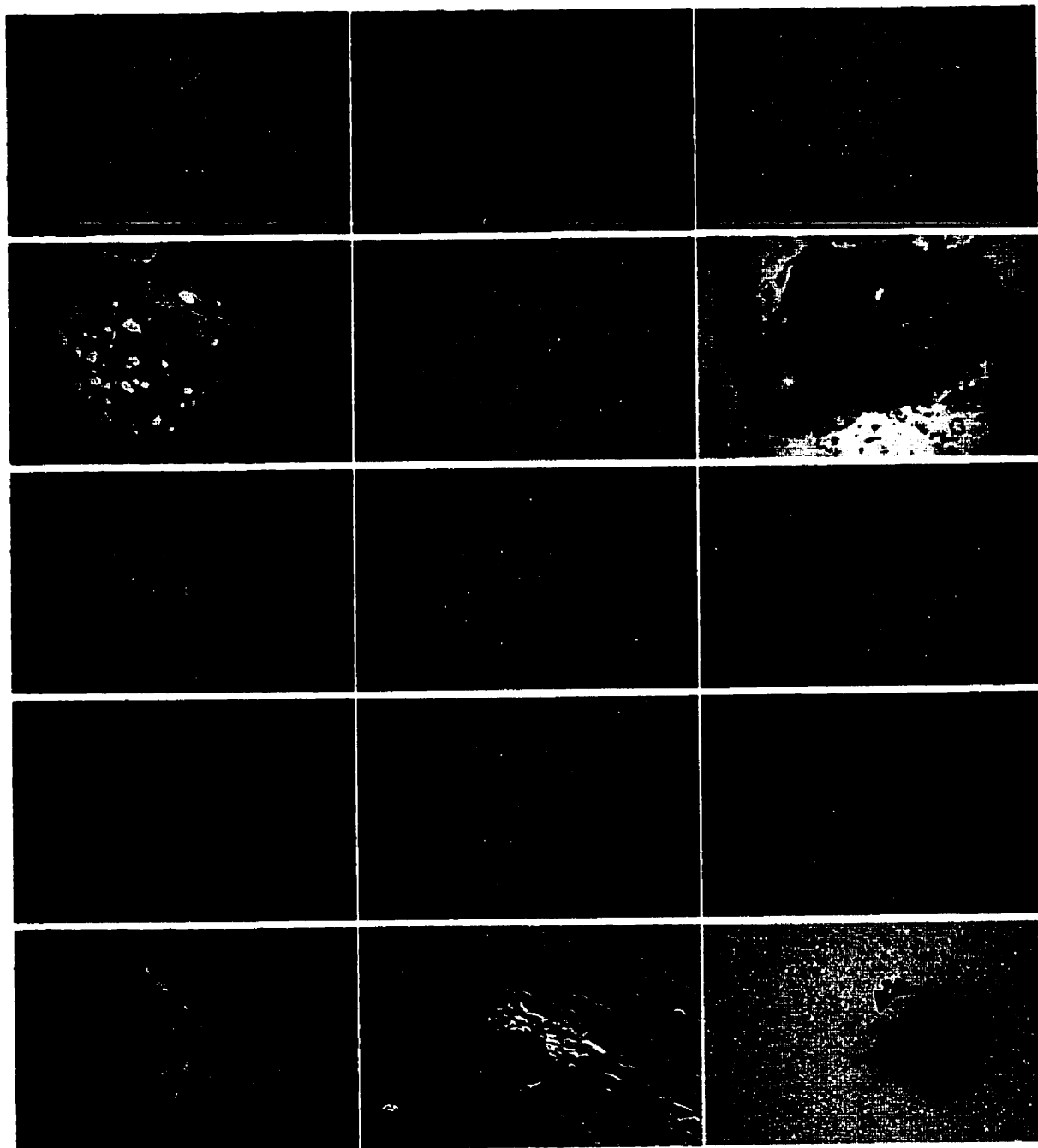


**Figure 1. Representative fields of radioisotopic *in situ* hybridization of ST3 expression.**

**(a):** The direct coculture of F9846 stromal cells with MCF-7 cells identified by anti-EMA mAb (brown color) reconstituted a mimic tumor tissue structure *in vitro* (x200); **(b):** Fibroblast-like cells (F9846) were identified by anti-vimentin mAb (brown color) ) in the reconstituted structure of coculture (x200); **(c):** Some myofibroblast-like cells were identified by anti-human  $\alpha$ -smooth muscle actin mAb (yellow color) (x200); **(d, g, & j):** Varying levels of stromal expression of ST3 mRNA by some primary breast carcinomas (d: T9846, g: T12820, j: T14700) (x200); **(e, h & k):** A very weak or negative ST3 level in purified tumor-associated fibroblasts (x 200) isolated from original cancer tissues (e: F9846 from T9846, h: F12820 from T12820, k: F14700 from T14700); **(f, i & l):** Stromal expression of ST3 mRNA was recovered in some tumor-associated stromal cells by the coculture with MCF-7 cells, and this expression exists only in the stromal cells located in stromal/epithelial interface (f: F9846, x200, i: F12820, x200). However, not all tumor-associated stromal cells gave response to tumor cell stimulation, F14700 (l, x100) gave merely this responsiveness. This difference of *in vitro* responsiveness seems to correspond with the *in vivo* difference of ST3 expression. **(m, n & o):** Stromal expression of ST3 mRNA in primary carcinoma T573 (m, x100). Culture of mixed cells from the same digested tumor led to a relocation of tumor and stromal cells on the culture slide forming spheroid epithelial cancer colonies surrounded by monolayer fibroblasts with well defined interface (n, x100). After relocating all mixed fibroblasts and cancer cells, ST3 overexpression was induced only in fibroblasts relocated close to the cancer colonies (o, x100).

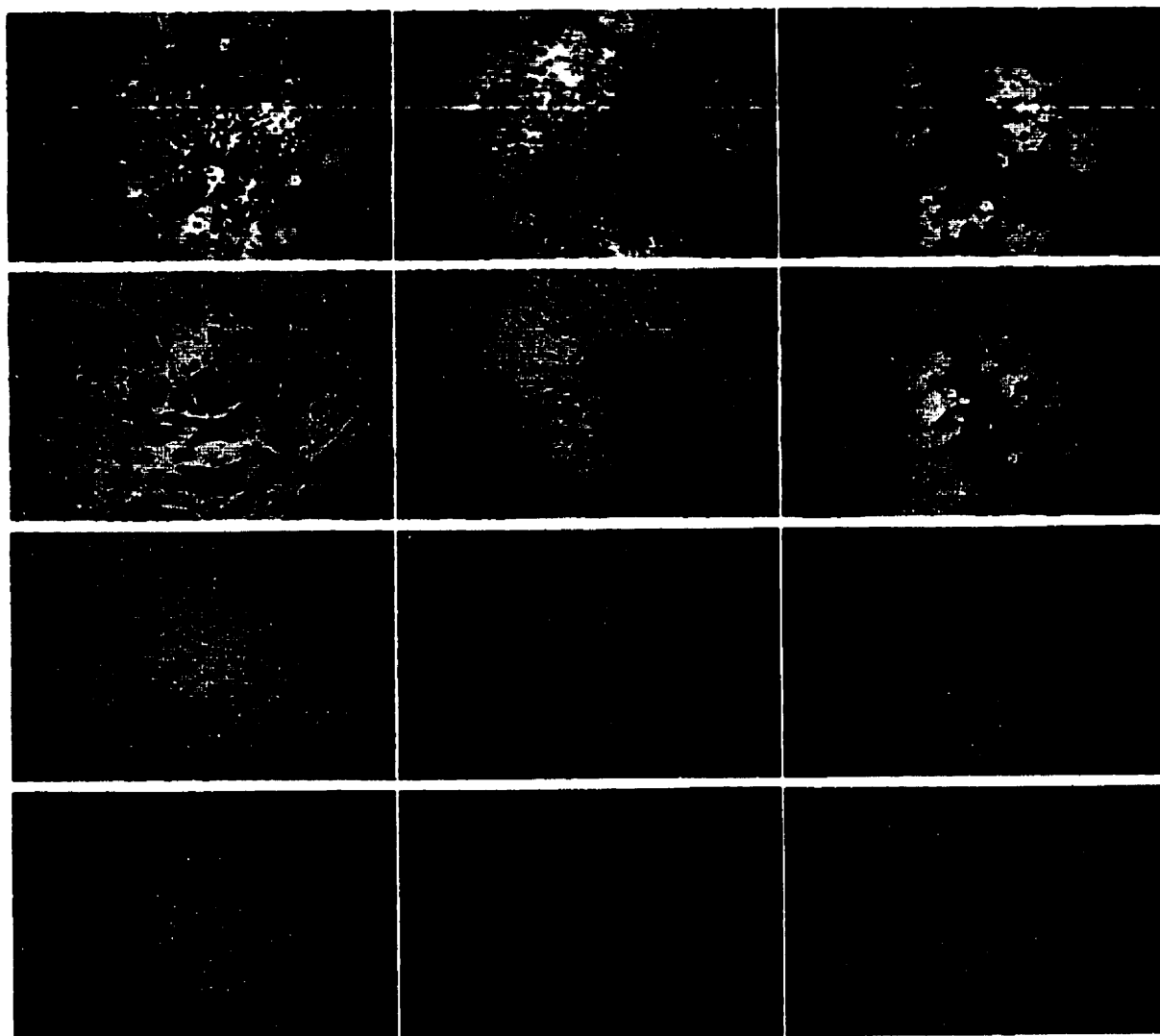
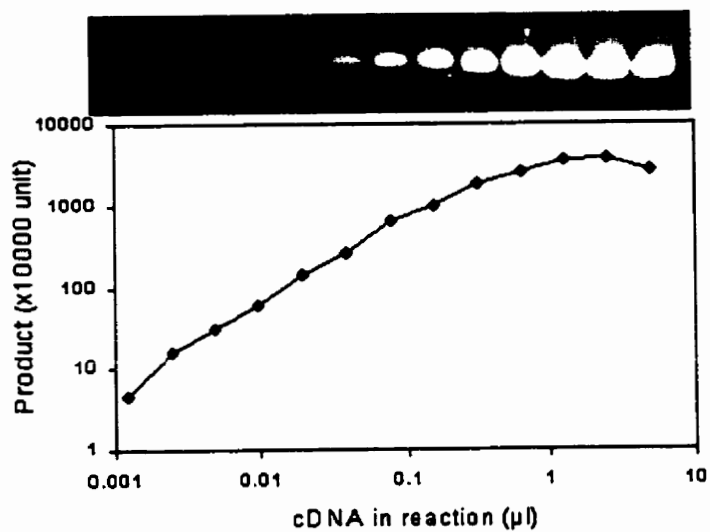


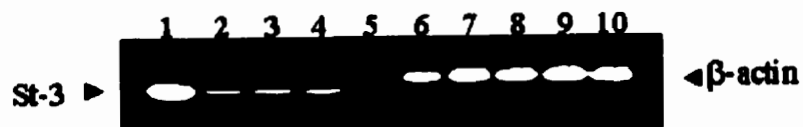
**Figure 2. ST3 mRNA expression by different tumor-associated stromal cells.**

(a, b & c): Stromal expression of ST3 mRNA was recovered in F9846 tumor-associated fibroblasts (x100) by the coculture respectively with MDA-MB-231 (a), SK-RB-3 (b), and MCF-7 (c); (d): The direct coculture resulted also in ST3 protein expression (F9846 with MCF-7), and the positive ST3 cells were located in close contact with cancer cell colonies (x200); (e & f): Coculture of F9846 fibroblasts with MCF-7 cells: the overexpression of protein (e, x100) or ST3 mRNA (f, x100) by stromal cells were not evidently modified by the administration of anti-bFGF mAb (e) or bFGF (f); (g): Weak and focal positive signal of ST3 protein expression was also observed in the coculture of stromal cells (F9846) with MGH-U4 cells (x200). (h): No expression of ST3 protein in stromal cells (F9846, x200) cocultivated with keratinocytes; (i & j): No expression of ST3 mRNA in stromal cells cocultivated with 184A1 cells (i, x100) or normal mammary epithelial cells (j, x100); (k): Control hybridization with sense probe in the coculture of F9846 fibroblasts with MCF-7 cells (x100); (l): A weak expression of ST3 mRNA was observed in NSF cocultivated with MCF-7 cells (x200).

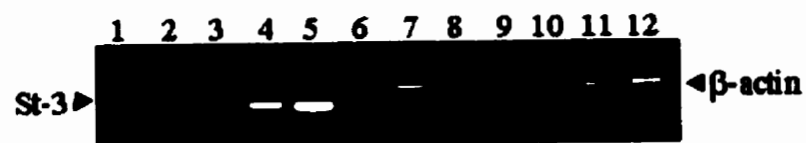
A.



B.



C.



D.

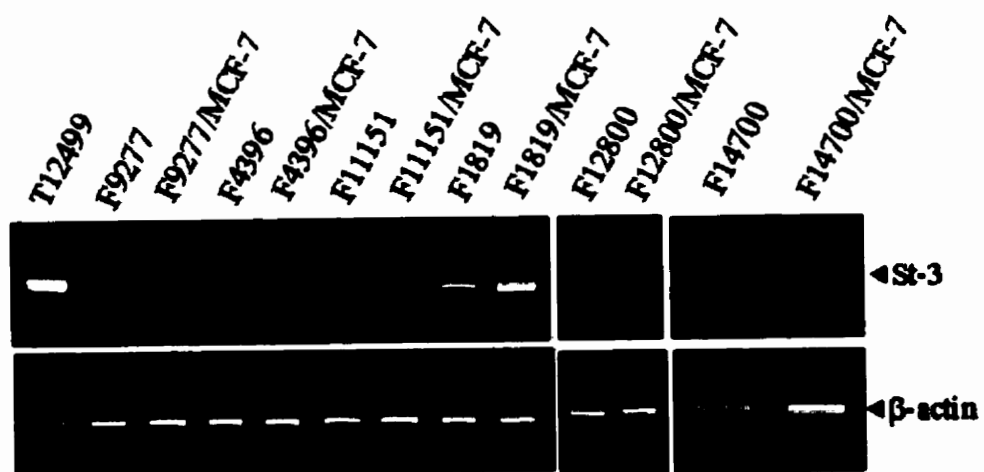


**Figure 3. Stromal expression of ST3 mRNA by indirect coculture.**

**(a): Optimization of PCR condition for  $\beta$ -actin amplification.** The amplified products with radioactivity was separated on 6% polyacrylamide gels and the level of expression was documented by ImageQuaNT. Log-log plot of RT-PCR product versus initial amount of input cDNA showing a linear distribution. The inputting of cDNA at 1:64 and 1:128 dilutions was situated in the linear range and chosen.

**(b): Effect of ST3 induction by cancer cells by indirect coculture with Transwell for 4 days.** 1 & 6: T12499, 2 & 7: F4059 stimulated by MDA-MB-231, 3 & 8: F4059 stimulated SK-BR-3, 4 & 9: F4059 stimulated by MCF-7, 5 & 10: F4059 only. The radioactive gels were exposed to phosphor screen, scanned and analyzed using ImageQuaNT program. The  $\beta$ -actin amplification was done with 1:64 dilution of inputting cDNA.

**(c): The same results were obtained also by cultures of those fibroblasts with the conditioned medium from cancer cell lines.** 1 & 7: F4059 only in culture for 2 days, 2 & 8: F4059 in culture for 2 days with MCF-7 conditioned medium, 3 & 9: F4059 only in the culture of 4 days, 4 & 10: F4059 cultivated with conditioned medium MCF-7 for 4 days, 5 & 11: T12499, 6 & 12: F4059 in culture with bFGF. The gel was stained with ethidium bromide and the  $\beta$ -actin amplification was done with 1:128 dilution of inputting cDNA.

**(d): The results of indirect coculture for 4 days with MCF-7 by Transwell.** T12499: positive control; F: tumor-associated fibroblasts. The  $\beta$ -actin amplification was done with 1:128 dilution of inputting cDNA.

## **CHAPITRE V**

### **VALEUR PRONOSTIQUE DE LA CATHEPSINE D STROMALE DANS LE CANCER MAMMAIRE**

## **AVANT-PROPOS.**

**Le rôle de la cathepsine D stromale comme facteur pronostique dans le cancer du sein a été initialement démontré par le Dr. Têtu et son équipe en 1993. Cette étude a en effet démontré pour la première fois que la cathepsine D stromale et non tumorale influençait la progression du cancer du sein.**

**Ce chapitre présente une étude clinique qui a confirmé la relation entre l'expression de la cathepsine D stromale et la survie à long terme chez des patientes porteuses de cancer du sein. Cet article a été publié dans la revue Breast Cancer research and Treatment (Breast Cancer Res. Treat. 55(2) : 137-147,1999).**

**RESUMÉ.**

En 1993, on a démontré pour la première fois que la cathepsine D stromale était associée à la progression du cancer du sein. Par la suite, plusieurs études ont confirmé ces observations. Dans une série de 1348 patientes porteuses de cancer du sein avec une période de surveillance à long terme ( $>5.2$  ans), on a trouvé une association entre l'expression de la cathepsine D stromale et la récurrence de la maladie et la survie de ces patientes. Les résultats montrent que l'expression de la cathepsine D par des cellules stromales et non les cellules tumorales corrèle significativement avec un taux élevé de récurrence précoce et un faible taux de survie. De plus, l'expression de la cathepsine D stromale corrèle avec d'autres protéases (stromélysine-3, gélatinase A et uPA) par les mêmes cellules stromales. Le résultat confirme le rôle de la cathepsine D stromale dans le cancer du sein.

**Cathepsin D expression by cancer and stromal cells in breast cancer:  
an immunohistochemical study of 1348 cases**

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**Running title: Cathepsin D in breast cancer.**

**ABSTRACT**

**Background:** This study was aimed at investigating the influence of cathepsin D (CD) expression by cancer cells and stromal cells on breast cancer prognosis.

**Methods:** This is a study of 1348 node-positive (NPBC) and node-negative (NNBC) breast cancers diagnosed between 1980 and 1986 and with a minimum follow-up of 5.2 years. CD expression was assessed by immunohistochemistry on archival material using a polyclonal antibody. The expression by cancer and stromal cells was assessed separately and correlated with distant metastasis free (DMFS) and overall survival (OS).

**Results:** CD was expressed by cancer cells in 38.9% of cases and by reactive stromal cells in 43.6%. CD expression by reactive stromal cells, and not cancer cells, correlated significantly ( $p < 0.05$ ) with factors of poor prognosis by cancer cells such as poor histologic and nuclear grades, DNA-anueploidy, c-erbB2 expression, lack of estrogen and progesterone receptors and abnormal p53 expression. A strong association was also found with expression of other proteases (stromelysin-3, gelatinase A and urokinase Plasminogen Activator) by these same reactive stromal cells. CD expression by cancer cells did not predict DMFS or OS but, by univariate analysis, CD expression by reactive stromal cells was associated with earlier recurrence and shorter survival in NNBC ( $p=0.0425$ ) and NPBC patients submitted to adjuvant chemotherapy ( $p=0.0234$ ). However, CD expression by reactive stromal cells remained a significant predictor of recurrence by multivariate analyses in a subgroup of NPBC submitted to adjuvant chemotherapy only.

**conclusion:** Overall, these data support the concept that protease production by reactive stromal cells is under the stimulatory influence of cancer cells. CD expression by stromal cells was however not found to be a significant prognostic factor.

## **Introduction**

Although recent literature regards cathepsin D (CD) as a controversial prognostic factor (Ravdin *et al.*, 1994), initial studies using Enzyme-linked immunosorbent (ELISA) assays suggested that CD overexpression by breast cancer might be a strong predictor of those patients at high risk of relapse (Duffy *et al.*, 1991; Romain *et al.*, 1990; Spyrtos *et al.*, 1989; Tandon *et al.*, 1990). However, data published by immunohistochemistry were controversial from the very beginning and most studies did not find any relation between CD expression and outcome (Domagala *et al.*, 1992; Kandalaf *et al.*, 1993). One study even one obtained a better prognosis with CD overexpression (Henry *et al.*, 1990). Most of those studies were however evaluating the prognostic role of protease expression by cancer cells only without paying attention to the role of CD expressed by stromal cells.

We demonstrated for the first time that, by assessing separately CD expression by cancer and reactive stromal cells, CD expression by stromal cells only predicted patients outcome (Têtu *et al.*, 1993). Since then, three different studies by three different groups reported similar findings on both node-positive and node-negative cases (Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995).

This prompted us to study the long term prognostic influence of CD expression of cancer and reactive stromal cells on a large cohort of node-positive (NPBC) and node-negative (NNBC) breast cancer patients.

## **Material and methods**

### **Population**

The patients included in this study met the following criteria: 1) they had node-positive or node-negative disease proven by histologic examination of axillary lymph nodes, but with absence of distant metastasis at diagnosis; 2) they had hormone receptor assays performed at the "Centre de Recherche en Cancérologie de l'Université Laval" at L'Hôtel-Dieu de Québec; 3) the surgery for breast carcinoma was performed between the 1st January 1980 and the 31st December 1986; the follow-up of surviving patients was available at least until the 1st July 1993.

Clinical information was obtained from the patients' charts by experienced research nurses. The tumor size, number of examined and positive lymph nodes were obtained from the pathology reports. The histologic types along with the histologic and nuclear grades (according to Fisher *et al.* (Fisher *et al.*, 1984)) were reassessed by one of us (B.T.).

### **Immunohistochemistry**

Immunohistochemical studies were performed on a representative section of formalin fixed-paraffin embedded breast carcinomas. In each case, a 5 micron section stained with hematoxylin and eosin was followed by 10 unstained sections for immunohistochemistry and 2 to 4 50 micron sections for DNA flow cytometry. A final 5 micron fixed section was stained with hematoxylin and eosin to insure the persistence of cancer despite the large number of sections performed.

The immunohistochemical study was performed using the avidin-biotin complex (ABC) method as described by Hsu *et al.* (Hsu *et al.*, 1981). Briefly, 5 micron unstained paraffin sections were deparaffinized, rehydrated in graded alcohols and incubated for 15 min with normal serum. The sections were incubated overnight at 4°C with a rabbit polyclonal primary antibody to cathepsin D (Novocastra Laboratories, Newcastle upon tyne, England) at a dilution of 1/150. The immunostaining was followed by a biotinylated secondary antibody and the ABC complex (Vector Laboratories, Burlingame, CA). The reaction was finally revealed with the use of 3-3' Diaminobenzidine (DAB) and the sections were counterstained with hematoxylin.

The sections were reviewed by one of us (B.T.) without knowledge of the clinical and other histopathologic information. The relative number of positive cells (0%, <10%, 10-50% and >50%) was also recorded. As defined in our previous publication (Têtu *et al.*, 1993) and by others (Nadji *et al.*, 1996), CD expression corresponded to the expression of more than 10% cells for the marker. A breast cancer with known strong cathepsin D expression was used as positive control while negative control was obtained by replacing the primary antibody by normal serum. In addition to cathepsin D expression, immunostaining to p53 (Signet Labs,

Dedham, Mass; ID labs, London, Ont., dilution: 1/50), the Heat-shock protein of 27 kDa (HSP-27, Hu27, Dr. Jacques Landry, Hôtel-Dieu de Québec, dilution: 1/200), c-erbB-2 (Triton Biosciences, Alameda, CA; dilution: 1/15) was also performed. For each antibody, positivity was defined by the presence of at least one cell expressing the marker. P53 expression was nuclear, c-erbB-2 was membranous and HSP-27 was cytoplasmic.

### **Flow cytometry**

DNA flow cytometric analysis was performed in all cases on formalin fixed-paraffin embedded tissues using the method described by Dressler *et al.* (Dressler and Bartow, 1989). Ploidy and SPF were determined on single parameter histograms with the use of modify® (Verity Software House inc., Topsham, ME). Debris were excluded with the use of the "Single Cut" algorithm. G0 G1 and G2 M were defined from a Gaussian curve while SPF was evaluated with the trapezoid model. All cases with a coefficient of variation (CV) exceeding 8% were excluded and the CV averaged 6.1% (range: 2.5 to 8.0%).

### **Hormone receptor assays**

Estrogen and progesterone receptor measurement was performed using the hydroxylapatite method (Erdos *et al.*, 1970). Positivity was defined by hormone levels above 10 fmol/mg proteins.

### ***In situ* Hybridization**

The expression of stromelysin-3, gelatinase A and urokinase plasminogen activator was evaluated by *In situ* Hybridization using the method of Wolf *et al.* (Wolf *et al.*, 1993).

### **Statistical analysis**

DMFS and OS curves were obtained according to Kaplan and Meier (Kaplan E.L and Meier P, 1958). The difference between the curves was assessed using the log-rank test (Armitage P, 1991). Correlation between CD immunostaining and other recognized prognostic variables in breast cancer was determined by the Chi-square test (Armitage P, 1991). The Cox proportional multivariate analysis was used to evaluate the relation of CD expression with DMFS and OS adjusting for other known or suspected prognostic factors (Cox D.R., 1972).

The Cox proportional hazard model was used to evaluate the relation of protease expression with recurrence and death adjusting for other known or suspected prognostic factors. Hazard Ratios (HR) for the occurrence of distant metastases or death of patients with or without marker expression were obtained by univariate and multivariate analyses. HR compare the rate of tumor recurrence or death of patients whose tumor expressed a protease to those whose tumor did not express the marker.

## **Results**

### **Population**

A total of 1469 patients were eligible for this study. Of those, the analysis has been performed on 1394. The other cases were excluded because the staining was not interpretable or the tissue did not stick on the slide. Furthermore 46 patients were excluded because they were lost to follow-up and were free of recurrence at last follow-up. Of the 1348 remaining patients, there were 841 NPBC and 507 NNBC. Of the NPBC, 214 (25.4%) received no other treatment than local radiation therapy following surgery. Adjuvant chemotherapy (mostly with cyclophosphamide, methotrexate and 5-fluorouracil) was given to 243 (28.9%), adjuvant hormone therapy (tamoxifen) to 190 (22.6%) and 194 (23.1%) patients received a combination of chemotherapy and hormone therapy. At diagnosis, the age ranged from 22 to 92 years (average: 57 years). The follow-up of surviving patients ranged from 5.2 to 14.7 years and averaged 9.8 years. Forty-six patients were lost to follow-up. At 5 years, 812/1347 (60.3%) of the patients were free of disease.

### **Tumor characteristics**

Most tumors (1162) were invasive ductal carcinomas (86.3%) and, in the NPBC category, 1 to 43 lymph nodes were involved at diagnosis (mean: 5.3). The first recurrence was local in 12.2% of cases, regional in 4.2% and to distant organs in 33.6%, mostly to bones, liver and lungs.

### **Cathepsin D immunostaining**

Of the 1348 cases, cancer cells expressed CD in 524 (38.9%) while reactive stromal cells showed positive immunostaining in 587 (43.6%) cases (figure 1). The immunostaining was

present mostly in stromal cells with morphologic features of macrophages located close to the tumor front.

### **Correlation with other prognostic factors**

Tables 1 and 2 summarize the relation between CD immunostaining by cancer and stromal cells and other well recognized prognostic factors in breast cancer. CD expression by cancer cells was associated with HSP27 expression only ( $p < 0.001$ ). However, overall, CD overexpression by reactive stromal cells was significantly associated with lack of hormone receptors, poor histologic and nuclear grades, ductal carcinoma subtype, aneuploidy, and c-erbB2/neu oncoprotein, p53, HSP27, Stromelysin-3, Gelatinase A and urokinase expression. Stromelysin-3 and Gelatinase A expression were significantly associated with CD expression by stromal cells in NNBC only.

### **Correlation with survival**

No association was found between tumors whose cancer cells expressed CD and survival, regardless of the stage of the disease (fig. 2). However, by univariate analysis, CD expression by reactive stromal cells was significantly associated with a poorer DMFS in NNBC (fig. 3) but not in NPBC (fig. 4) breast cancers. In NPBC, the association between CD and DMFS was significant only for patients submitted to adjuvant chemotherapy (fig. 5). By multivariate analyses, the HR of developing metastases or dying of the disease in patients with NNBC was 30% greater for those with CD expression by reactive stromal cells but CD expression by stromal cells (Table 3) was not an independent factor of recurrence in this subgroup of patients (CI: 0.923-1.1811;  $p = 0.135$ ). In NPBC submitted to adjuvant chemotherapy, the HR was 37% greater for patients with CD expression by reactive stromal cells (Table 3). By multivariate analysis, the difference of risk of developing metastases between cases with and without CD expression was not significant (CI: 0.975-1.938;  $p = 0.0697$ ). The HR of developing recurrence was however 70% greater for CD positive tumors, beginning two years after the diagnosis and remained statistically significant by multivariate analysis (CI: 1.098-2.654;  $p = 0.0175$ ).

## Discussion

This study confirms our prior findings (Têtu *et al.*, 1993) and adds stronger evidence that CD expression by reactive stromal cells and not cancer cells is associated with most factors of poor prognosis by cancer cells. By univariate analysis, we also confirmed our prior findings that CD expression by stromal cells, but not cancer cells, was associated with recurrences in a subgroup of NPBC patients treated by adjuvant chemotherapy and we add new information on NNBC. Three recent publications (Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995) confirmed the prognostic role of reactive stromal cells. Those findings explain most immunohistochemical studies published to date which focused on CD expression by cancer cells, disregarding the influence of CD expression by reactive stromal cells, and which failed to significantly relate CD expression with prognosis (Castiglioni *et al.*, 1994; Charpin *et al.*, 1993; Domagala *et al.*, 1992; Kandalaft *et al.*, 1993; Ravdin *et al.*, 1994; Winstanley *et al.*, 1993). In fact, one immunohistochemical study found a prognostic advantage with cancer cell CD expression (Henry *et al.*, 1990) whereas another correlated significantly CD expression by cancer cells with a poor prognosis (Isola *et al.*, 1993). Our findings explain at least in part that most studies using cytosolic assays consistently related a high cathepsin D level expression with a poor prognosis (Duffy *et al.*, 1991; Romain *et al.*, 1990; Spyrtos *et al.*, 1989; Tandon *et al.*, 1990).

However, most recent literature and our own experience show that CD is not a major prognostic factor. Initial data published by Tandon *et al.* using Western blotting and a polyclonal antibody reported CD as the strongest variable of relapse and survival (Tandon *et al.*, 1990). Those data were later revisited using Western blotting and immunohistochemistry with a monoclonal antibody and the prognostic role of CD was no more significant (Ravdin *et al.*, 1994). The use of a different antibody cannot explain as such the lack of correlation between these two studies since the levels of CD expression by Western blot and immunohistochemistry using the monoclonal antibody correlated with each other and since CD levels measured by Western blotting with two different (polyclonal and monoclonal) antisera also correlated (Ravdin *et al.*, 1994).

Our multivariate analysis confirms our prior study (Têtu *et al.*, 1993) that significantly relates

CD expression by reactive stromal cells with prognosis in the subgroup of NPBC submitted to adjuvant chemotherapy, but only after two years of follow-up. This suggests that tumors whose stromal cells failed to express CD better responded to adjuvant chemotherapy. It is interesting to note that CD expression by stromal cells correlates with HER2/neu expression by cancer cells, which is thought to be linked to drug resistance (Ravdin and Chamness, 1995; Têtu and Brisson, 1994). However, a similar correlation was found by univariate analysis in NNBC while no patient received any adjuvant therapy. Additional studies are clearly needed to better understand the role of CD on breast cancer behavior.

Our data strongly suggest that protease expression by reactive stromal cells is a response to a stimulation of cancer cells on reactive stromal cells. They show that protease expression correlates with several markers of poor prognosis by cancer cells. Interestingly, Roger *et al.* (Roger *et al.*, 1994) demonstrated that CD is mostly produced by macrophages close to cancer cells and Pupa *et al.* (Pupa *et al.*, 1996) reported, in a phenotypic analysis of the leukocytic infiltrate, a preponderance of macrophages in high-grade or c-erbB-2 positive tumors. Furthermore, Leek *et al.* (Leek *et al.*, 1996) reported that an increased macrophage count was an independent marker of poor prognosis. The strong association of CD expression with three other proteases known to be overexpressed essentially by reactive stromal cells confirms that proteases produced by reactive stromal cells are under a common stimulatory influence.

*In vitro* studies confirmed that protease production is at least partly under a paracrine influence of cancer cells. For example, Gelatinase A activity by fibroblasts was enhanced in the presence of MDA-MB 231 or SKBR3 cells, which are known to have a strong invasive potential while MCF-7, a less invasive cell line, had only a weak influence (Noel *et al.*, 1994). Furthermore, the human MCF-7 breast adenocarcinoma cells, but not normal mammary epithelial cells, increased the production of pro-MMP-1,2 and 3 and TIMP-1 by human normal dermal fibroblasts (Ito *et al.*, 1995).

The exact mechanism by which cancer cells induce protease production by stromal cells is not clearly elucidated. However, a 58 kDa plasma membrane glycoprotein produced by cancer cells has been found to induce collagenase I and gelatinase A expression (Biswas *et al.*, 1995).

This shows that certain cancer cells may synthesize a number of diffusible factors with a stimulatory influence on protease production by stromal cells.

Our data clearly indicate that reactive stromal cells are not a reaction of the host aimed at preventing the progression of the disease. Other stromal elements were found to correlate with prognosis in breast cancer. Angiogenesis was recently identified as a major actor in breast cancer behavior (Gasparini and Harris, 1995; Weidner *et al.*, 1991) and a protease produced by stromal cells, urokinase plasminogen activator, was found to be significantly associated with this factor (Hildenbrand *et al.*, 1995). Although the exact role of stromal cells is not clear, it is however evident that those cells do not protect the host against the aggressive potential of the disease.

We conclude that CD expression by reactive stromal cells, and not cancer cells, is associated with factors of poor prognosis by cancer cells, suggesting that CD production by the former is under a paracrine influence of the latter. CD was found, however, to be a weak prognostic marker.

### **Acknowledgements**

This study was financially supported the Cancer Research Society Inc. and the FRSQ-Hydro-Québec joint program of the Fonds de la Recherche en Santé du Québec (FRSQ). Doctor Têtu was recipient of a research clinical scholarship of the FRSQ and of a MRC (Canada)-INSERM (France) exchange award.

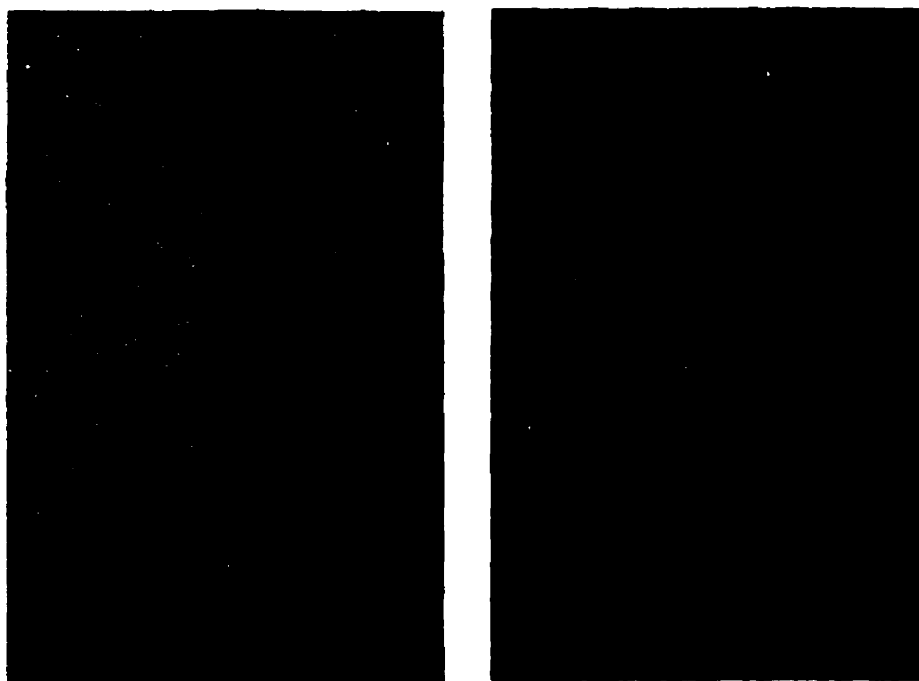


**Figure 1. Immunohistochemical staining of cathepsin D limited to cancer cells (a) or reactive stromal cells (b).**

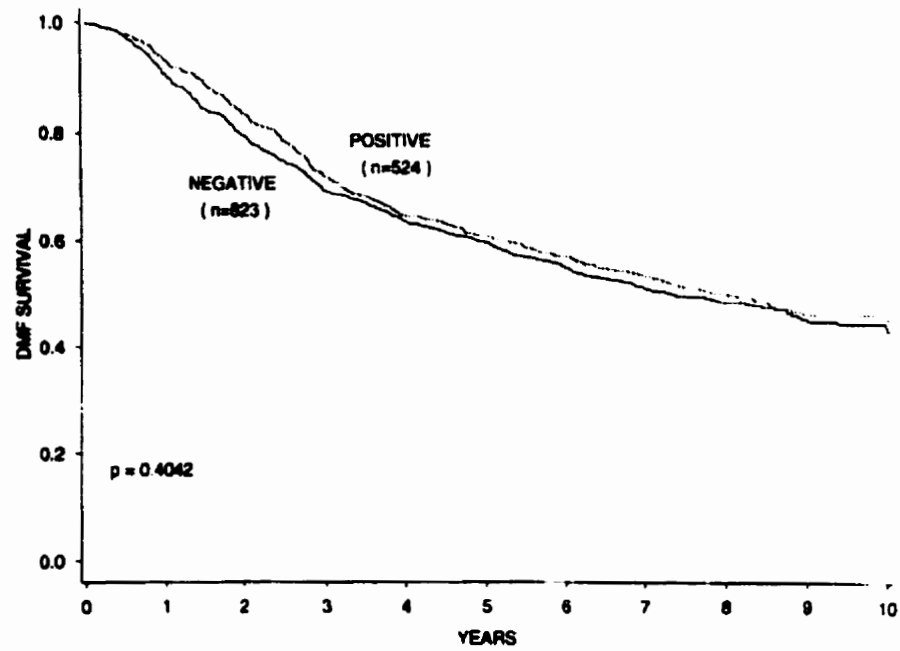


Figure 2. DMFS of patients with node-negative and node-positive disease according to immunostaining for CD by cancer cells.

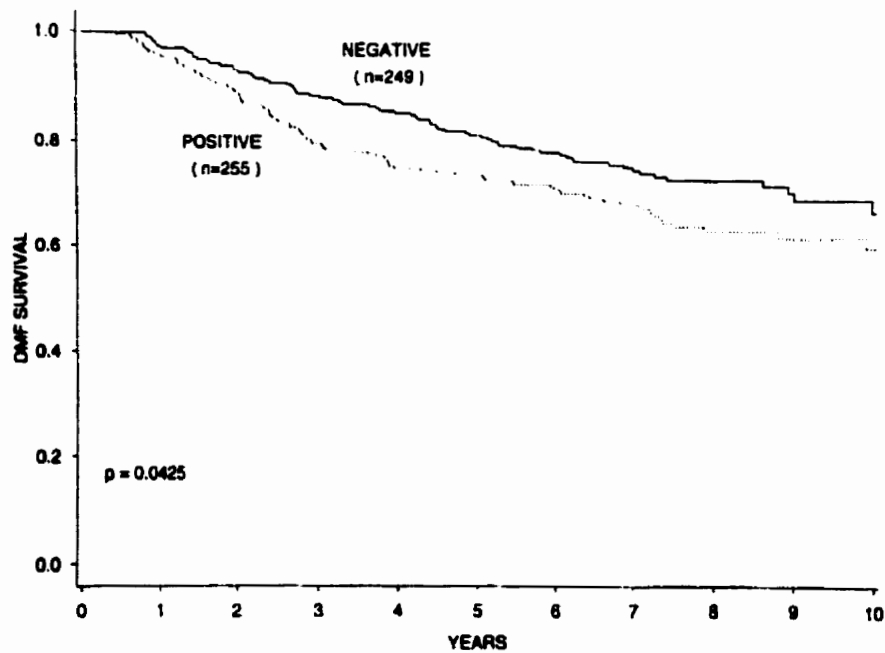


Figure 3. DMFS of patients with node-negative disease according to immunostaining for CD by reactive stromal cells.

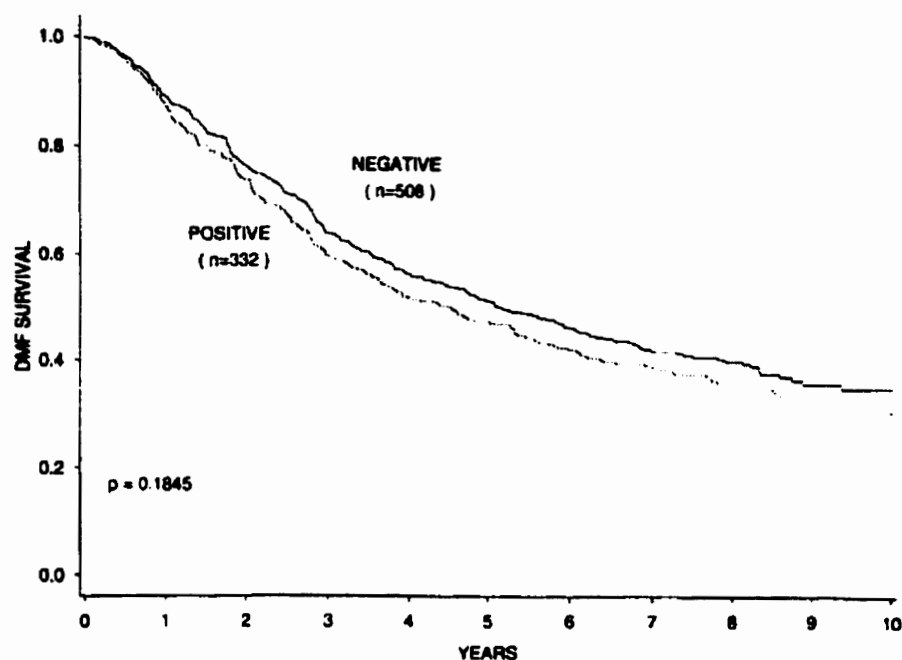


Figure 4. DMFS of patients with node-positive disease according to immunostaining for CD by reactive stromal cells.

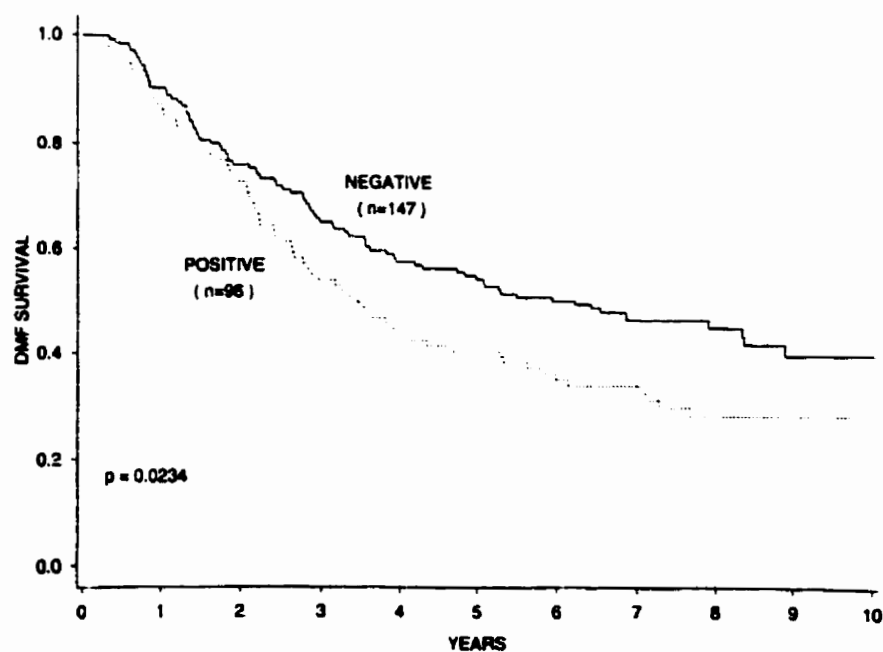


Figure 5. DMFS of patients with node-positive disease who received adjuvant chemotherapy according to immunostaining for CD by reactive stromal cells.

Table 1. Association of CD expression by cancer cells and recognized prognostic factors in breast cancer

| Factor                 | All cases   |         | Node-positive |         | Node-negative |         |
|------------------------|-------------|---------|---------------|---------|---------------|---------|
|                        | N (%)       | p       | N (%)         | p       | N (%)         | p       |
| Age (years)            |             |         |               |         |               |         |
| < 50                   | 382 (39.3)  | 0.852   | 243 (38.7)    | 0.457   | 139 (40.3)    | 0.533   |
| > 50                   | 966 (38.7)  |         | 598 (36.0)    |         | 368 (43.2)    |         |
| Tumor size (cm)        |             |         |               |         |               |         |
| < 3                    | 699 (41.1)  | 0.149   | 350 (36.3)    | 0.834   | 349 (45.8)    | 0.113   |
| > 3                    | 569 (37.1)  |         | 454 (37.0)    |         | 115 (37.4)    |         |
| Vascular invasion      |             |         |               |         |               |         |
| Yes                    | 315 (38.4)  | 0.714   | 252 (40.5)    | 0.535   | 63 (30.2)     | 0.480   |
| No                     | 260 (36.9)  |         | 176 (37.5)    |         | 84 (35.7)     |         |
| Histologic grade       |             |         |               |         |               |         |
| 1                      | 152 (37.5)  | < 0.001 | 69 (29.0)     | 0.024   | 83 (44.6)     | 0.005   |
| 2                      | 712 (43.5)  |         | 463 (40.8)    |         | 249 (48.6)    |         |
| 3                      | 467 (32.5)  |         | 293 (32.4)    |         | 174 (32.8)    |         |
| Nuclear grade          |             |         |               |         |               |         |
| 1                      | 305 (39.7)  | 0.336   | 175 (34.3)    | 0.694   | 130 (46.9)    | 0.066   |
| 2                      | 758 (40.1)  |         | 499 (37.9)    |         | 259 (44.4)    |         |
| 3                      | 268 (35.1)  |         | 151 (36.4)    |         | 117 (33.3)    |         |
| Histologic sub-type    |             |         |               |         |               |         |
| Ductal                 | 1162 (39.2) | 0.630   | 735 (37.0)    | 0.675   | 427 (42.9)    | 0.698   |
| Others                 | 185 (37.3)  |         | 106 (34.9)    |         | 79 (40.5)     |         |
| DNA-ploidy             |             |         |               |         |               |         |
| Diploid                | 379 (35.7)  | 0.822   | 215 (31.6)    | 0.268   | 164 (45.1)    | 0.561   |
| Aneuploid              | 718 (38.2)  |         | 467 (36.0)    |         | 251 (42.2)    |         |
| S-phase fraction       |             |         |               |         |               |         |
| < 10%                  | 243 (39.5)  | 0.119   | 109 (29.4)    | 0.881   | 134 (47.8)    | 0.151   |
| > 10%                  | 119 (31.1)  |         | 89 (30.3)     |         | 30 (33.3)     |         |
| Estrogen receptors     |             |         |               |         |               |         |
| Negative               | 401 (36.4)  | 0.274   | 244 (34.0)    | 0.295   | 157 (40.1)    | 0.559   |
| Positive               | 909 (39.6)  |         | 597 (37.9)    |         | 312 (42.9)    |         |
| Progesterone receptors |             |         |               |         |               |         |
| Negative               | 569 (38.0)  | 0.718   | 358 (35.8)    | 0.620   | 211 (41.7)    | 0.984   |
| Positive               | 737 (38.9)  |         | 481 (37.4)    |         | 256 (41.8)    |         |
| c-erbB-2               |             |         |               |         |               |         |
| Negative               | 1125 (37.6) | 0.031   | 703 (35.0)    | 0.018   | 422 (41.9)    | 0.638   |
| Positive               | 223 (45.3)  |         | 138 (45.7)    |         | 85 (44.7)     |         |
| HSP-27                 |             |         |               |         |               |         |
| Negative               | 623 (27.6)  | < 0.001 | 476 (26.9)    | < 0.001 | 147 (29.9)    | < 0.001 |
| Positive               | 473 (51.4)  |         | 350 (50.0)    |         | 123 (55.3)    |         |
| P53                    |             |         |               |         |               |         |
| Negative               | 967 (38.8)  | 0.748   | 651 (36.9)    | 0.998   | 316 (42.7)    | 0.423   |
| Positive               | 302 (37.7)  |         | 160 (36.9)    |         | 142 (38.7)    |         |
| Stromelysin-3          |             |         |               |         |               |         |
| Negative               | 286 (35.7)  | 0.282   | 142 (35.2)    | 0.684   | 144 (36.1)    | 0.185   |
| Positive               | 349 (39.8)  |         | 217 (37.3)    |         | 132 (43.9)    |         |
| Gelatinase A           |             |         |               |         |               |         |
| Negative               | 289 (36.0)  | 0.100   | 181 (33.7)    | 0.299   | 108 (39.8)    | 0.117   |
| Positive               | 259 (42.9)  |         | 177 (39.0)    |         | 82 (51.2)     |         |
| Urokinase              |             |         |               |         |               |         |
| Negative               | 383 (40.2)  | 0.457   | 255 (37.6)    | 0.444   | 128 (45.3)    | 0.728   |
| Positive               | 163 (36.8)  |         | 102 (33.3)    |         | 61 (42.6)     |         |

**Figure 2. Association of CD expression by reactive stromal cells and recognized prognostic factors in breast cancer**

| Factor                 | All cases   |         | Node-positive |         | Node-negative |         |
|------------------------|-------------|---------|---------------|---------|---------------|---------|
|                        | N (%)       | p       | N (%)         | p       | N (%)         | p       |
| Age (years)            |             |         |               |         |               |         |
| < 50                   | 382 (43.7)  | 0.972   | 243 (39.9)    | 0.868   | 139 (50.4)    | 0.948   |
| > 50                   | 963 (43.6)  |         | 598 (39.3)    |         | 365 (50.7)    |         |
| Tumor size (cm)        |             |         |               |         |               |         |
| < 3                    | 698 (45.1)  | 0.558   | 350 (40.0)    | 0.919   | 348 (50.3)    | 0.115   |
| > 3                    | 568 (43.5)  |         | 454 (39.6)    |         | 114 (58.8)    |         |
| Vascular invasion      |             |         |               |         |               |         |
| Yes                    | 315 (45.4)  | 0.642   | 252 (43.7)    | 0.424   | 63 (52.4)     | 0.886   |
| No                     | 260 (43.5)  |         | 176 (39.8)    |         | 84 (51.2)     |         |
| Histologic grade       |             |         |               |         |               |         |
| 1                      | 151 (33.8)  | < 0.001 | 69 (27.5)     | 0.007   | 82 (39.0)     | 0.001   |
| 2                      | 711 (40.4)  |         | 463 (37.8)    |         | 248 (45.2)    |         |
| 3                      | 466 (52.8)  |         | 293 (46.1)    |         | 173 (64.2)    |         |
| Nuclear grade          |             |         |               |         |               |         |
| 1                      | 303 (29.0)  | < 0.001 | 175 (25.7)    | < 0.001 | 128 (33.6)    | < 0.001 |
| 2                      | 758 (46.0)  |         | 499 (41.7)    |         | 259 (54.4)    |         |
| 3                      | 267 (55.1)  |         | 151 (50.3)    |         | 116 (61.2)    |         |
| Histologic sub-type    |             |         |               |         |               |         |
| Ductal                 | 1161 (46.3) | < 0.001 | 735 (41.5)    | 0.002   | 426 (54.7)    | 0.698   |
| Others                 | 183 (26.8)  |         | 106 (25.5)    |         | 77 (28.6)     |         |
| DNA-ploidy             |             |         |               |         |               |         |
| Diploid                | 379 (32.7)  | < 0.001 | 215 (26.5)    | < 0.001 | 164 (40.9)    | < 0.001 |
| Aneuploid              | 717 (47.1)  |         | 467 (41.3)    |         | 250 (58.0)    |         |
| S-phase fraction       |             |         |               |         |               |         |
| < 10%                  | 243 (30.5)  | 0.278   | 109 (21.1)    | 0.137   | 134 (38.1)    | 0.124   |
| > 10%                  | 119 (36.1)  |         | 89 (30.3)     |         | 30 (53.3)     |         |
| Estrogen receptors     |             |         |               |         |               |         |
| Negative               | 400 (53.8)  | < 0.001 | 244 (48.4)    | < 0.001 | 156 (62.6)    | < 0.001 |
| Positive               | 907 (38.4)  |         | 597 (35.8)    |         | 310 (43.2)    |         |
| Progesterone receptors |             |         |               |         |               |         |
| Negative               | 567 (50.3)  | < 0.001 | 358 (45.0)    | 0.005   | 209 (59.3)    | < 0.001 |
| Positive               | 736 (37.4)  |         | 481 (35.3)    |         | 255 (41.2)    |         |
| c-erbB-2               |             |         |               |         |               |         |
| Negative               | 1125 (41.3) | < 0.001 | 703 (37.4)    | 0.006   | 419 (47.4)    | 0.004   |
| Positive               | 223 (55.6)  |         | 138 (50.0)    |         | 85 (64.7)     |         |
| HSP-27                 |             |         |               |         |               |         |
| Negative               | 623 (34.1)  | < 0.001 | 476 (30.7)    | < 0.001 | 146 (45.2)    | 0.010   |
| Positive               | 473 (53.7)  |         | 350 (51.1)    |         | 123 (61.0)    |         |
| P53                    |             |         |               |         |               |         |
| Negative               | 966 (40.3)  | < 0.001 | 651 (36.1)    | < 0.001 | 315 (48.9)    | 0.091   |
| Positive               | 301 (57.5)  |         | 160 (57.5)    |         | 141 (57.4)    |         |
| Stromelysin-3          |             |         |               |         |               |         |
| Negative               | 284 (37.0)  | < 0.001 | 142 (37.3)    | 0.193   | 142 (36.6)    | < 0.001 |
| Positive               | 349 (51.9)  |         | 217 (44.2)    |         | 132 (64.4)    |         |
| Gelatinase A           |             |         |               |         |               |         |
| Negative               | 287 (36.9)  | < 0.001 | 181 (37.0)    | 0.074   | 106 (36.8)    | < 0.001 |
| Positive               | 259 (52.1)  |         | 177 (46.3)    |         | 82 (64.6)     |         |
| Urokinase              |             |         |               |         |               |         |
| Negative               | 382 (36.4)  | < 0.001 | 255 (35.3)    | < 0.001 | 127 (38.6)    | < 0.001 |
| Positive               | 162 (61.7)  |         | 102 (56.9)    |         | 60 (70.0)     |         |

**Table 3. Hazard Ratios for distant metastases or death of breast cancers with CD expression by reactive stromal cells.**

| <b><u>Category</u></b> | <b><u>Univariate</u></b>  | <b><u>P value</u></b> | <b><u>Multivariate*</u></b> | <b><u>P value</u></b> |
|------------------------|---------------------------|-----------------------|-----------------------------|-----------------------|
|                        | <b><u>HR (95% CI)</u></b> |                       | <b><u>HR (95% CI)</u></b>   |                       |
| <b>All patients</b>    | 1.083 (0.931-1.260)       | 0.2995                | 1.101 (0.938-1.292)         | 0.2410                |
| <b>NNBC</b>            | 1.383 (1.010-1.894)       | 0.0435                | 1.293 (0.923-1.811)         | 0.1351                |
| <b>NPBC</b>            | 1.125 (0.945-1.339)       | 0.1849                | 1.058 (0.881-1.270)         | 0.5491                |
| <b>NPBC chem</b>       | 1.450 (1.050-2.004)       | 0.0242                | 1.374 (0.975-1.938)         | 0.6970                |
| <b>0-2 YFU**</b>       | 1.168 (0.703-1.940)       | 0.5494                | 0.970 (0.557-1.687)         | 0.9131                |
| <b>2-10 YFU</b>        | 1.687 (1.109-2.565)       | 0.0145                | 1.707 (1.098-2.654)         | 0.0175                |

\* Adjusted for Lymph node invasion, histologic and nuclear grades and hormone receptors.

\*\* Years of Follow-up

## **CHAPITRE VI**

### **LA PRODUCTION DE LA CATHEPSINE D PAR LES CELLULES STROMALES ASSOCIÉES À LA TUMEUR MAMMAIRE**

**AVANT-PROPOS.**

Le rôle de la cathepsine D dans la progression du cancer mammaire demeure controversé à ce jour. Différents résultats contradictoires rendent l'utilisation difficile de cette protéase comme facteur pronostique.

Ce chapitre présente une étude *in vitro* portant sur la production de cathepsine D par des cellules stromales cocultivées avec des cellules cancéreuses mammaires. Cet article a été soumis pour fin de publication dans la revue Human Pathology.

**RESUMÉ.**

Utilisant une coculture *in vitro* des cellules stromales purifiées de carcinomes mammaires avec des lignées cellulaires de cancer du sein, nous avons observé pour la première fois la production de cathepsine D par les cellules stromales en présence de cellules cancéreuses mammaires. Par coculture directe et culture avec milieu conditionné de cellules cancéreuses, on observe une augmentation de l'expression de cathepsine D dans les cellules stromales. L'analyse par Western blot montre que la cathepsine D augmentée dans les cellules stromales est toujours sous sa forme mature. Ces résultats encourageants devront être complétés par des cocultures tridimensionnelles et de recherches au niveau de la transcription.

**Cathepsin D in tumor-associated fibroblasts  
cocultivated with breast cancer cells**

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**Running title: cathepsin D in tumor-associated fibroblasts**

## **Abstract**

Despite conflicting results, the prognostic value of cathepsin D in breast cancer patients revealed by different studies of large series suggests that this enzyme play an important role in breast cancer invasion. We recently showed that therapeutic outcome was significantly associated with cathepsin D expression by stromal cells and but not by tumor cells, indicating unnegligible role of stromal cathepsin D contributed to this cancer progression. In order to investigate if stromal cathepsin D production is changed in cancer situation, we have analyzed the cytoplasmic cathepsin D in tumor-associated fibroblasts purified from primary breast carcinomas in the presence of breast cancer cells MDA-MB-231, SK-BR-3 and MCF-7. By cocultivating tumor-associated fibroblasts and breast cancer cells *in vitro*, the cathepsin D was analyzed using same method as that used in our published clinical studies. The cathepsin D was found elevated in stimulated tumor-associated fibroblasts in simple non-glycosylated mature form. No precursor was found elevated in tumor-associated or normal fibroblasts by cancer cell stimulation in any case. The results confirm the inductive effect of cancer cells over expression of cathepsin D in tumor-associated fibroblasts. Further study should be focus to elucidate whether this elevated stromal cathepsin D could be implicated directly in the degradation of extracellular matrix.

## **Introduction**

The place of cathepsin D as a prognostic factor has been well discussed in clinical research of the human breast cancer (see review (Westley and May, 1996). The cathepsin D was reported to be an independent prognostic marker in women with stage I breast cancer (Rochefort, 1990; Spyrtos *et al.*, 1989; Tandon *et al.*, 1990; Thorpe *et al.*, 1989). Despite discrepancies of results and different analytic methods used, some recent studies with large population reported and confirmed prognostic value of cathepsin D in breast cancer patients (Ferrandina *et al.*, 1997; Foekens *et al.*, 1999; Têtu *et al.*, 1999). Overexpression of cytosolic cathepsin D of tumor tissues (Ferrandina *et al.*, 1997; Foekens *et al.*, 1999) or the cathepsin D by stromal cells (Têtu *et al.*, 1999) in the tumor tissues were respectively to correlate with breast cancer progression and poor survival rate of patients, indicating that cathepsin D participates in breast cancer invasion by degrading extracellular matrix.

Principally, two distinguished methods are used in cathepsin D prognostic value analyses in breast cancer: analysis of cytosol of total tumor tissue or analysis of cathepsin D in different cell types by immunohistochemistry. The analysis of cytosol of total tumors by Western Blot, IRMA and ELISA measures total cathepsin D in tumor tissue including secreted precursor by cancer cells and the cathepsin D by both cancer and stromal cells. Secreted cathepsin D precursor has been considered one specific pattern of cathepsin D for breast cancer (Rochefort, 1990). It is reported that secreted precursor can be autoactivated into a 51 kDa cathepsin D at acidic pH 4,5 and this 51 kDa cathepsin D can digest extracellular matrix *in vitro* (Briozzo *et al.*, 1988). Although acidic pH microenvironment may be created in cancer situation, one *in vitro* study fails to show association of secreted cathepsin D by several breast cancer cell lines with invasiveness (Johnson *et al.*, 1993). In fact, extracellular proteolysis by secreted cathepsin D in extracellular matrix may not be only extracellular matrix degradation pattern of cathepsin D. The phagocytose, endocytose by large acidic vesicles (Montcourrier *et al.*, 1990) in breast cancer cells, as well as internalization of secreted cathepsin D by cancer and stromal cells (Laurent-Matha *et al.*, 1998; Rochefort and Liaudet-Coopman, 1999) were also observed. Moreover, phagocytose and endocytose are rather activities specific to macrophages, monocytes and polynuclear neutrophiles, as phagocytic cancer cells seem rare (Spivak, 1973).

Immunohistochemistry approaches with separate analyses of cancer or stromal cell cathepsin D was initiated in 1993 (Têtu *et al.*, 1993), when some other studies have produced conflicting results regarding prognostic value of the cathepsin D in breast cancer patients (Domagala *et al.*, 1992; Ravdin *et al.*, 1994). Some authors even reported good correlation between the expression of cathepsin D in the tumor tissue and increased survival (Henry *et al.*, 1990). Although this method rule out the putative secreted cathepsin D in the analysis, the approach permits to analyze the cathepsin D by different cell types responsible for poor prognostic. In fact, by this method, some separate groups have found and respectively confirmed stromal cell cathepsin D associated with breast cancer progression (Gonzalez-Vela *et al.*, 1999; Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995; Têtu *et al.*, 1993; Têtu *et al.*, 1999; Visscher *et al.*, 1993). If intracellular phagocytose and endocytose, as well as internalization of secreted cathepsin D are principal patterns of cathepsin D proteolytic function, these events can be identified also by cellular analysis with immunohistochemistry.

The fact that cathepsin D by stromal cells are associated with tumor progression puts a question for us: do cathepsin D of stromal cells promote cancer progression or do stromal cells have more important phagocytose or endocytose activities by cathepsin D in a malignant situation of breast cancer? It is known now that cancer invasion can be considered as a tissue remodeling process that involved a proteolytic degradation of extracellular matrix in the surrounding normal but tumor-associated tissue. Most of the components regulating proteolysis are made by nonmalignant stromal cells and not by the epithelial cancer cells in invasion of breast cancer (Johnsen *et al.*, 1998). To date, no *in vitro* studies have been reported in this aspect. In order to investigate if stromal cathepsin D can be over-produced in the breast cancer, we have analyzed the *in vitro* production of cathepsin D by normal or tumor-associated fibroblasts cocultivated with breast cancer cells. This is a first report.

## **Materials and methods**

### **Stromal cells**

The fibroblasts used in this study were purified from three primary breast carcinomas and one normal mammary gland tissue. Table 1 shows the histological characteristics and clinical data of these tissues samples. Briefly, fresh sterile tissue specimens from primary breast tumors or normal mammary tissue were obtained after surgical resection. The diagnosis of carcinomas was confirmed by routine histopathological examination. The tumor tissue specimens were then trimmed and placed in Dulbecco's modified Eagle's medium (DMEM) with antibiotics at 4 °C. Then, the tissue specimen was minced with crossed surgical blades into about 1 mm cubes and then digested with collagenase (0,14%) at 37°C overnight with shaking. The single-cell suspension was obtained and the cell viability was determined by the trypan blue exclusion technique.

Tumor-associated fibroblasts were obtained from all 3 primary breast cancers and the fibroblasts from normal tissue by serial passages with their predominant overgrowth in cultures. Only cells obtained between passage 5 and 10 were used for this study. Cells were maintained in DMEM with 10% fetal bovine serum (FBS). Cell lines were incubated at 37°C with 5% CO<sub>2</sub>. The fibroblastic natures of those cells were confirmed by their morphology, the

expression of vimentin, and the absence of cytokeratins.

### **Cancer cell lines**

Human breast cancer lines used in the study are MDA-MB-231, SK-BR-3 and MCF-7. All cells were maintained in DMEM with 10% FCS and incubated at 37 °C with 5% CO<sub>2</sub>.

### **Direct coculture with cell lines**

2 x 10<sup>5</sup> fibroblasts were seeded in the chamber slides (Nunc, Naperville, IL), 1 x 10<sup>5</sup> cancer cell lines were added when those fibroblasts were confluent. The cells were cultivated with DMEM plus 10% FCS during 24 hrs then washed twice by sterile PBS and cocultivated in DMEM during 2 to 4 days according to assay design. Parallel culture of same tumor-associated stromal cell without cancer cells served as control. In the end of coculture, the chamber slides with cells were washed twice by PBS and store in -80 °C until use.

### **Indirect coculture**

Indirect coculture was performed using Transwell culture apparatus (Corning Costar, Cambridge, MA). 1 x 10<sup>5</sup> fibroblasts were first seeded in the lower chamber and 24 hrs after, 1 x 10<sup>5</sup> epithelial cell lines in the upper chamber of the Transwell systems (0.4 µm pore size). The coculture was performed for the first 24 hrs with DME and 10% FCS and then washed twice by sterile PBS and cocultivated for 2 to 6 days in DME without FCS in 5% CO<sub>2</sub> incubator. Parallel culture of same tumor-associated stromal cell without cancer cells served as control. The coculture was repeated in duplicate for each cell line studied.

Indirect coculture was also performed by using daily changed conditioned medium. The conditioned medium was obtained by culturing cancer cell lines in DMEM without FBS during 24 hrs. The culture media were collected and centrifuged in 250 x g during 10 min. The supernatant was then stored in 4°C until use. The coculture was also repeated in duplicate for each cell line studied.

### **Immunocytochemistry**

Tumor-like cells grown on coverslips or chamber slides were washed in PBS for 5 minutes,

fixed for 15 min at -20°C with cold methanol then quenched for 5 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), slides were covered with normal horse serum during 20 minutes and incubated overnight with each monoclonal antibody at 4°C. Afterwards, slides were rinsed with PBS and exposed for 30 minutes to a secondary biotinylated anti-mouse antibody. After another wash with PBS, slides were incubated with the avidin-biotin-peroxidase complex (Vector laboratory, Burlingame, CA) and the reaction was revealed with diaminobenzidine (DAB). The coverslips and slides were finally counterstained with Mayer's haematoxylin for 3 minutes and mounted in permount.

Monoclonal antibodies used for epithelial markers were anti-epithelial membrane antigen (EMA) (Dako, Mississauga, Ontario, Canada), anti-human cytokeratin identifying cytokeratin 8 and 18 (CAM 5.2, Becton Dickinson, Mississauga, Ontario). Other antibodies used were anti-vimentin (Dako), anti-CD68 (DAKO) and anti-human  $\alpha$ -smooth muscle actin (Dako).

Cathepsin D analysis was performed using double immunoenzyme labeling based on two separate, immunoenzymatic procedures performed on the same slide containing the cells in culture. The technique consisted of two sequential immunohistochemical reactions in our study, and horseradish peroxidase with diaminobenzidine (DAB) and alkaline phosphatase with new fuchsin substrate were used. The first labeling used anti-epithelial membrane antigen (EMA) (Dako) at room temperature for 1 h and the reaction with DAB gave brown color. After the reaction with DAB, the slides were treated with pronase A for 15 min at room temperature. After washes with PBS and reblocked with normal horse serum, the slides were incubated overnight at room temperature with the second immunolabeling antibody: anti-cathepsin D mAb (Ab-1, Oncogene Research Products, Cambridge, MA). The slides were then washed and incubated with phosphatase-conjugated goat anti-mouse polyclonal antibody (Dako). The immunoreaction was finally detected by enzymatic reaction with new fuchsin substrate, which gave the red color. The slides were counterstained with Mayer's haematoxylin for 3 minutes and mounted in Glycergel (Dako).

### **Western blot analysis**

The cells in culture were washed twice with ice-cold PBS, and the proteins were extracted with lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecylsulfate and 50 mM Tris pH 8.0) for 1 h at 4 °C. The supernatants were centrifuged at 10,000 x g for 30 min at 4 °C and the quantity of the protein was measured using DC Protein Assay kit (Bio-Rad, Mississauga, Ontario). Fifty µg of each protein extract were heated with β-mercaptoethanol, separated by electrophoresis in a 10% polyacrylamide gel and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec). The membrane was soaked for 1 h in 5% non-fat dry milk then incubated at room temperature overnight in Tris-buffered saline (TBS) containing anti-human cathepsin D polyclonal antibody (Ab-2, Oncogene Research Products, Cambridge, MA) and monoclonal antibody (Ab-1, Oncogene Research Products), washed and finally incubated with peroxidase-conjugated goat anti-mouse polyclonal antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) for one hour at room temperature. After washes, antibody binding was detected by chemiluminescence (Renaissance<sup>R</sup>, NEN Life Science Products, Boston, MA). Molecular weights of the bands were estimated by their mobilities relative to known molecular weight standards. In the selective cases, membranes were incubated by TBS containing secondary antibody labeled by <sup>125</sup>I and the membranes were subjected to phosphor screen and then analyzed by ImageQuaNT (Molecular Dynamics). The treatment with endoglycosidase was performed by incubation of 50 µg of protein sample with N-glycosidase F (Boehringer Mannheim, Laval, Quebec) at 3.6 u/ml in extraction buffer overnight at 37°C then heated with β-mercaptoethanol and separated by electrophoresis in a 10% polyacrylamide gel as cited above.

### **Results**

All purified tumor-derived fibroblasts were fibroblast-like cells in culture. The mesenchymal origin of fibroblast-like cells purified from primary breast carcinomas and that of fibroblasts purified from the normal breast tissues were confirmed by the lack of human cytokeratin expressions (8 & 18), presence of vimentin and their spindle-cell morphology (Fig. 1a). Unexpectedly, immunoreaction was positive for CD68 (KP1) in all cases (Fig. 1b).

In the direct coculture assay, cell proliferation resulted in a reconstituted structure reminiscent of the tumor seen on the original section. In general, cancer epithelial cells formed colonies, surrounded by a monolayer of fibroblasts. The epithelial cancer cells were identified by anti-EMA mAb. The expression of intracellular cathepsin D in epithelial cancer cells was masked by brown color due to reaction by horseradish peroxidase with diaminobenzidine (DAB). While stromal cathepsin D was identified as red color due to enzymatic reaction with new fuchsin substrate (Fig. 1c).

Intercellular Cathepsin D expression was weak or absent by immunocytochemistry in unstimulated tumor-associated fibroblasts purified and isolated from each primary tumor (Fig. 1d). After direct coculture with malignant cancer cells, immunocytochemical analysis showed that in tumor-associated stromal cells or normal skin fibroblasts, intracellular cathepsin D was overexpressed. In each positive case, cathepsin D expression was observed in fibroblasts located not only in close contact but remote with cancer cells and colonies. All stromal positive signals were red and granular representing cytoplasmic/lysosomal cathepsin D. This over-expression of cathepsin D after the coculture with cancer cells was found in three tumor-associated stromal cells as well as in the non-tumor-associated mammary stromal cells. Among three cancer cell lines, MDA-MB-231 and SK-BR-3 have induced over-expression of cathepsin D in each stromal cell lines used, while MCF-7 has induced only in one tumor-associated stromal cell line. The representative results are shown in Figure 1.

Western blot showed that in tumor-associated stromal cells and normal mammary fibroblasts, there was a base of cathepsin D principally in mature form, while in cancer cell lines, both mature form and precursor were expressed (Fig. 2a & 2b). All natural cytoplasmic cathepsin D is in form of N-glycosylation, this is shown by treatment with N-glycosidase F (Fig. 2b). Chemiluminescence method was used in the initial assays and gave often a band in about 38 kDa.

By culture with conditioned media of cancer cells, cathepsin D in the stromal cells were found changed, resulting in overexpression of intracellular cathepsin D in mature form by Western blot analysis (Fig 3a). The maximum change was in 6<sup>th</sup> days of culture (Fig. 3c). No evident

precursor was found after culture with conditioned medium. However, by indirect coculture with Transwell, only a minor change of cathepsin D expression was observed (Fig. 3b).

## Discussion

Prognostic value of cathepsin D is an undefined issue in breast cancer research work. Despite different methods used, its prognostic value has been reported to be associated with either total cytosolic enzyme in tumor tissue (Ferrandina *et al.*, 1997; Foekens *et al.*, 1999; Spyrtatos *et al.*, 1989; Thorpe *et al.*, 1989), or with stromal cell cathepsin D (Gonzalez-Vela *et al.*, 1999; Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995; Têtu *et al.*, 1993; Têtu *et al.*, 1999), or cancer cell cathepsin D (Isola *et al.*, 1993), or both stroma and malignant cell cathepsin D (Visscher *et al.*, 1993). However, some other reports failed to show this prognostic value (Domagala *et al.*, 1992; Ravdin *et al.*, 1997; Ravdin *et al.*, 1994). Taking account of some large series with great population, we find these different results persist. By measuring cytosolic cathepsin D, Ferrandina (Ferrandina *et al.*, 1997) and Foekens (Foekens *et al.*, 1999) have confirmed prognostic value of cytosolic cathepsin D by analyzing respectively 2690 and 2810 patients. By immunohistochemical analysis of 1348 patients, we have recently confirmed the prognostic value of cathepsin D by stromal cells and not by cancer cells (Têtu *et al.*, 1999). However, by both cytosolic and cytoplasmic cathepsin D analyses, the same group of Ravdin has always not confirmed prognostic value of cathepsin D by IRMA cytosolic cathepsin D measuring with 1984 patients (Ravdin *et al.*, 1997) and by Western Blot analysis with 927 patients (Ravdin *et al.*, 1994), or by immunocytochemical analysis of cytoplasmic cathepsin D of all cells with 562 patients (Ravdin *et al.*, 1994).

From 1993 and by using immunohistochemical methods, which permitted to study cathepsin D from different cell types, some investigators have addressed the question of the cellular source of cathepsin D in breast cancer (Castiglioni *et al.*, 1994; O'Donoghue *et al.*, 1995; Visscher *et al.*, 1993). Many authors found that macrophages identified by CD 68 expression in tumors were the responsible tumor-associated parenchyma cell source of cathepsin D to the therapeutic failure. All these results prompted us to investigate if cathepsin D by stromal cells in breast cancer contribute to tumor invasion. As the first step, we do like to observe eventual quantity change of stromal cytoplasmic cathepsin D in malignant situation.

By purifying tumor-associated stromal cells from primary breast cancers and cocultivating these stromal cells *in vitro* directly or indirectly with mammary cancer cells, we tried to understand whether the expression of intracellular cathepsin D by these tumor-associated stromal cells could be influenced by presence of breast cancer cells. The *in vitro* model was aimed to mimic partially the *in vivo* situation, and the immunocytochemistry method was used principally in order to have the same similarities of analysis approaches and criteria as in clinical studies (Têtu *et al.*, 1993; Têtu *et al.*, 1999). In order to rule out the influence by different proliferation in the presence of cancer cells, the coculture was arranged to begin when fibroblasts were confluent, as contact inhibitory effect could be implicated.

It is very interesting for us to find all these tumor-associated stromal cells from mammary origin had CD 68 expression by KP1 monoclonal antibody. CD 68 is a marker of pan-macrophage origin cells or myeloid origin cells (Micklem *et al.*, 1989). Many clinical analysis of CD 68 used immunohistochemistry method by KP1 and reported the results as macrophage contribution in cathepsin D studies. In fact, tumor-associated macrophages constitute a large proportion (up to 80% in breast carcinoma) of the cells infiltrating solid tumors (O'Sullivan *et al.*, 1993). In our study, all tumor-associated fibroblasts were fibroblast-like cells *in vitro* culture. It may be postulated that these tumor-associated and fibroblast-like cells *in vitro* could be transdifferentiated from macrophages *in vivo* and could conserve this pan-macrophage antigen, as this transdifferentiation was reported in human monocytes (Reuter *et al.*, 1995). However, some studies have reported findings that CD 68 was expressed also in non macrophage cells, such as in tumor cells of glandular cell neoplasms, sarcomas, lymphomas, malignant melanomas and carcinomas (Cassidy *et al.*, 1994; Tsang and Chan, 1992). One report concluded that CD 68 was only a lysosome marker (Tsang and Chan, 1992). So another possibility may be that those so-called macrophage cells *in vivo* identified by K1 may be not real macrophages but other stromal cells as fibroblasts.

It is well known that cathepsin D is an ubiquitous enzyme found in the lysosomes of many cell types such as macrophages and other connective tissue cells, and in epithelial cells (Reid *et al.*, 1986). Because of this wide distribution, it would appear to have no specificity as tumor

marker only by tumor cells. It has, however, a role in enhanced proteolysis of extracellular matrix remodeling of tumor invasion. Many clinical research work used immunohistochemical methods (Gonzalez-Vela *et al.*, 1999; Isola *et al.*, 1993; Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995; Têtu *et al.*, 1993; Visscher *et al.*, 1993), with which not every stromal cells were positive for cathepsin D in tissue sections. Using Western Blot in the analysis of intracellular cathepsin D in purified and unstimulated tumor-associated fibroblasts, we have found expression of cathepsin D in every tumor-associated fibroblast cell lines, suggesting a different sensibility of analysis by these two methods. The ~38 kDa band was often in the assays with chemiluminescence detection method, a little "over-exposition" resulted in this unidentified band, giving difficulty of comparison analysis of cathepsin D expression. It is why we have adopted radioactive approaches to have a semi-quantitative analysis. However, The expression of cathepsin D by tumor-associated cells was found only in mature form, while the expression by tumor cells contained not only mature form but also precursor. HDQ-P1 is a newly established breast cancer cell line in our group from a primary ductal infiltrating breast carcinoma. Particularly, the HDQ-P1 cells showed also a precursor expression as other commercialized breast cancer cell lines, moreover, the HDQ-P1 cells have only a weak expression of mature form of cathepsin D. The different forms of cathepsin D expression between tumor and stromal cells from our results were in agreement with the conclusive fact that in cancer cells, there is a misrouting of precursor in the processes of maturation of cathepsin D, which causes an accumulation of precursor and secretion of excess precursor (Capony *et al.*, 1989; Rochefort, 1992).

Expression of cathepsin D was found only in mature form before and after coculture with cancer cells. The augmentation of quantity confirms the positive stimulation effects of breast cancer cells. Among three cancer cell lines used, MCF-7 gave only its positive induction effect in one stromal cell line, while MDA-MB-231 and SK-BR-3 induced over-expression of cathepsin D in each stromal line. It is well known that MCF-7 is estrogen dependent cell line. As stromal cells have only minor estrogen receptor level and no response to the 17- $\beta$ -estradiol treatment (Malet *et al.*, 1991), we have not used 17- $\beta$ -estradiol in coculture assays in order to rule out its possible influence. It is possible that this weak induction effect of cathepsin D production is due to absence of estrogen for MCF-7 cells.

The fact that indirect coculture with conditioned medium gave also positive stimulation effects indicates some possible soluble factor(s) implicated in over-expression of cathepsin D. The stimulation by presence of cancer cells induced over-expression of cathepsin D in all stromal cells, close or remote to cancer cells, it is not as stromelysin-3, for which, the stromal expression was found only in the epithelial cancer/stromal cell interface (Basset *et al.*, 1990). Indirect coculture with Transwell failed to stimulate fibroblasts to over-produce cathepsin D, this may be explained by a possibly weak stimulation effect of cancer cells separated with fibroblasts by one mm distance; while in direct coculture or coculture with conditioned medium, the cancer cells and stromal cells have a better communication in their interaction processes.

In summary, by *in vitro* coculture, we have observed the changes of cathepsin D by stromal cells of mammary origin in the presence of breast cancer cells. Although these cells have not many precursors of cathepsin D produced as epithelial cancer cells do, the mature form of cathepsin D was indeed augmented. The cathepsin D participates in the tumor invasion by not only proteolytic activity, but also some favorite effects on cell proliferation and cell migration (Westley and May, 1996). Cathepsin D can function as ligand to some receptor such as mannose-6-phosphatase/IGF II. (Mathieu *et al.*, 1990). All those results suggest that it is worth to continue the investigation of stromal cathepsin D in breast cancer, including possible changes of phagocytic and endocytic macrophages, in order to resolve clinical discrepancies.

### **Acknowledgment**

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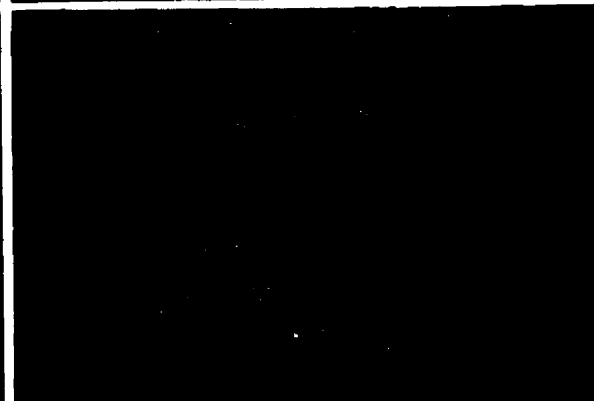
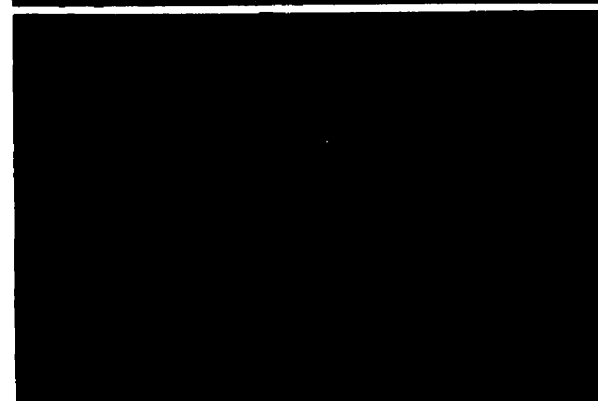
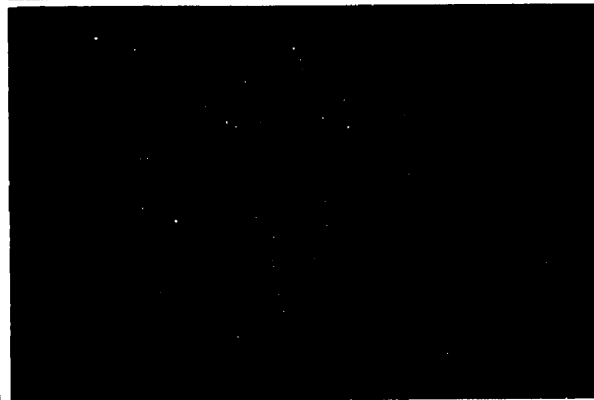
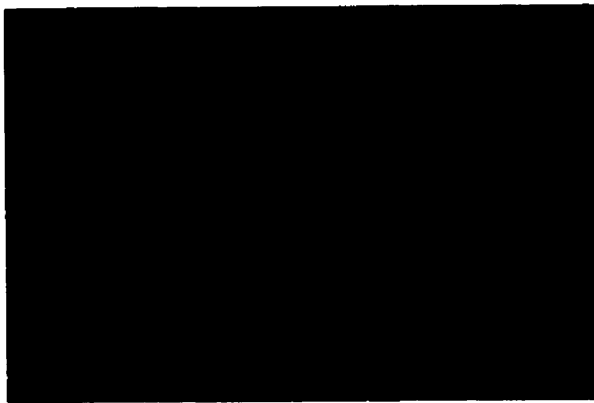


**Figure 1. Immunocytochemical detection of stromal CD by direct coculture.**

**(a). Positive expression of vimentin in purified fibroblasts from primary breast cancer; (b). Positive expression of CD68 identified by KP1 in purified tumor-associated fibroblasts (F12820); (c). Positive stromal expression in a breast cancer section. The epithelial cancer cells are in brown color identified by enzymatic reaction of horseradish peroxidase with diaminobenzidine, while cytoplasmic cathepsin D is granular and red by enzymatic reaction of alkaline phosphatase with new fuchsin; (d). Negative cathepsin D expression in unstimulated F9846 tumor-associated fibroblasts; (e) Some positive signals of cathepsin D in F9846 tumor-associated fibroblasts cocultivated with MCF-7 cells; (f & g). Positive expression of cathepsin D in F4143 tumor-associated fibroblasts cocultivated with SK-BR-3 cells (f) and with MDA-MB-231 cells (g); (h). Normal fibroblasts F573N give positive expression of cathepsin D when cocultivated with SK-BR-3 cells.**

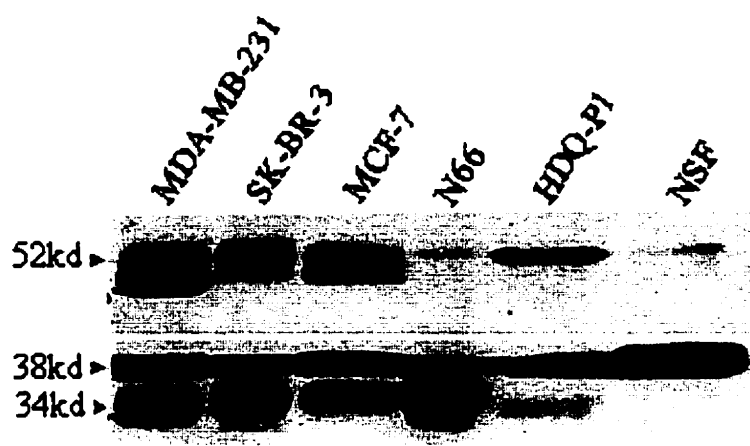
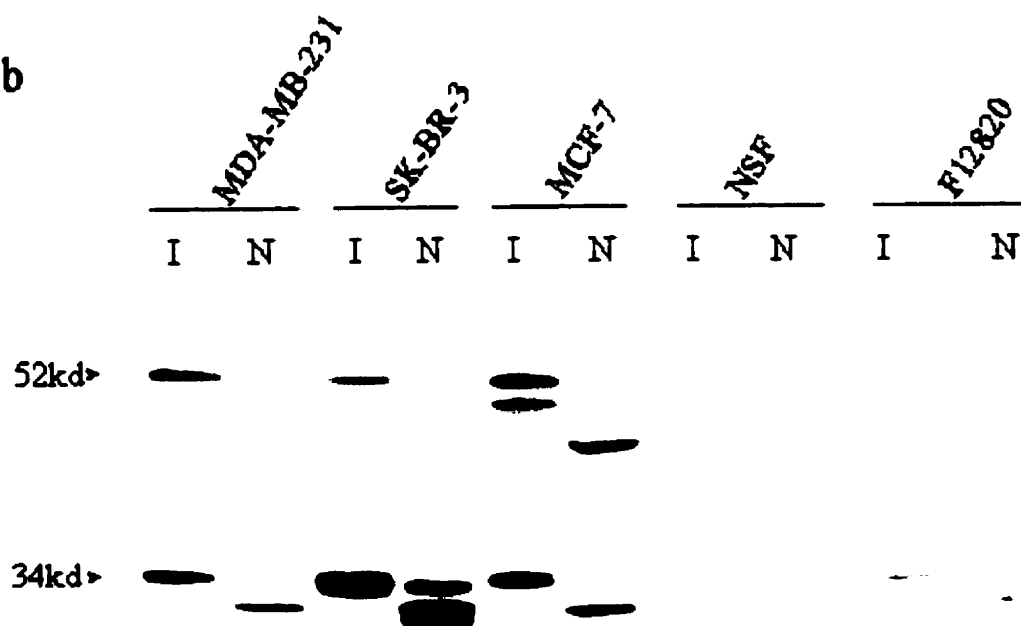
**a****b**

**Figure 2. Western blot analysis of cellular cathepsin D**

(a). Western blot analysis of cathepsin D in different cells analyzed by chemiluminescence method. Three breast cancer cell lines MDA-MB-231, SK-BR-3 and MCF-7 show cytoplasmic mature cathepsin D and precursor. The HDQ-P1 is a newly established breast cancer cell line in our group, the HDQ-P1 cells have also cathepsin expression of precursor as commercial breast cancer cell lines and a weak mature form. Normal or tumor-associated fibroblasts show only weak expression of mature cathepsin D without precursor. The band about 38 kDa is unknown and this band was easily observed by chemiluminescence detection., thus gave some difficulty of comparison analysis. (b). Western blot analysis of cellular cathepsin D by  $^{125}\text{I}$  and ImageQuaNT. All breast cancer cells have precursor expression. The natural form of cytoplasmic cathepsin D is in form of N-glycosilation. I: intact; N: N-glycosidase F treated.



## **CHAPITRE VII**

### **CONSTRUCTION DE TISSU CANCÉREUX *IN VITRO*: CULTURE TRIDIMENSIONNELLE D'ÉQUIVALENT TISSULAIRE**

## **AVANT-PROPOS.**

Plusieurs recherches en biologie de cancer nécessitent l'utilisation d'un bon modèle de culture *in vitro*. Ce chapitre porte sur la mise au point d'une nouvelle méthode de culture tridimensionnelle, aussi appelée culture par équivalent tissulaire, et devant servir à étudier les interactions stromales-épithéliales dans le cancer du sein. Le travail a été réalisé principalement dans le Laboratoire d'Organogenèse Expérimentale. Cet article a été soumis pour publication dans la revue *In vitro Cellular and developmental Biology*.

## RÉSUMÉ.

Un bon modèle de culture *in vitro* est utile et parfois même essentiel pour la recherche en oncologie. L'objectif est de simuler le mieux possible la situation *in vivo*. Utilisant du collagène bovin, l'élément principal de la matrice extracellulaire, nous avons développé un modèle de coculture tridimensionnelle. Ce modèle est comparable à un équivalent tissulaire. La coculture a été réalisée par construire un gel contenant des cellules stromales de différentes sources puis ensemer de différentes cellules épithéliales y compris les cellules épithéliales cancéreuses mammaires au-dessus du gel. L'invasion du gel et la migration des cellules cancéreuses mammaires ont été analysées. La sécrétion de différentes gélatinases a été aussi documentée par zymogramme. Cette étude montre que les cellules stromales associées à la tumeur sont phénotypiquement différentes des cellules normales. Ces cellules stromales associées à la tumeur favorisent la migration et l'invasion des cellules hautement invasives comme les cellules de MDA-MB-231. L'interaction épithéliale-stromale influençait la production et la sécrétion de certaines gélatinases. Ce modèle peut être utilisé pour étudier les interactions stromales-épithéliales dans le cancer du sein et pour comprendre le rôle de certaines protéases (cathepsine D, stromélysine-3 et uPA) par les cellules stromales sur l'invasion des cellules tumorales.

**Production of bioengineered cancer tissue constructs *in vitro*:  
Epithelium-mesenchyme heterotypic interactions**

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**Running title: Breast cancer bioengineered cancer tissue construct *in vitro*.**

**Abstract**

A few models have been established to study cancer cells *in vitro*. However, rare reports studied specifically cellular interactions with the use of bioengineered cancer constructs combining human carcinoma cells and tumor-associated fibroblasts. We developed a three-dimensional bioengineered cancer tissue constructs (bCTC) by seeding mammary epithelial cancer cells or normal keratinocytes over a mesenchymal layer containing tumor-associated fibroblastic cells or normal skin fibroblasts. Constructs containing only epidermal cells and fibroblasts from non-cancerous sources (skin samples from health donors) were used as normal controls. All constructs were cultured for 10 days. Histologic analyses showed that carcinoma cell lines could invade the subjacent mesenchymal layer and that the capacity to migrate was associated with the invasive potential of cancer cells while non-invasive populations did not. Zymography showed the selective secretion of gelatinases according to the phenotype of the cells seeded in the various bCTC. Specifically, a 50-kDa gelatinase band has been observed in bCTC with non-invasive epithelial cells only.

**Key words:** human cancer tissue construct, tissue engineering, cellular interactions, collagen matrix.

## Introduction

Current literature shows that stromal-epithelial interactions play a key role on breast cancer progression. Several *in vitro* studies showed that mesenchyme-epithelium interactions may exert mutual physiological regulation through growth factors secretion (Atula *et al.*, 1997; Dong-Le *et al.*, 1997; van Roozendaal *et al.*, 1996). There is increasing evidence that host cells such as fibroblasts influence cancer proliferation and tumor growth through heterotypic signaling between the diverse cell types (van Roozendaal *et al.*, 1996). In absence of extracellular matrix, cells grown alone in monolayers or with other cell types in coculture fails to adopt an organization comparable to the native tissue. In contrast, a three-dimensional matrix provides an adequate microenvironment to promote tissue organization. Indeed, three-dimensional cocultures with collagen or matrigel have been developed *in vitro* to provide a good matricial support to cancer cell growth to study epithelium-mesenchyme interactions (Atula *et al.*, 1997; Leighton, 1991; Leighton, 1997). Moreover, various parameters can be monitored to assess the influence of one cell population on another, within the same tissue construct. Cell growth and morphologic features, functional properties such as migration, growth factors and enzyme secretion, maintenance of expression of specific markers and receptors, and tissue organization, can be observed upon culture time.

Considerable evidence from *in vitro* and *in vivo* studies suggests that certain matrix metalloproteases play an important role in cancer invasion and metastasis. Tumor cells may either secrete matrix metalloproteases themselves or induce host cells within tumor stroma to produce them. In fact, the expression of a majority of MMP family members is by stromally derived host cells in response to the presence of cancer cells. Stromal cells were shown to produce stromelysin-3 (Basset *et al.*, 1990; Mari *et al.*, 1998) and gelatinase A (Ito *et al.*, 1995; Noel *et al.*, 1994) in invasive breast cancer. Similarly, matrix degradation and remodeling seems associated with their metastatic potential. In healthy tissues, matrix involves enzyme secretion stimulated through cell-matrix interaction to maintain tissue integrity. Collagenases are enzymes secreted by the cells *in situ* that degrade collagen and stimulate its post-injury remodeling. For instance, gelatinase A or MMP-2 (72 kDa) and gelatinase B or MMP-9 (92 kDa) are regarded as specific secretion products of dermal fibroblasts (Oikarinen *et al.*, 1993; Raynaud *et al.*, 1992) and keratinocytes (Petersen *et al.*, 1987), respectively.

The aim of this study was to create a breast cancer tissue equivalent construct using breast cancer cells and breast tumor associated fibroblasts, and to observe the epithelial-stromal interaction in this tissue-equivalent coculture. The cancer epithelial cell invasive properties have been evaluated in response to the microenvironment created by different fibroblastic cell populations seeded in the underlying mesenchymal layer of the bCTC. Meanwhile, gelatinase activity in the supernatants have been analyzed by zymography.

## **Materials and methods**

### **Cells**

Normal human skin epithelial cells (keratinocytes) and dermal fibroblasts (F23) were isolated from normal breast biopsies obtained from volunteers during breast reconstructive surgery according to the method established in our laboratory (Auger *et al.*, 1995). Tumor-associated fibroblastic cell populations were isolated from primary infiltrating mammary carcinomas using the same method as to isolate normal skin dermal fibroblasts (Auger *et al.*, 1995). The fibroblastic nature of those cells was confirmed by their morphology, the expression of vimentin, and the absence of cytokeratins. Two of these were used to produce bCTC, F1 and F2, from two infiltrating ductal primary mammary carcinoma of poor and moderate differentiation, respectively. All normal and tumor tissues have been obtained from our bank of breast tissues which has been approved by the hospital ethical committee. Each patient whose tissue is recorded in our tissue bank is asked to sign an informed consent form. All cells were maintained in DMEM with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO<sub>2</sub>.

Two human breast cancer cell lines, MDA-MB-231 and MCF-7, were used to produce bCTC. HDQ-P1 is a new cell line recently established by our group from a primary ductal infiltrating mammary carcinoma (Wang *et al.*, 2000). The HDQ-P1 cell line was well characterized and also used in the present study. MDA-MB and MCF-7 cells were maintained in DMEM with 10% BFS and antibiotics. HDQ-P1 cells were maintained in the same medium but supplemented with 5 µg/ml bovine crystallized insulin.

### **Production of the bCTC mesenchymal layer**

The bCTC were produced according to the method published by Auger *et al.* (Auger *et al.*, 1995) with a few modifications. Briefly, a mixture of bovine Type I collagen (1.5 mg/ml) was prepared by dissolving the powder overnight at 4°C in sterile 0.017 M acetic acid. A solution of 1.68 ml of DMEM 2.7x containing 200 IU/ml penicillin G and 50 µg/ml of gentamicin, pH 8.0, was mixed with a second solution containing 1.02 ml of FBS, 2.86 ml of the stock collagen solution, 60 µl of NaOH 0.7 N and 0.3 ml of a fibroblastic cell suspension ( $1 \times 10^6$  cells/ml). The final concentration of collagen in all the mesenchymal layers was always 0.715 mg/ml. This mixture was quickly distributed (3 ml/petri dish) in bacteriological petri dishes (35 mm, Falcon, Becton Dickinson Inc, Mississauga, Ontario) already containing a peripheral anchorage, to produce the mesenchymal layers of each bCTC. The peripheral anchorage method that prevents collagen lattice contraction by the cells, was used as previously described (Auger *et al.*, 1995; Lopez *et al.*, 1992). The mesenchymal layers of the bCTC were covered with 2 ml of DMEM supplemented with 10% FBS, 100 IU/ml penicillin G and 25 µg/ml gentamicin following collagen polymerization and cultured in this medium until their epithelialization. All experiments were done in duplicate.

### **Epithelialization of the bCTC**

Four days later, the epithelialization was performed by seeding human epithelial carcinoma cells or normal keratinocytes ( $8 \times 10^5$  cells/bCTC) on the mesenchymal constructs. A confluent epithelial cell layer was obtained after 4-6 days. All bCTC were cultured for 10 days in a culture medium as previously reported (Goulet *et al.*, 1996) consisted of combination of DMEM with Ham's F12 in a 3:1 proportion (Gibco-BRL, Burlington, Ontario), supplemented with 10 ng/ml human epidermal growth factor (EGF, Chiron Corp., Emeryville, CA), 5 ng/ml 17-β-estradiol, 500 ng/ml progesterone, 24.3 µg/ml adenine, 5 µg/ml crystallized bovine insulin, 5 µg/ml human transferrin,  $2 \times 10^{-9}$  M 3,3',5',-triiodo-L-thyronine (Sigma Chemicals, St Louis, MO, USA), 0.4 µg/ml hydrocortisone (Calbiochem, La Jolla, CA, USA),  $10^{-10}$  M cholera toxin (Schwarz/Mann, Cleveland, OH, USA), 10% FCS, 100 IU/ml penicillin G and 25 µg/ml gentamicin.. All cultures were kept in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The culture medium was changed daily.

### **Histological Analysis**

Small sections of all bCTC biopsies were fixed with Bouin's solution and formalin and were embedded in paraffin. The 4 µm thick Bouin's fixed sections were stained using the *Masson's trichrome* staining methods (Auger *et al.*, 1995).

### **Immunohistochemistry**

Four 4 µm thick formalin fixed sections were prepared for immunoreaction. The bCTC sections were deparaffined, washed in phosphate-buffered saline (PBS, pH 7.4) for 5 min and then quenched for 5 minutes in 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. After washing with PBS, the bCTC sections were covered with normal horse serum during 20 minutes and incubated with first antibody at 4°C overnight. Afterwards, the sections were rinsed with PBS and exposed for 30 minutes to a secondary biotinylated anti-mouse antibody. After another wash with PBS, the sections were incubated with the avidin-biotin-peroxidase complex (Vector laboratory, Burlingame, CA) and the reaction was revealed with diaminobenzidine (DAB). The sections were finally counterstained with Mayer's haematoxylin for 3 minutes and mounted in Permount (Dako, Mississauga, Ontario).

Anti-vimentin (Dako) and anti-human 8 and 18 cytokeratins primary monoclonal antibodies (CAM 5.2, Becton Dickinson) were used. The slides were analyzed under a Nikon Optiphot microscope and photographed using a Kodak Tmax 400 ASA film.

### **Gelatinase activity assay**

The active gelatinases secreted in the bCTC culture supernatants were semi-quantitatively analyzed by zymography, according to the method described previously (Auger *et al.*, 1995; Remacle *et al.*, 1998). Briefly, some samples of bCTC culture supernatants were collected daily, filtered and immediately frozen at -70°C until use. A constant volume (25 µl) of each sample diluted in equal volume of 2x sample buffer was resolved under non-reducing conditions by SDS-PAGE using 10% polyacrylamide gels containing 3.5% gelatin. The gels were then rinsed and equilibrated in the proper buffer systems and put overnight at 37°C in digestion buffer (50 mM Tris, pH 7.4, containing 10 mM CaCl<sub>2</sub> and 100 mM NaCl), under

slow agitation. The gels were fixed with a 30% methanol solution containing 10% acetic acid and stained with 0.05% Coomassie blue prepared in the fixative. All experiments were done in triplicate.

## **Results**

### **Structural analyses of the bCTC**

Phase contrast microscopy analyses showed confluent layers of carcinoma cells on mesenchymal constructs as early as 4 days after epithelialization in all bCTC. Three types of mesenchymal layers were constructed using F23, F1 and F2 (Fig. 1). Histological bCTC sections showed the migration of the epithelial cancer cells MDA-MB-231 in all types of the mesenchymal layers on which they were plated (Fig. 1. A, E & I). Similarly, HDQ-P1 cells also showed an invasive but weaker behavior in all conditions tested, notably when grown over the mesenchymal layers seeded with tumor-associated fibroblasts F1 and F2 (Fig. 1. B, F & J). No obvious invasion was observed with MCF-7 cells in any condition tested (Fig. 1. C, G & K). Indeed, normal skin keratinocytes never showed any sign of migration towards any mesenchymal layer as expected (Fig. 1. D, H & L). Striking findings were repeatedly observed when epithelial cells of tumor origins were seeded over mesenchymal layers containing normal skin fibroblasts, in these cases, the mesenchymal layers were degraded twice faster than those containing tumor-associated fibroblastic cells.

Immunohistochemistry analyses were performed to verify the maintenance of cellular phenotypic associated markers upon culture time in the various bCTC. The anti-keratin antibody identified all epithelial cell populations, as shown in Figure 1, N and P (two representative labeling). The immunoreaction of the mesenchymal cells with the anti-vimentin antibody showed typical elongated fibroblast morphology in all bCTC (Fig. 1. M & O). Thus, all the cells included in the various groups of bCTC remained alive and metabolically active until the end of all experiments.

### **Gelatinases secretion in bCTC supernatants**

The active gelatinases secreted in all bCTC supernatants were analyzed and compared by zymography (Fig. 2). Some baseline level of 92 kDa and 72 kDa gelatinases was also found

in the culture supernatants of acellular mesenchymal layers. The serum added to the bCTC (added also in the culture of acellular mesenchymal layers) is supposed to contain traces of this gelatinase. However, 92 kDa and 72 kDa gelatinases and their activated forms as well as a 50 kDa gelatinase band were also found in the supernatants of bCTC only containing fibroblasts.

Gelatinase B of 92 kDa (MMP-9) was detected in the supernatants of bCTC epithelialized with normal breast skin keratinocytes and all other epithelial cell populations. However, bCTC with MDA-MB-231 was associated with the highest level of gelatinase B and comparable to the bCTC with keratinocytes, while the bCTC with MCF-7 and HDQ-P1 were associated with much lower levels. Interestingly, bCTC with the highly invasive epithelial cells, MDA-MB-231, induce higher amounts of the 92 kDa gelatinase when grown on mesenchymal layers seeded with F1 and F2. Moreover, the activated form of gelatinase B of 82 kDa, while never detected in any supernatant of bCTC seeded with epithelial tumor cells, was induced at significant levels in the keratinocyte-seeded bCTC containing F1 and F2 but not F23. Thus, F1 and F2 under keratinocyte stimulation induce activated gelatinase B secretion.

Higher amounts of gelatinase A, 72 kDa, were detected in the supernatants of non-epithelialized bCTC containing F1 and F2 than those seeded with F23. However, MDA-MB-231 seems to be the only the epithelial cancer cell population able to reduce the expression of gelatinase A by F1 and F2 through intercellular interactions. The activated form of gelatinase A of 62 kDa was highly secreted under the keratinocyte stimulation in all bCTC supernatants, independently of the fibroblast population seeded in the mesenchymal counterpart. Interestingly, the supernatants of bCTC epithelialized with cells of tumor origin on mesenchymal layers containing F1 and F2 populations contained much lower amounts of this 62 kDa enzyme, compared to those containing F23. These observations strongly suggest direct modulation of gelatinase expression through epithelium-mesenchyme interactions in the various groups of bCTC.

Finally, a gelatinase of 50 kDa was observed in all bCTC containing fibroblasts only and more important in bCTC containing only F23 than in bCTC containing only F1 and F2.

Interestingly, coculture of epithelial cells in bCTC modulated the level of expression of this gelatinase. No more 50 kDa gelatinase was observed in the supernatants of bCTC epithelialized with the most invasive cancer cell line, MDA-MB-231. A significant reduction of its expression was also observed in the supernatants of bCTC epithelialized with MCF-7. In bCTC epithelialized with HDQ-P1 cells, the 50 kDa gelatinase was much reduced in the coculture with F1 and especially with F2, but weakly reduced in the coculture with F23. Larger amounts of 50 kDa gelatinase was secreted in bCTC seeded with normal keratinocytes.

### **Discussion**

The study of interactions between tumor epithelial cells and mesenchymal cells is difficult *in vivo*, considering the complexity of the cascade of events involved in the development of tumors. The literature often reports the use of the Boyden's chambers (chemoinvasion assay) or Transwell modified Boyden's chamber techniques to evaluate the invasion potential of tumor cells *in vitro* in presence of other factors (Nabeshima *et al.*, 1999; Pilkington *et al.*, 1997; Sugiura *et al.*, 1996). The advantage of our bCTC over this system is the opportunity to partially reproduce the epithelium-mesenchyme interactions, and the capacity to assess histological and functional parameters.

Immunohistochemical analyses confirmed that all respective phenotypes of the cells seeded in our bCTC (epithelial or mesenchymal) were maintained in culture. For instance, all epithelial cells expressed keratins while mesenchymal cells (fibroblast-like cells) expressed vimentin throughout the whole duration of the experiments. These results suggest that the cells included in our bCTC keep at least some of their typical features in coculture and after being seeded in our bCTC.

Our data suggest that in our bCTC, the invasive potential of mammary carcinoma cells is maintained *in vitro*, and consistent with their respective phenotypes documented by ATCC and their tissues of origin. For instance, normal skin keratinocytes and the cell line MCF-7 which are known to be non-invasive, do not migrate into the mesenchymal layer when integrated in our bCTC. Similarly, MDA-MB-231, characterized as potentially invasive cell, and HDQ-P1 cells always migrate into the mesenchymal layers over which they are seeded in

all groups of bCTC. Such observations suggest that our bCTC could be a useful indicator of the invasive potential of epithelial cells *in vitro*. Indeed, this conclusion will have to be supported by additional experiments using several other cell lines or freshly isolated epithelial tumor cell populations. However, we have performed assays with two additional cell lines, SK-BR-3 and BT-20, and similar conclusions were drawn (data not shown).

Interestingly, the three-dimensional organization of the epithelial cells showed differences according to the mesenchymal layers on which they were seeded. When keratinocytes were seeded over layers containing normal skin fibroblasts, they were made of fewer flattened stratified layers, compared to the bCTC produced with mesenchymal cells of tumor origin. In other word, intercellular interactions occur in our bCTC, which influence the structural features of the reconstructed tissues. These data suggest that our bCTC is a good tool to study epithelium-mesenchyme interactions that occur between normal and tumoral cells as well as between cells isolated from the same or different tumor specimens. The bCTC allows the monitoring of three-dimensional constructs using cell populations of different origins and phenotypes. In addition, the accelerated degradation of the extracellular matrix of the mesenchymal layers seeded with F23 suggests that some mechanisms of tissue repair including collagen remodeling may be involved in presence of normal fibroblasts only. This hypothesis needs to be confirmed on bCTC seeded with normal fibroblasts of various origin.

Prior studies have documented the role of matrix metalloproteases, especially collagenases and gelatinases, on invasion of normal tissues by breast cancer cells (Davies *et al.*, 1993; Noel *et al.*, 1994; Remacle *et al.*, 1998). Immunohistochemistry or hybridization *in situ* methods have been used to identify which enzymes could play a role on matrix remodeling by tumor cells. Zymography may help to identify most collagenases/gelatinases secreted *in vitro* by living cells. In our study, zymography allowed the identification, in supernatants, of a 92 kDa gelatinase under keratinocyte stimulation and a 72 kDa gelatinase produced by normal skin fibroblasts.

As expected, the gelatinases expressed under normal epithelial and dermal cell stimulation were secreted in the bCTC supernatants. Our results suggest that the amounts and types of

gelatinases secreted by the cells are modulated according to their phenotypic features. For instance, the bCTC with normal keratinocytes induces significantly more collagenase secretion, especially active and inactive gelatinase B, in presence of tumor-associated fibroblasts, F1 and F2, compared to F23. In contrast, the highly invasive epithelial cell line, MDA-MB-231, induces lower amounts of gelatinase A (72 kDa) and its active form (62 kDa) in presence of tumor-associated fibroblasts (F1 & F2). Thus, the origin of the mesenchymal cells and overlying tumor cells seems influence protease secretion. Moreover, the bCTC with the non-invasive epithelial cells, MCF-7, induced lower levels and fewer types of gelatinases than all other epithelial cancer cell lines. Taken together, these observations may explain in part the low level of mesenchymal invasion by MCF-7 cells in bCTC.

Interestingly, a significant amount of a 50 kDa gelatinase has repeatedly been detected in the keratinocyte-dermal fibroblast tissue constructs. However, this gelatinase was absent in bCTC containing MDA-MB-231 cells and low levels were found in those seeded with HDQ-P1 and MCF-7 cells. This enzyme has rarely been reported in the literature. Despite the matrix degradation associated with the activity of gelatinases, we may postulate that some of them play a beneficial role in normal tissue repair and post-injury collagen remodeling. Remacle *et al.* (Remacle *et al.*, 1998) have reported in 1998 that breast cancer patients with high levels of 50 kDa gelatinase had a significantly better survival than those with low gelatinase levels. Our results concur with those observations, and support the hypothesis raised by Remacle *et al.* regarding its association with an improved prognosis of cancer patients. In addition, this 50 kDa gelatinase was observed *in vivo* during cutaneous wound healing in mice (unpublished data). Therefore, our bCTC could be useful to identify the collagenases and other enzymes involved in matrix organization and tissue remodeling processes *in vitro*.

Thus, *in vitro* models such as our bCTC may provide an interesting alternative. The development of such bCTC may pave the way to the new breakthrough in cancer research.

### **Acknowledgments**

The authors are grateful to Danielle Murray for technical assistance, Claude Marin for photographic assistance, Kathleen Baker, M.Sc., for her collaboration on zymography

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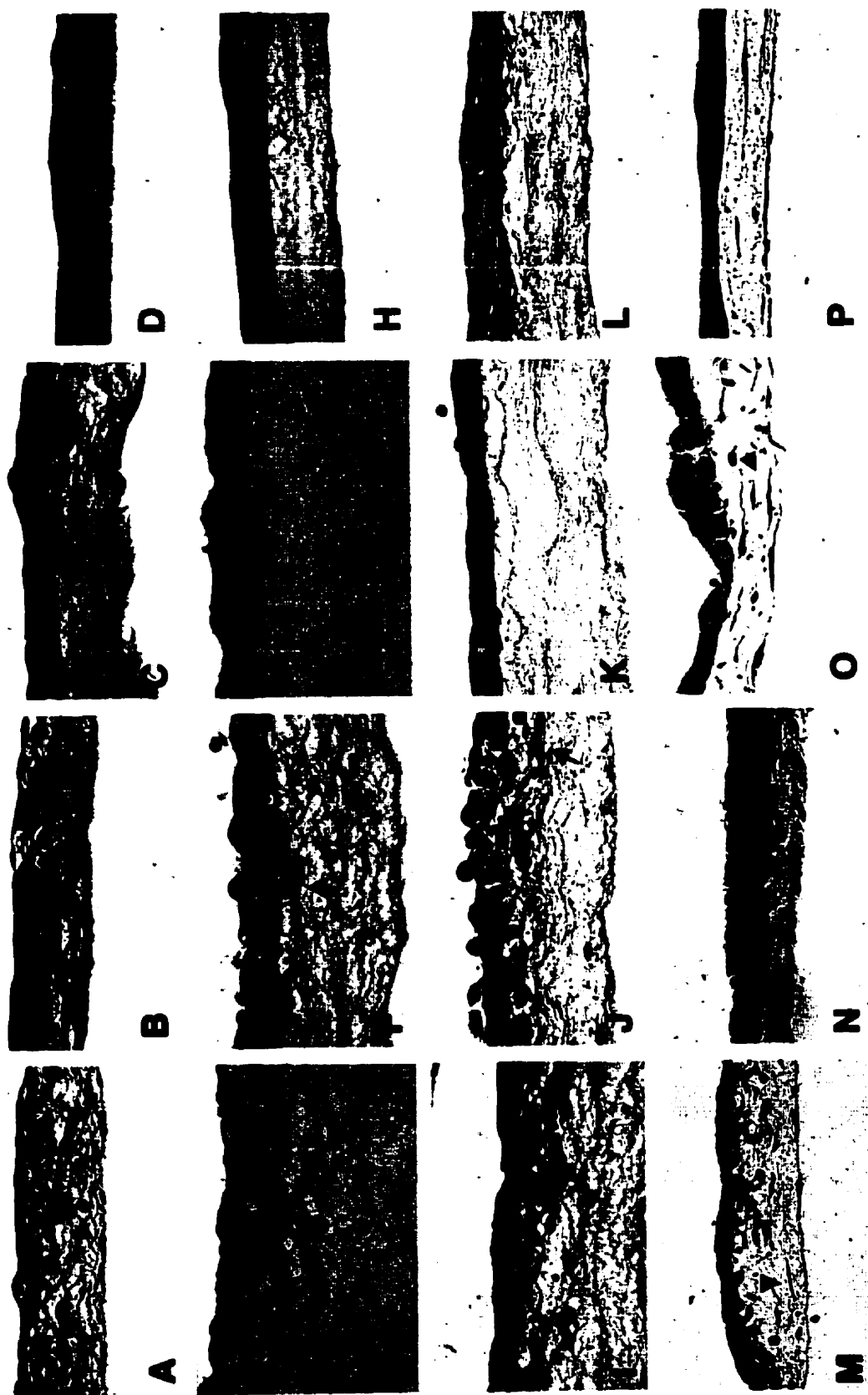


**Figure 1. Photomicrographs of histological bCTC sections produced with mesenchymal cells. F23 (A-D), F1 (E-H) and F2 (I-L), combined to epithelial cells : MDA-MB-231 (A, E, I & M), HDQ-P1 (B, F, J & N), MCF-7 (C, G, K & O) and normal skin keratinocytes (D, H, L & P). Black arrowheads indicate the cells that migrated into the mesenchymal layers of the various bCTC (A-L). Some bCTC sections were stained with the Masson's trichrome method (A-L) while others were used for immunohistochemistry using the anti-keratin antibody (N & P) and anti-vimentin antibody (M & O). Blue arrowheads show the positively labeled cells in each case.**

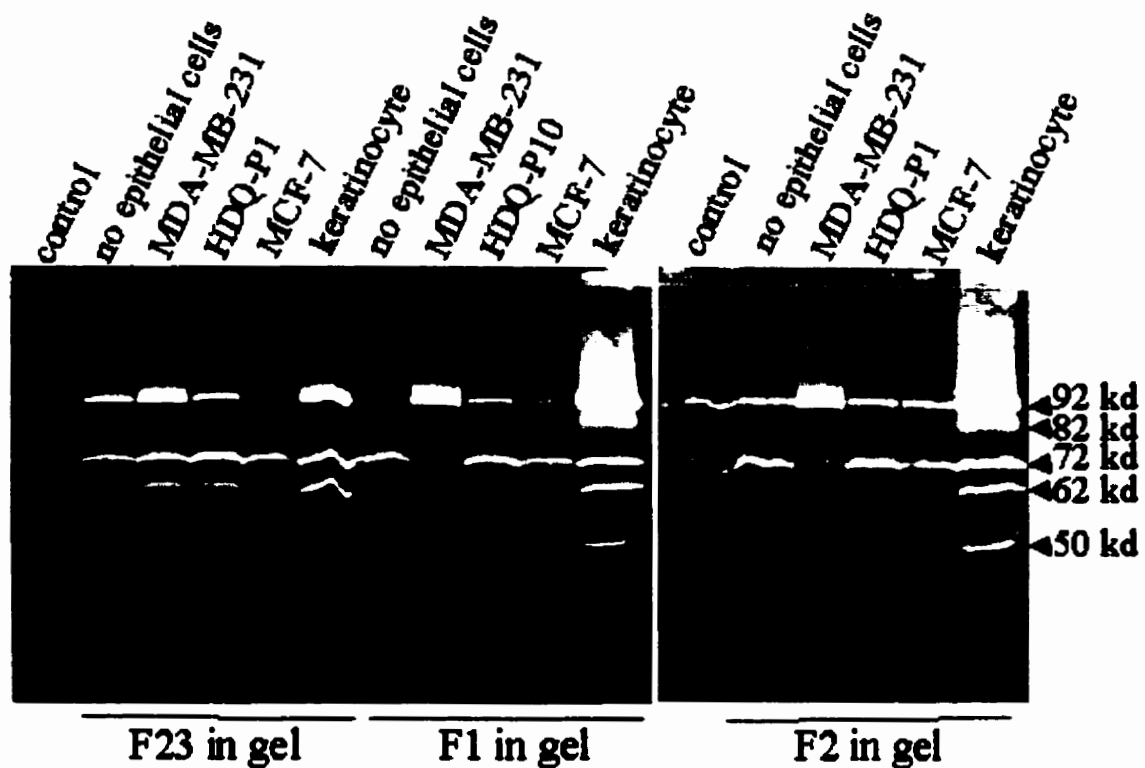


Figure 2. Gelatinase activities in supernatants of bCTC.

Zymograms showing the relative gelatinase activities analyzed from 40  $\mu$ l of supernatants of bCTC maintained in culture for ten days. The respective molecular weights of the gelatinases observed are indicated on the figure. The control corresponds to the culture medium only, in absence of cells. Some bCTC were not epithelialized, they contained only fibroblasts, F1, F2 and F23, in order to allow the visualization of their respective secretion products.

## **CHAPITRE VIII**

### **ANALYSE INTÉGRALE DE TROIS PROTÉASES PAR LES CELLULES STROMALES SUR LA PROGRESSION DU CANCER DU SEIN**

## **AVANT-PROPOS.**

Il est connu que l'invasion tumorale implique plusieurs protéases. Cependant, la plupart de ces études sur le rôle des protéases par les cellules stromales porte sur un ou deux facteurs indépendamment. Afin de comprendre l'interdépendance des protéases stromales sur le pronostic du cancer mammaire, une étude a été réalisée pour analyser le rôle combiné de l'expression de l'uPA, la stromélysine-3 et la cathepsine D dans le cancer du sein. Cet article a été soumis pour fin de la publication à la revue Cancer.

## RÉSUMÉ.

Des études *in vitro* montrent que l'invasion tumorale implique l'intervention de plusieurs protéases agissant en cascade dans le processus d'invasion tumorale. Comme des protéases stromales jouent aussi un rôle dans la progression tumorale, on suppose que ces protéases stromales sont interdépendantes et agissent également en cascade. Une étude a été effectuée afin d'analyser le rôle de l'expression stromale combinée de l'uPA, la cathepsine D et la stromélysine-3 dans une série de 557 patientes avec une survie moyenne de plus de 10 ans. En effet, les trois protéases étaient exprimées simultanément par les cellules stromales de 80 patientes. 134 patientes n'avaient aucune expression stromale de l'une ou l'autre des trois protéases. L'expression combinée de 3 protéases par les cellules stromale était significativement associée à un faible taux de survie sans métastase à distance (DMFS) ( $p=0.0001$ ). Les patientes dont la tumeur exprimait seulement une ou deux de ces trois protéases avait le même pronostic que celles n'ayant aucune expression stromale de ces trois protéases. Le résultat confirme que les protéases produites par les cellules stromales du cancer du sein sont interdépendantes et qu'une brèche dans cette cascade peut compromettre le processus de progression tumorale.

**Expression Of Cathepsin D, Stromelysin-3 and Urokinase  
By Reactive Stromal Cells On Breast Cancer Prognosis**

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**Running title: Proteases and prognosis in breast cancer**

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March 22, 1999**

## ABSTRACT

**Background:** Current literature supports the concept that proteases act in a cascade manner to mediate remodeling of the extra-cellular matrix (ECM) and favor cancer progression. We and others recently identified cathepsin D, stromelysin-3 and urokinase plasminogen activator (uPA) expression by reactive stromal cells as significant factors of poor prognosis in breast cancer. We evaluated the joint effect of protease expression on cancer aggressiveness.

**Methods:** Protease expression was analyzed by immunohistochemistry (Cathepsin D) and *in situ* hybridization (Stromelysin-3 and uPA using anti-sense RNA probes) on formalin-fixed paraffin-embedded specimens from 557 breast cancers without distant metastasis at diagnosis and with an average of 10 years of follow-up.

**Results:** Of the 557 breast cancers, 80 expressed all three proteases and 134 expressed none of them. The expression of all three proteases strongly correlated with each other ( $p < 0.001$ ). An adjusted Cox model revealed that distant-metastasis-free survival (DMFS) was significantly worse for patients whose tumor expressed all three proteases ( $p < 0.0001$ ). The DMFS of patients whose tumor lacked at least one of the three proteases was similar to that of patients without any protease expression, irrespective of the type or number of proteases missing.

**Conclusion:** This study suggests that proteases expressed by reactive stromal cells are interdependent and that a breach in the protease pathway may impair breast cancer progression.

**Key Words:** Stromelysin-3, cathepsin-D, urokinase, breast cancer, Proteases

## INTRODUCTION

There is increasing evidence that reactive stromal cells play a key role on breast cancer progression (Basset *et al.*, 1993; Gregoire and Lieubeau, 1995; Zipori *et al.*, 1987), at least through protease production. In 1993, we demonstrated that cathepsin D produced by stromal cells, and not cancer cells, was associated with a poor prognosis in node-positive breast cancer (Têtu *et al.*, 1993). Those data have been confirmed by three different studies from three different groups of investigators (Chenard *et al.*, 1996; Joensuu *et al.*, 1995; Nadji *et al.*, 1996; O'Donoghue *et al.*, 1995; Thewes *et al.*, 1996). Other proteases produced primarily by stromal cells have also been linked to disease progression. More specifically, of those tested, high expression of urokinase Plasminogen Activator (uPA) (Duffy, 1996; Duffy *et al.*, 1994)

(Athanassiadou *et al.*, 1998) and stromelysin-3 (Chenard *et al.*, 1996; Engel *et al.*, 1994; Ravdin *et al.*, 1997) were also found to be prognostically significant.

It has been well recognized that several proteases interact with each other in a cascade manner in which certain are activated and others inhibited (Nagase, 1997). Endogenous tissue inhibitors of metalloproteinases (TIMP) modulate matrix metalloproteinase (MMP) activity. For example, TIMP-2 is the major inhibitor of gelatinase A and TIMP-1 is the major inhibitor of gelatinase (Chambers and Matrisian, 1997). However, proteases with no specific inhibitor activity may also interact with other proteases. The three proteases that we identified separately as prognostic factors may in fact part be part of the same cascade. Cathepsin D was found to play a role in the activation of cathepsin B and L (van der Stappen *et al.*, 1996) and both cathepsin B (Kobayashi *et al.*, 1991) and L (Goretzki *et al.*, 1992) can activate uPA. In turn, uPA is known to be an activator of MMPs through the plasmin system (Nagase, 1997) and stromelysin-3 is a member of the MMP family. Stromelysin-3 and uPA are both expressed by myofibroblasts (Nadji *et al.*, 1996; Nielsen *et al.*, 1996; Uden *et al.*, 1996), further supporting the possibility of an interaction between those molecules.

We recently demonstrated that proteases expressed by reactive stromal cells are significantly related with each other (Têtu *et al.*, 1998), supporting the idea that the expression of one protease depends, in part, on the expression of the others. This interdependence, in turn, suggests the hypothesis that their effects on breast cancer progression may also be complementary. This prompted us to investigate the combined role of those three proteases on breast cancer progression.

## **MATERIAL AND METHODS**

### **Population**

The patients included in this study had node-positive (NPBC) or node-negative (NNBC) breast cancer proven by histologic examination of axillary lymph nodes, but with absence of distant metastasis at diagnosis. The surgery for breast carcinoma was performed between the 1st January 1980 and the 31st December 1986. The project has been approved by the Laval University Ethical committee. Clinical information was obtained from the patients' charts by

experienced research nurses. The tumor size, number of examined and positive lymph nodes were obtained from the pathology reports. The histologic types along with the histologic and nuclear grades were reassessed by one of us (B.T.).

### **Immunohistochemistry**

For each breast cancer case, a 5 micron section stained with hematoxylin and eosin was followed by 10 unstained sections for immunohistochemistry and *in situ* hybridization. For cathepsin D, an immunohistochemical study was performed using the avidin-biotin complex (ABC) method as described before (Têtu *et al.*, 1993). A primary polyclonal antibody to cathepsin D (Novocastra, Newcastle, England; dilution: 1/200) was used.

### ***In situ* Hybridization**

Stromelysin-3 and uPA expression has been analyzed by *In situ* hybridization. The technique used was a modification of the method of Wolf *et al.* (Wolf *et al.*, 1993) as described before (Têtu *et al.*, 1998). S<sup>35</sup> labeled antisense RNA probes for Stromelysin-3 and uPA were used. Plasmids were kindly provided by Pr. Paul Basset, IGBMC, Illkirch, France. cDNA inserts were prepared from breast cancer cDNA libraries, subcloned in pBluescript II vectors and were used as templates for *in vitro* transcription to generate sense and anti-sense probes. The 1759 base pairs (bp) Stromelysin-3 cDNA was subcloned in the Eco RI site and extended from nucleotides (nt) 346 to 2105 and the uPA cDNA was subcloned in the Pst I site and extended from nt 300 to 1300. Reduction of the probe length was achieved by partial alkaline hydrolysis. Optimal *in situ* hybridization was obtained with 100 bp probes for stromelysin-3 and uPA. The quality of RNA preservation has been assessed with the use of antisense RNA probes for beta-actin and negative controls were obtained with the use of sense probes.

### **Interpretation**

Immunohistochemistry and *in situ* hybridization have been interpreted by one of us (BT) without knowledge of the clinical information. The scoring was assessed separately on cancer and stromal cells using a semi-quantitative scale. For each cell compartment, the percentage of cancer or stromal cells expressing the marker (0%, <10%, 10-50%, >50%) was evaluated on the whole tumor surface of one representative section.

### **Statistical analysis**

Distant-metastasis-free (DMFS) and overall (OS) survival curves were obtained for each protease expressed by reactive stromal cells according to Kaplan and Meier. The difference between the curves was assessed using the log-rank test. DMFS and OS curves were generated using 10% as cutoff point between those expressing low (negative) or high (positive) protease levels by stromal cells, as defined before (Okamura *et al.*, 1997; Têtu *et al.*, 1993; Têtu *et al.*, 1998). Three categories were defined: 1) Tumors expressing all three proteases; 2) Tumors expressing none of those proteases; 3) Tumor with expression of any other combination of proteases. The Cox proportional hazard model was used to evaluate the relation of protease expression with recurrence and death adjusting for other known or suspected prognostic factors. Hazard Ratios (HR) for the occurrence of distant metastases or death of patients with any of the above combinations were obtained by univariate and multivariate analyses. HR compare the rate of tumor recurrence or death of patients whose tumor expressed a set of proteases to those whose tumor did not express the markers.

### **RESULTS**

In our tumor bank, information was available for cathepsin D by reactive stromal cells in 1348 breast cancers, in 676 for stromelysin-3 and in 585 cases for uPA. Results for all three proteases were available on 557 cases. One hundred ninety-two (192) were node-negative (NNBC) and 365 had node invasion (NPBC). At last follow-up, 266 (47.8%) were alive and the follow-up of surviving patients averaged 9.6 years and ranged from 5.2 to 14.6. Twenty (20) were lost to follow-up.

Figure 1 shows representative examples of positive expression of all three markers by reactive stromal cells. Of the 557 cases, using 10% as cut-off point, stromal cells expressed cathepsin D in 241 (43.3%) cases, stromelysin-3 in 329 (59.1%) cases and uPA in 167 (30.0%) cases. Three categories have been defined: tumors expressing all three proteases (80 cases; 14.4%), tumors expressing no protease (134 cases; 24.1%) and those expressing 1 or 2 proteases (343 cases; 61.5%).

Figure 2 shows the DMFS curves of each of the three categories. At five years, 42.5% of patients whose tumor expressed all three proteases were alive and free of distant disease whereas 63.1% of those whose tumor lacked at least one protease were alive and metastasis-free. The difference between the curves was statistically significant ( $p=0.0026$ ). No difference in DMFS was noted whether 0, 1 or 2 proteases were expressed. Therefore, when the category representing tumors expressing no protease was grouped with that expressing 1 or 2 proteases, the difference in DMFS with tumors expressing all three proteases was even more significant ( $p=0.0006$ ). Similar results were found with OS (54% alive at 5 years for cases expressing three proteases and 72% for those expressing 0, 1 or 2 proteases).

The table shows the HR of developing distant metastases when all three categories (three proteases, no protease, 1 or 2 proteases expressed) were included in the model and when the cases lacking at least one protease were grouped together. By multivariate analysis, after adjustment for lymph node status and tumor size, the HR of developing distant metastases was 83% greater for those expressing all three proteases compared to those expressing none ( $HR=1.83$ ;  $p=0.002$ ). The HR for those expressing 1 or 2 proteases was comparable to that of tumors expressing no protease ( $HR=0.99$ ;  $p=0.96$ ). The influence of concomitant expression of all three proteases was even more significant when tumors expressing 1 or 2 proteases were grouped with those expressing no protease ( $HR=1.84$ ;  $p<0.0001$ ).

Further analyses were performed to confirm our findings. We focused successively on NNBC, NPBC, and on tumors smaller or larger than 3 cm. In each subgroup, as described above, the prognosis of tumors expressing no protease was significantly more favorable than that of tumors expressing all three proteases. The prognosis of tumors expressing no protease was comparable to that of tumors expressing one or two proteases in all but the NNBC subgroup. Indeed, the survival of NNBC patients whose tumor expressed 1 or 2 proteases was intermediate between the other two categories.

## DISCUSSION

In this study, the prognosis of breast cancer patients was significantly worse for those tumors whose reactive stromal cells overexpressed all three proteases compared to those lacking at

least one. Furthermore, although a lower recurrence rate was observed when one or two proteases were missing, the recurrence rate was comparable to that of tumors expressing none of the three proteases. Those findings are in line with the concept that several proteases, including those produced by reactive stromal cells, are interdependent (Chambers and Matrisian, 1997; MacDougall and Matrisian, 1995; Nagase, 1997; Santavicca *et al.*, 1995; Solomayer *et al.*, 1998) and suggest that a breach in that protease pathway may impair breast cancer progression.

Our data also stress the stronger clinical relevance of assessing the prognostic value of several proteases jointly rather than separately. However, this concept is better established for proteases that have specific inhibitors. For example, clinical studies suggest that the ratio of gelatinase A and TIMP-2 expression may play a critical role on cancer progression. Patients with bladder cancer (Gohji *et al.*, 1996) and uterine cervix cancer (Nuovo *et al.*, 1995) with higher gelatinase A/TIMP-2 ratios were more likely to follow a poor outcome. In a study on 14 breast cancer patients, Onisto *et al.* (Onisto *et al.*, 1995) report that a high gelatinase A/TIMP-2 ratio correlated with lymph node metastases. The study of Foekens *et al.* (Foekens *et al.*, 1995) even reported no significant association of Plasminogen Activator type-2 Inhibitor (PAI-2) with the prognosis in uPA-low breast tumors while in uPA-high tumors, low inhibitor levels were strongly associated with a short DMFS (Foekens *et al.*, 1995), further supporting our hypothesis that an intact protease cascade is necessary to maintain tumor invasiveness.

While several proteases may be inhibited by specific inhibitors, they may also be activated by other proteases. For example, there are *in vitro* evidences that Pro-gelatinase A can be activated by other MMPs such as MMP-1 and MMP-7 (Crabbe *et al.*, 1994) and progelatinase B can be activated by interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), matrilysin (MMP-7) and gelatinase A (Nielsen *et al.*, 1997). Cathepsin D was found to indirectly activate uPA since cathepsin D can activate cathepsin B and L (van der Stappen *et al.*, 1996) which in turn can activate uPA (Goretzki *et al.*, 1992; Kobayashi *et al.*, 1991; Takei *et al.*, 1997). Very few studies, however, investigated the prognostic influence of combined proteases that lacked protease inhibitor activity.

Unexpected findings were observed in NNBC. In this subgroup, tumors expressing 1 or 2 proteases followed a clinical course that was intermediate between those expressing none or all three proteases. Those findings are difficult to explain but may be due to a relatively small number of patients in this subcategory (192 cases) or some unrecognized biologic factors.

In conclusion, our study further demonstrates the clinical relevance of assessing the joint effect of several proteases when studying their expression in relation to the risk of recurrence in breast cancer. This is consistent with the fact that many proteases are strongly interrelated and appear to act in a cascade. It also adds support to the concept that proteases produced by stromal cells play a key role on tumor progression.

#### **ACKNOWLEDGEMENTS**

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**Figure 1. Representative examples of positive expression of all three markers by reactive stromal cells.**

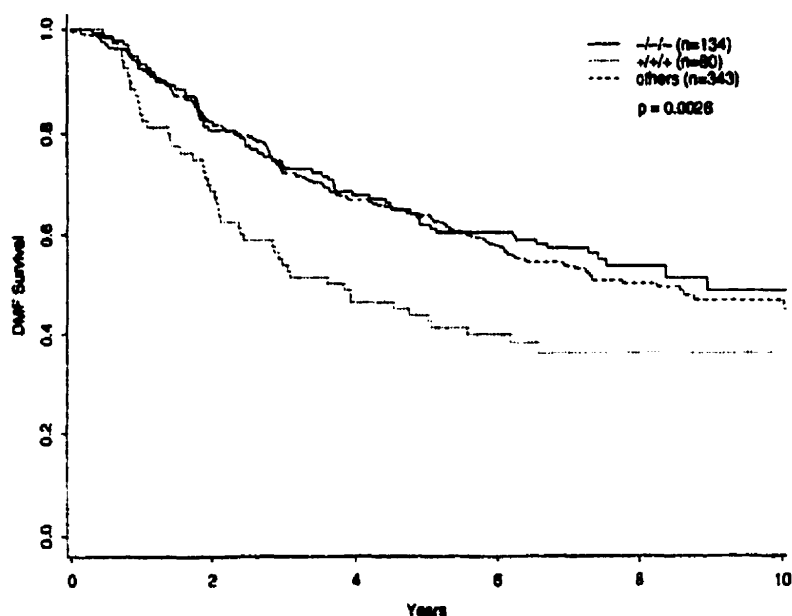


Figure 2. DMFS of patients with expression of three proteases.

DMFS of patients with tumors expressing either all three (+/+/+), or any other combination of the three proteases (Cathepsin D, urokinase Plasminogen activator, stromelysin-3); positivity being defined as  $\geq 10\%$  positive cells.

Table 1. Hazard Ratios for the association of protease expression on occurrence of metastases and death.

| Marker           | Univariate<br>HR** (95% CI) | P value | Multivariate*<br>HR (95% CI) | P value |
|------------------|-----------------------------|---------|------------------------------|---------|
| -/-/-            | 1.0                         |         | 1.0                          |         |
| +/+/+            | 1.800 (1.239-2.616)         | 0.0020  | 1.829 (1.247-2.683)          | 0.0020  |
| Other            | 1.087 (0.812-1.455)         | 0.5747  | 0.992 (0.732-1.344)          | 0.9582  |
| Combinations     |                             |         |                              |         |
| -/-/- and others | 1.0                         |         | 1.0                          |         |
| +/+/+            | 1.695 (1.247-2.304)         | 0.0008  | 1.840 (1.345-2.516)          | 0.0001  |

\* adjusted for lymph node involvement and tumor size.

\*\* HR: Hazard ratio

## **CHAPITRE IX**

### **DISCUSSION ET CONCLUSION**

## Discussion

Cette étude qui a duré quatre ans soit de 1995 à 1999 a porté sur les interactions épithéliales-stromales dans le cancer mammaire primaire plus particulièrement sur le rôle des cellules stromales. Elle visait à vérifier l'hypothèse que les cellules stromales jouent également un rôle non négligeable en favorisant la progression tumorale mammaire ouvrant la voie à de nouvelles avenues de thérapie anticancéreuse. Cette hypothèse découle de la découverte du docteur Têtu en 1993 que la survie dans un sous-groupe de patientes porteuses de cancer mammaire avec atteinte ganglionnaire lymphatique était liée significativement à l'expression de la cathepsine D par les cellules stromales et non par les cellules cancéreuses. Ce résultat implique que les protéases stromales produites par les dans le cancer du sein influencent la progression tumorale.

Les résultats des études réalisées et présentées dans cette thèse apportent un nouvel éclairage sur le rôle des cellules stromales dans le cancer mammaire humain, et répondent à une partie de l'hypothèse proposée.

### 1. Les fibroblastes associés à la tumeur mammaire sont différents des fibroblastes normaux.

Les fibroblastes utilisés dans cette étude ont été purifiés à partir de tumeurs primaires mammaires. Les fibroblastes présentent deux aspects morphologiques *in vitro*, soit les aspects *fibroblast-like* et *myofibroblast-like*. La nature de cellules stromales a été confirmée par l'expression de vimentine et la morphologie en culture. Dans le cas de myofibroblastes, on observe une expression de l' $\alpha$ -actine.

Dans le cancer mammaire, on a démontré que 70% (Ronnov-Jessen *et al.*, 1992) à 80% (Sappino *et al.*, 1988) des cellules stromales sont des myofibroblastes. Les myofibroblastes sont impliqués dans plusieurs processus pathologiques. Ils induisent la desmoplasie (Barsky *et al.*, 1984) et sont une source importante de protéases (Nielsen *et al.*, 1996) impliquées dans la dégradation protéolytique. La fonction des myofibroblastes n'est pas complètement élucidée dans le cancer mammaire.

Toutes les cellules stromales purifiées de tumeurs mammaires présentent une expression de CD68 identifié par KP-1. On ne sait pas si ces cellules stromales sont vraiment d'origine macrophagique avec persistance de CD68 malgré une transdifférentiation en cellules fibroblastiques ou myofibroblastiques ou si le CD68 identifié par KP-1 n'est pas un marqueur spécifique des cellules d'origine myéloïde. Le CD68 est couramment utilisé sur coupes tissulaires en clinique pour identifier les macrophages. Il serait peut-être préférable de caractériser ces cellules stromales associées à la tumeur par une analyse ultrastructurale ou à l'aide d'anticorps dirigés contre d'autres épitopes de CD68, afin de confirmer la véritable nature de ces cellules.

Ces cellules stromales associées à la tumeur sont différentes des fibroblastes purifiés de la peau normale. Dans la coculture de tissu-équivalent bCTC), ces fibroblastes favorisent la migration ou l'invasion des cellules de cancer mammaire et participent à la modulation de la production ou sécrétion de protéases comme les gélatinases liées au phénotype des cellules épithéliales, suggérant qu'ils jouent un rôle dans la migration des cellules cancéreuses et dans le remodelage tissulaire.

Par coculture *in vitro* et avec une stimulation tumorale comparable, le degré de réponse des cellules stromales à la stimulation cancéreuse est variable selon la tumeur dont les fibroblastes sont issus. En effet, l'expression de la stromélysine-3 par ces cellules stromales associées à la tumeur après la stimulation par une même source maligne est différente pour chaque tumeur individuelle dont ces cellules stromales ont été purifiées.

Tous ces résultats indiquent que les cellules stromales d'origine tumorale sont différentes des cellules normales puisqu'elles produisent des protéases absentes chez les cellules non stimulées. Elles méritent le nom déjà proposées de cellules activées, réactionnelles, et anormales. Comme la stromélysine-3 exprimée à l'interface de cellules épithéliales cancéreuses-stromales par ces cellules associées à la tumeur est impliquée dans l'invasion tumorale et que ces cellules favorisent la dégradation de la matrice extracellulaire et la migration des cellules cancéreuses, l'approche visait à inactiver ces cellules associées à la tumeur devrait avoir un impact majeur sur la recherche de nouvelles voies de thérapie

anticancéreuse.

## **2. La prolifération des cellules épithéliales cancéreuse mammaires a besoin du support stromal.**

Les cellules épithéliales cancéreuses mammaires ne peuvent proliférer facilement en culture primaire quand ces cellules épithéliales sont isolées de l'organe *in vivo*. Au cours de l'étude initiale, les cellules épithéliales cancéreuses mammaires ont été sélectionnées par prolifération en culture sur agar. Cependant, quand ces cellules épithéliales purifiées sont transférées en culture simple, la prolifération de ces cellules est complètement perturbée et même arrêtée, bien qu'un milieu de culture riche en nutriments et avec sérum à 10% ait été utilisé.

On sait que la prolifération de ces cellules épithéliales cancéreuses ainsi que la croissance tumorale dépendent de facteurs de croissance, du support stromal, de stimulations paracrinienes, et de l'angiogenèse *in vivo*. En culture *in vitro*, cet environnement n'existe plus. Il est donc logique de chercher un moyen pour assurer le support stromal *in vitro*. En effet, l'utilisation d'une couche nourricière de cellules 3T3 irradiées sert non seulement à contrôler la surcroissance des fibroblastes, mais aussi à améliorer et accélérer la prolifération des cellules épithéliales cancéreuses mammaires *in vitro*.

La purification de cellules épithéliales mammaires humaines à partir de tumeurs mammaires est un travail difficile (McCallum and Lowther, 1996). Deux facteurs expliquent cette difficulté: la surcroissance de fibroblastes (Cailleau *et al.*, 1974; Engel *et al.*, 1978b; Soule *et al.*, 1973; Speirs *et al.*, 1998) et la prolifération lente des cellules épithéliales lorsqu'isolées de l'organe vivant (von Fournier *et al.*, 1980). En effet, sans inhibition sélective de la prolifération des fibroblastes, la culture primaire suite à la digestion enzymatique de tissus tumoraux a entraîné une surcroissance de fibroblastes dans chaque cas dans l'expérience initiale (résultats non montrés). Nous avons au début essayé également l'utilisation d'un flacon de culture (Primaria®, Falcon) spécifiquement pour inhiber la prolifération stromale. Bien que la vitesse de prolifération des fibroblastes ait été relativement ralentie, les cellules épithéliales ne pouvaient être purifiées et utilisées. L'administration de généticine a été tentée également, et l'effet d'inhibition des cellules stromales était très faible. Nous avons ensuite

utilisé la sédimentation au lieu de la centrifugation pour récupérer sélectivement les cellules épithéliales. En général, dans la suspension cellulaire après digestion enzymatique, les cellules stromales étaient isolées les unes des autres, tandis que les cellules épithéliales se regroupaient en agrégat, et cet agrégat épithélial formait le sédiment plus vite que les cellules stromales. Cependant, il était très difficile d'optimiser le bon moment pour une pure séparation sélective.

La plupart des méthodes rapportées se concentrent uniquement sur les moyens de contrôler la surcroissance des fibroblastes. La culture en milieu sélectif est une méthode couramment utilisée à ce jour. Cette méthode utilise un milieu riche en nutriments et permet de réduire la concentration de sérum en culture, car le sérum pourrait favoriser la surcroissance des fibroblastes (Dairkee *et al.*, 1995; Ethier *et al.*, 1993). Bien que cette méthode puisse diminuer la proportion des fibroblastes en culture cette inhibition n'est jamais complète. En plus, cette méthode nécessite une concentration très faible de sérum, tandis que ce dernier contient des peptides, des facteurs de croissance, des hormones etc. qui sont également nécessaires pour maintenir une culture primaire de cellules épithéliales.

La couche nourricière de cellules 3T3 irradiées est couramment utilisée par le groupe de recherche du LOEX (CHA, Pavillon Saint-Sacrement, Québec) pour maintenir les kératinocytes en culture. Les résultats de notre étude indiquent que cette méthode fonctionne également pour la culture de cellules épithéliales cancéreuses mammaires. Cette méthode semble résoudre en même temps les deux problèmes rencontrés couramment dans la culture sélective de cellules épithéliales de tumeurs mammaires soit la trop grande prolifération de fibroblastes et la faible prolifération des cellules épithéliales.

Au moment de commencer ce travail, nous n'avions pas trouvé d'étude semblable dans la littérature. En 1998, Matouskova (Matouskova E *et al.*, 1998) a présenté, par communication courte, une étude utilisant la couche nourricière par cellules 3T3. Dans son étude, les 3T3 ont été irradiée à 10 Gy, et l'auteur a observé, à court terme, une expansion clonale importante des cellules épithéliales de cancers mammaires. Cependant, les auteurs ne s'intéressaient pas à la purification des cellules épithéliales. Bien que l'on n'ait pas comparé l'effet d'une dose d'irradiation entre 10 et 60 Gy, il semble que l'irradiation à la dose de 60 Gy supprime le plus

la capacité de prolifération des cellules 3T3 irradiées tout en conservant leur métabolisme biologique. Les cellules 3T3 irradiées à une dose de 60 Gy sont incapables de se proliférer. Dans notre étude, nous ne voulions nous limiter à la culture à court terme. En effet, la culture à long terme a été réussie chez une des six cas étudiés.

Le karyotype est une technique sophistiquée qui nécessite une quantité importante de cellules purifiées. Pour cette raison, l'analyse du karyotype a été seulement réalisée dans un cas de culture à long terme, et a confirmé la malignité de ces cellules.

Au total, l'utilisation de la couche nourricière 3T3 irradiées à 60 Gy est une méthode simple et pratique qui permet non seulement une purification de cellules épithéliales en culture à court terme mais permet aussi d'accroître les chances de réussir une culture à long terme et l'établissement de lignées cellulaires. Cette méthode pourrait être combinée avec d'autres méthodes sélectives. On se demande si la combinaison d'une couche nourricière de cellules 3T3 irradiées et un milieu sélectif pourrait améliorer la sélection de cellules épithéliales cancéreuses mammaires.

### **3. L'intérêt de la nouvelle lignée cellulaire mammaire.**

Purifiée en utilisant la couche nourricière de cellules 3T3 irradiées, la lignée HDQ-P1 a été établie et bien caractérisée. L'établissement de cette nouvelle lignée mammaire a plusieurs intérêt pour l'étude du cancer du sein.

D'abord, la méthode utilisée pour réussir l'établissement de cette nouvelle lignée a été un succès et représente une alternative intéressante. De plus, la plupart des lignées mammaires commercialisées sont d'origine métastatique (Cailleau *et al.*, 1974; Kurebayashi *et al.*, 1995; Siwek *et al.*, 1998; Smith *et al.*, 1987; Zoli *et al.*, 1997). Dans la littérature, deux grandes séries ont rapporté des lignées à partir des tumeurs primaires mammaires. Le taux de réussite est de 1/136 tumeurs et de 10 sur 135 tumeurs (McCallum and Lowther, 1996) par milieu sélectif. Parmi les 6 cas présentés dans cette thèse, une lignée a été établie, suggérant une grande probabilité d'avoir de nouvelles lignées cellulaires à partir de tumeurs primaires pouvant servir à la recherche dans le cancer du sein.

Les caractéristiques cytogénétiques du cancer du sein ne sont pas claires à ce jour. Il n'y a pas encore d'anomalies spécifiques identifiées, sauf dans de rares études. Certains auteurs ont rapporté qu'une altération du chromosome 6 était la plus fréquente (McCallum and Lowther, 1996) et que le chromosome 1 montrait souvent un réarrangement de structure (Amadori *et al.*, 1993; McCallum and Lowther, 1996). L'analyse cytogénétique de HDQ-P1 a révélé dans ces cellules un karyotype hyperploïdique réarrangé et stable. Les chromosomes les plus réarrangés sont les 1, 7, 9, et le chromosome 6 est celui le plus souvent dupliqué. Ces résultats s'ajoutent aux connaissances actuelles sur les changements cytogénétiques du cancer du sein. En plus du karyotype réarrangé, les cellules HDQ-P1 présentent une mutation de p53 au codon 213 et une amplification de l'oncogène c-myc. Ces deux événements sont connus dans une population des cancers mammaires. Ces caractéristiques des cellules HDQ-P1 sont comparables au cancer *in vivo* et cette lignée a donc un bon modèle pour étudier le cancer mammaire.

#### **4. Intérêt de valeurs pronostiques des protéases dans le cancer du sein.**

Un facteur pronostique constitue un des paramètres importants en cancérologie clinique, et surtout dans le cancer mammaire. L'amélioration des modalités thérapeutiques permet à ce jour à des patientes de mieux bénéficier de la thérapie adjuvante par une amélioration de la survie (Early Breast Cancer Trialists' Collaborative Group, 1992). La plupart des patientes sans envahissement ganglionnaire ont effectivement une excellente survie à long terme. Cependant, toutes ces patientes ne bénéficient de la thérapie adjuvante et une proportion de ces patientes à bon pronostic ont une récurrence de la maladie, malgré l'absence d'envahissement ganglionnaire au moment du diagnostic. Le dépistage et l'avancement des technologies diagnostiques permettent en réalité un diagnostic précoce du cancer du sein. Par conséquent, une grande proportion des lésions découvertes sont petites et sans envahissement ganglionnaire. Donc, selon la classification classique, toutes ces lésions ont un bon pronostic. Cependant, une certaine proportion des patientes avec ces petites lésions développeront tout de même une récurrence précoce. Si on pouvait cibler ces patientes, elles pourront être traitées par des modalités plus adéquates avant la récurrence de la maladie. Nous croyons que les protéases produites par les cellules stromales jouent un rôle dans l'invasion tumorale mammaire, et que

les cellules stromales sont un cible intéressant pour de nouvelles thérapies, parce que moins susceptibles de développer des résistances.

Les facteurs pronostiques cliniques établis permettent d'identifier les patients avec cancer à faible ou à haut risque, et donc d'adapter le traitement. Dans deux études cliniques, l'intérêt pronostique de la CD, l'uPA et la ST3 a été étudié chez des patientes porteuses de cancer du sein. Une bonne corrélation entre la forte expression stromale de ces protéases et la faible survie chez ces patientes confirme que ces protéases stromales (CD, uPA et ST3) participent à la progression du cancer mammaire, et l'expression stromale de ces protéases peut prédire la survie à long terme chez ces patientes avec cancer mammaire.

Dans une étude clinique du Dr Têtu (Têtu *et al.*, 1998), l'expression de la ST3 dans les fibroblastes était associée à certains facteurs cliniques, tels qu'un grade nucléaire pauvre, un âge plus jeune, un envahissement ganglionnaire, une histologie canalaire, l'aneuploïdie et l'expression de HSP-27. L'expression de ST3 pouvait prédire un faible taux de survie sans récurrence. Certains autres auteurs ont rapporté une association entre l'expression de ST3 et l'histologie canalaire (Chenard *et al.*, 1996; Linder *et al.*, 1997), au degré nucléaire pauvre (Hahnel *et al.*, 1993) et à un faible taux de survie de patientes (Ahmad *et al.*, 1998; Chenard *et al.*, 1996; Delany and Canalis, 1998).

Dans notre étude clinique sur l'intérêt pronostique de ST3 à long terme, l'analyse nous montre que l'expression de ST3 ne pouvait pas prédire toute seule une survie sans métastase à long terme. Par contre, l'expression stromale combinée de la CD, l'uPA et la ST3 montre une bonne corrélation avec un faible taux de survie sans métastase à long terme. Par contre, la survie est la même, quelque soit le type et le nombre de protéases manquantes. L'importance de ces résultats est la mise en évidence en clinique que ces protéases sont interdépendantes pour favoriser la progression tumorale, suggérant que ces protéases agissent en cascade dans le processus d'invasion par les cellules cancéreuses.

### **5. L'expression stromale de la ST3 est cancer-spécifique et représente une réaction individuelle de l'hôte.**

Par le modèle de coculture *in vitro*, l'expression de ST3 a été examinée dans des fibroblastes dérivés de tumeur mammaire et dans des fibroblastes cutanés normaux en présence de cellules épithéliales cancéreuses mammaires de différente agressivité. Ce modèle de coculture reproduit un tissu-équivalent *in vitro* simulant une tumeur mammaire *in vivo*. L'objectif était de localiser, sur un tissu-équivalent, les fibroblastes répondants à la stimulation cancéreuse. La technique *in situ* a permis d'étudier l'expression de protéases par des fibroblastes de diverses sources et de localiser le signal (distal ou proximal), répondant ainsi à la recommandation par Dano (Johnsen *et al.*, 1998).

A l'aide d'une coculture avec des fibroblastes cutanés normaux, nous avons confirmé l'hypothèse de Basset *et al.* à l'effet que les cellules cancéreuses mammaires pouvaient stimuler les fibroblastes normaux à produire la ST3 *in vitro* (Ahmad *et al.*, 1997). Cependant, la coculture directe ainsi que la méthode *in situ* nous a permis d'observer une surexpression évidente de ST3 par ces fibroblastes normaux en présence de cellules cancéreuses des lignées MCF-7 et MDA-MB-231 déjà rapportées, mais aussi de SK-BR-3.

Cocultivés avec les cellules cancéreuses d'origine mammaire, les fibroblastes d'origine tumorale ont été stimulés pour produire de la ST3. Cet effet de l'induction était très évident dans les trois lignées malignes utilisées dans l'étude (MDA-MB-231, SK-BR-3 et MCF-7); par contre, la coculture avec la lignée non tumorale (184A1) et les cellules épithéliales normales mammaires ne montrait pas d'effet d'induction sur l'expression de ST3. Nos résultats montrent que les cellules stromales d'origine tumorale ou normales, cellules réceptives, expriment la ST3 uniquement en présence de cellules cancéreuses, cellules stimulantes. Nos résultats ont confirmé que l'induction par les cellules épithéliales sur la production de ST3 par les fibroblastes était spécifique aux tumeurs malignes.

Il est intéressant de commenter également l'effet d'induction par la lignée MGH-U4. Cette lignée a été obtenue à partir d'une lésion atypique sévère de l'urothélium (Lin *et al.*, 1985). La coculture avec cette lignée a induit une production de ST3 dans les fibroblastes, bien que cette

expression de ST3 soit faible et focale. Les résultats supportent l'observation clinique que la ST3 était fréquemment exprimée dans les cancers *in situ* et les lésions précancéreuses comme la dysplasie (Basset *et al.*, 1990; Bolon *et al.*, 1996).

Il est intéressant également de noter que, dans le cas d'une coculture mettant en présence des cellules cancéreuses ou stromales d'une même tumeur, l'expression de ST3 par les fibroblastes se situe au voisinage des cellules cancéreuses. Nos résultats confirment que l'expression de ST3 par les fibroblastes était ré-inductible, que la stimulation venait de la présence de cellules cancéreuses et que seuls les fibroblastes situés à proximité des cellules tumorales répondaient à la stimulation.

L'effet d'induction est très probablement surtout via un contact direct de cellules épithéliales cancéreuses et de cellules stromales, car cette induction est toujours limitée aux fibroblastes situés à l'interface. Bien que l'effet d'induction soit aussi observé par la coculture indirecte, c'est dans la coculture directe qu'on observe l'expression la plus importante dans les cellules stromales voisines. Ceci nous fait présumer que le contact cellules-cellules serait possiblement la condition essentielle pour l'induction de ST3 ou que les fibroblastes répondaient à certains facteurs solubles très limités et concentrés près des cellules cancéreuses. Certains auteurs dans la littérature ont présumé que les cellules cancéreuses pouvaient produire un gradient de concentration des facteurs diffusibles qui stimuleraient la production de ST3 par les fibroblastes situés à une distance efficace de l'activité biologique de ces facteurs présumés (Basset *et al.*, 1990). Il serait souhaitable d'utiliser une coculture tridimensionnelle afin de simuler la situation *in vivo* et d'étudier la relation directe entre la production stromale de stromélysine-3 et l'invasion des cellules cancéreuses mammaires.

Parmi les facteurs solubles et responsables de l'induction de la production de ST3, on retrouve le bFGF. Cependant, l'administration de bFGF exogène ou anti-bFGF n'a pas changé significativement l'effet d'induction par cellules cancéreuses sur l'expression de ST3. Dans le carcinome mammaire, les cellules cancéreuses peuvent produire des facteurs/cytokines qui pourraient activer les gènes des MMPs des fibroblastes voisins (Basset *et al.*, 1990; Gullick W.J, 1990; Lippman and Dickson, 1989). Cependant, ce n'est pas le cas pour la ST3. Dans une

étude récente (Ahmad *et al.*, 1997), ces facteurs, EGF, bFGF et PDGF n'ont pas stimulé le promoteur de ST3, et les auteurs ont suggéré que l'activation de l'activité du promoteur de ST3 n'était pas une conséquence de cytokines. Nos résultats correspondent à ceux récemment publiés par Mari *et al.* (Mari *et al.*, 1998) où le bFGF n'influence pas l'induction du niveau d'ARNm ni du niveau de protéine, mais participe à la modification post-traductionnelle seulement. On suggère que les cellules cancéreuses produisent des facteurs diffusibles dont bFGF, et que ce bFGF active certaines autres MMP qui modifient la forme mature de ST3 de 45 kDa en un fragment de 35 kDa, vérifiable par la technique de Western blot. Par immunohistochimie dans notre étude, nous ne pouvons détecter cette modification post-traductionnelle.

Dans la littérature, seulement deux études *in vitro* ont analysé l'effet de l'induction de ST3 par les cellules cancéreuses. Une étude a utilisé des cellules cancéreuses pulmonaires (NSCLC) dans une étude de coculture indirecte et directe (Mari *et al.*, 1998) et l'autre a utilisé des cellules cancéreuses mammaires (MDA-MB-231, MCF-7 et ZR-75) (Ahmad *et al.*, 1997). Dans ces deux études, les cellules stromales étaient des fibroblastes normaux de poumon et de peau, et l'analyse s'est faite par Northern blot. Notre étude cherchait pour la première fois la réponse *in vitro* par des fibroblastes d'origine tumorale à une stimulation par des cellules cancéreuses mammaires. A la différence des fibroblastes normaux, les fibroblastes d'origine tumorale ont répondu différemment à la stimulation tumorale. En effet, les fibroblastes n'ont pas répondu à la stimulation cancéreuse de la même façon. Par la coculture *in vitro* et devant la même stimulation tumorale, le degré de réponse à la stimulation cancéreuse était variable. Les cellules F9846 ont le mieux répondu à l'effet d'induction, tandis que les fibroblastes F14700 ont très peu répondu, et même après une amplification de 25 cycles, l'expression de ST3 dans les cellules F14700 ont été observée presque négative. Le degré d'expression de ST3 dans chaque lignée de fibroblastes a été corrélé avec l'aspect histologique de la tumeur original, sa différenciation et l'envahissement ganglionnaire. Limité par le nombre de cas, nous ne pouvons pas tirer une conclusion définitive. Cependant, le fait que l'expression variable de ST3 se retrouve uniquement parmi les fibroblastes d'origine tumorale suite à une stimulation par des cellules malignes suggère l'hypothèse que les fibroblastes d'origine tumorale sont phénotypiquement différents des fibroblastes normaux de peau. Ces fibroblastes

associés à la tumeur répondent de façon différente à la stimulation cancéreuse par production variable de ST3 suggérant que la réponse par ces fibroblastes à la stimulation de cellules cancéreuses mammaires *in vitro* représente une réaction propre à l'hôte. En plus, non seulement les cellules cancéreuses mais aussi les cellules stromales joueraient un rôle également actif dans le processus d'invasion.

Les divers types de tumeurs n'expriment pas ST3 de façon comparable. L'expression de ST3 n'est pas observée dans les lymphomes et on croit que l'induction de ST3 est *epithelial specific* (Kossakowska *et al.*, 1996). Dans une autre étude sur 12 carcinomes de la thyroïde (Zedenius *et al.*, 1996), l'expression de ST3 était absente chez 12 tumeurs étudiées. Une hypothèse peut être tirée de nos résultats, c'est que la réponse de l'hôte à l'induction de cellules cancéreuses pourrait dépendre du phénotype des fibroblastes de différentes sources, et du type de cytokines produites par la tumeur.

Il n'est pas clair si l'activité enzymatique de certaines protéases telle ST3 est absolument nécessaire pour l'invasion et la métastase des cellules cancéreuses. La ST3 semble plutôt impliquée dans la formation tumorale et, surtout, son effet protéolytique semble minime. Des chercheurs dans le domaine se demandent (Blasi and Stoppelli, 1999) si la ST3 est essentielle à la progression tumorale? Comme les substrats de la ST3 sont encore inconnus, on peut croire qu'ils sont des facteurs de croissance sous une forme immature. On peut également présumer que le site de l'hémopexine C-terminal est impliqué dans une interaction fonctionnelle au lieu du site protéolytique ?

En utilisant l'approche très sensible par RT-PCR, l'expression de ST3 a été identifiée dans des lignées cellulaires cancéreuses mammaires telles que MCF-7, MDA-MB-231, SK-BR-3, etc. Bien que la ST3 soit surtout présente dans les cellules stromales des carcinomes, son expression est aussi observée dans des cellules épithéliales cancéreuses de certains carcinomes (Bolon *et al.*, 1997). Dans les carcinomes pulmonaires, cette expression épithéliale existe surtout dans les carcinomes malpighiens et basaloïdes et est liée de façon inverse au degré de différenciation malpighienne (Bolon *et al.*, 1997). L'expression épithéliale de ST3 est aussi observée dans des cellules cancéreuses épithéliales du pancréas (6/12) (von Marschall *et al.*,

1998). L'expression de ST3 a été également retrouvée dans des lignées cellulaires cancéreuses, tels que MCF-7 et MDA-MB-231 dans le cas mammaire, et CCF-STTG1 (astrocytome), HepG2 (hépatome), SW620 (adénocarcinome de côlon), et Tera-2 (teratome) (Giamb Bernardi *et al.*, 1998). L'expression épithéliale de ST3 dans le cancer du sein est liée uniquement à la transition histologique du phénotype épithéliale à mésenchymateux (Ahmad *et al.*, 1998). Il est possible que cette expression épithéliale reste absente à cause de la sensibilité faible du Northern blot, la technique utilisée dans la plupart des études. Par PCR, toutes les lignées épithéliales dans notre étude montrent une expression variable de l'ARNm de ST3, suggérant qu'il existe un niveau de base de la ST3 dans ces cellules épithéliales. A ce jour, le rôle de ST3 par les cellules épithéliales n'est pas clair, et il pourrait s'agir d'une expression non-adéquate liée au phénotype malin.

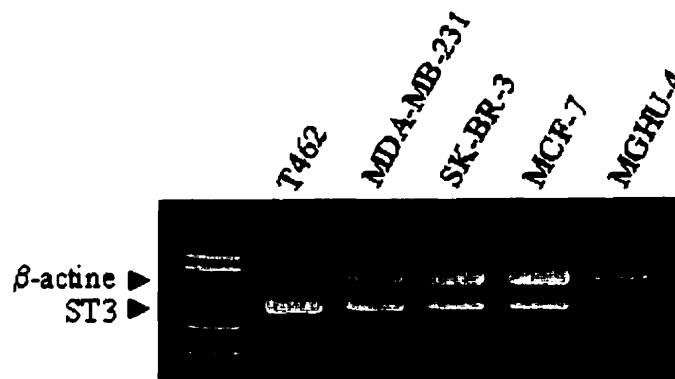


Figure 1. L'expression d'ARNm de la ST3 par les cellules épithéliales.

L'expression d'ARNm de la ST3 est observée également dans des lignées cancéreuses mammaires (MDA-MB-231, SK-BR-3 et MCF-7). Cette expression est faiblement positive dans la lignée MGHU-4, une lignée cancéreuse de l'urothélium. T462 est un tissu tumoral du sein. (Résultat de RT-PCR, dilution à 1 :256 pour  $\beta$ -actine).

#### 6. La Cathepsine D stromale favorise la progression du cancer mammaire.

Une bonne corrélation a été observée entre l'expression de la cathepsine D par les cellules stromales, et non les cellules cancéreuses, et un faible taux de survie des patientes porteuses de cancer mammaire. Ces observations confirment que la cathepsine D par les cellules stromales

contribue à cette progression tumorale. Dans la littérature, deux principales catégories de techniques analytiques ont été étudiées : analyse du cytosol par Western blot, IRMA et ELISA ; et analyse de la cathepsine D cytoplasmique par immunohistochimie. Outre des différences de technique, des anticorps utilisés et des seuils de positivité choisis, une différence essentielle est que l'analyse du cytosol considère la cathepsine D du tissu tumoral *in toto*, sans différencier la contribution des différents compartiments de cellules tandis que l'analyse par immunohistochimie permet de localiser les différentes cellules exprimant la cathepsine D.

L'analyse de la cathepsine D par méthode cytosolique est préférée par les chercheurs de l'école européenne, et la technique est maintenant standardisée pour IRMA et ELISA. Les chercheurs qui préfèrent cette analyse estiment que le précurseur sécrété est typique et représentatif des cellules cancéreuses, et que le précurseur sécrété peut être autoactivé dans le milieu acide à l'extérieur des cellules tumorales puis agit par dégradation protéolytique. Selon eux, l'activation du précurseur et la dégradation de la matrice extracellulaire serait la seule voie de la fonction protéolytique de cathepsine D dans la situation cancéreuse. Une méta-analyse récente a confirmé que l'expression de la cathepsine D dans le cytosol corrèle avec un faible taux de survie chez 2810 patientes avec cancer mammaire (Foekens *et al.*, 1999).

Il est vrai que la sécrétion du précurseur de cathepsine D est spécifique au cancer mammaire (Rochefort, 1990). Le milieu extracellulaire tumoral est souvent acide. Le précurseur sécrété peut donc être activé et participer à la dégradation de la matrice extracellulaire (Briozzo *et al.*, 1988; Sloane *et al.*, 1987). Cependant, la sécrétion de précurseur n'est pas le seul aspect de la cathepsine D dans le cancer mammaire. On observe dans le cancer du sein une accumulation de précurseurs et une maturation importante de cathepsine D cytoplasmique. De plus, de grandes vésicules intracellulaires contenant un haut niveau de cathepsine D mature ont été retrouvées dans les cellules cancéreuses *in vitro* et dans les sections tumorales de sein (Roger *et al.*, 1994), signifiant que la dégradation protéolytique se fait par la phagocytose cellulaire (Montcourrier *et al.*, 1990). Une expérience a également étudié le rôle du précurseur sécrété dans le milieu de culture sur l'invasion des cellules mammaires à l'aide de plusieurs lignées cellulaires : MCF-7, MDA-MB-231, MDA-MB-231-435, MDA-MB-231-435s, SK-BR-3 et

MCF-7-ADDr. Aucune corrélation n'a été observée entre la sécrétion de cathepsine D et l'invasion de ces cellules cancéreuses (Johnson *et al.*, 1993). Ces résultats supportent le rôle du processus de maturation de la cathepsine D dans l'invasion tumorale (Liaudet *et al.*, 1994) et que la procathepsine D, sécrétée ou intracellulaire, ne participe pas directement au développement de métastases des cellules cancéreuses. Les auteurs ont considéré que la cathepsine D des cellules stromales devait plutôt participer à la progression tumorale mammaire (Johnson *et al.*, 1993). Des auteurs s'interrogent également à savoir si le pH à l'extérieur des cellules peut être tellement acide que le précurseur serait autoactivé, car le pH optimal de l'activité de cathepsine D est entre 3 à 5. En effet, les macrophages et autres cellules stromales sont une source importante d'enzymes lysosomiales, y compris la cathepsine D. C'est pourquoi une coculture de cellules stromales d'origine tumorale mammaire avec des cellules malignes mammaires a été réalisée. L'objectif était de vérifier si la présence de cellules cancéreuses peut modifier la quantité de cathepsine D stromale, car l'analyse par immunohistochimie a confirmé la valeur pronostique de la cathepsine D d'origine stromale.

Par coculture *in vitro*, la cathepsine D mature et cytoplasmique a été augmentée dans les cellules stromales mammaires en présence des cellules malignes. Le résultat supporte les résultats cliniques. Il serait souhaitable d'effectuer une étude au niveau de l'ARNm et des niveaux de sécrétion, ainsi qu'une coculture tridimensionnelle pour vérifier si cette augmentation de la cathepsine D cytoplasmique et stromale influence la migration/invasion des cellules cancéreuses mammaires.

### **6.1. Quelle méthode est la meilleure ?**

La plupart des études sur la cathepsine D ont utilisé un anticorps monoclonal pour l'analyse de la concentration de cathepsine D dans le cytosol de tumeur. Cependant, compte tenu des diagnostics de plus en plus précoces, les tumeurs sont plus petites et le tissu est peut-être insuffisant pour l'analyse cytosolique. De plus, la méthode par Western blot, ELISA et IRMA ne permettent pas d'avoir d'information sur l'influence des différentes cellules qui expriment la cathepsine D. Donc, l'analyse par l'immunohistochimie semble une bonne alternative et utilise peu de tissu et peut donner une information utile sur l'expression par différents

compartiments cellulaires.

Cependant, le fait que les études par immunohistochimie aient donné des résultats plus variables que ceux par IRMA pose des interrogations sur l'analyse immunohistochimique. Les résultats variables par immunohistochimie pourraient être partiellement dus aux différents anticorps utilisés (anticorps monoclonal et polyclonal), qui reconnaissent différents épitopes ou même différentes formes (mature ou précurseur) de cathepsine D. Cependant, les études qui utilisent le même anticorps arrivent au même résultat. En effet, les études de Domagala (Domagala *et al.*, 1992), Têtu (Têtu *et al.*, 1993) et O'Donoghue (O'Donoghue *et al.*, 1995) ont confirmé la valeur pronostique de la cathepsine D par les cellules stromales et non par les cellules cancéreuses. Les études de Kandalaft (Kandalaft *et al.*, 1993) et Winstanley (Winstanley *et al.*, 1993) avec un autre anticorps ont retrouvé une valeur pronostique de la cathepsine D par les cellules cancéreuses. Une étude utilisant différents anticorps contre la cathepsine D mais en s'intéressant uniquement aux cellules tumorales a démontré une association différente selon l'anticorps avec la survie sans métastase (Eng *et al.*, 1994).

Au lieu de définir l'expression de la cathepsine D en positif et négatif, certains auteurs ont défini des seuils de positivité soit par utilisation d'un histoscore pour définir les cas positifs et négatifs en fonction du nombre et de l'intensité de coloration des cellules exprimant la cathepsine D (Kandalaft *et al.*, 1993), soit par mesure simple de la proportion de cellules colorées (Têtu *et al.*, 1993), et de la mesure relativement subjective de la positivité totale (Henry *et al.*, 1990; Winstanley *et al.*, 1993). Des auteurs ont rapporté des méthodes pour quantifier les résultats d'immunohistochimie par analyse d'images assistée par ordinateur (Charpin *et al.*, 1994; Lehr *et al.*, 1997), ce qui pourrait aider à standardiser la mesure par immunohistochimie.

Au total, l'étude de la cathepsine D pourrait aider à élucider le mécanisme impliqué dans la régulation de l'expression du gène des œstrogènes et son rôle pronostique dans le cancer du sein. Des études de grandes séries de type prospectif seraient souhaitables pour établir définitivement son rôle pronostique dans le cancer mammaire, et ainsi répondre à des questions cliniques. La source de la cathepsine D mesurée par méthode cytosolique n'est pas

encore bien définie et la valeur pronostique de l'expression de cathepsine D par différents types de cellules dans une tumeur reste à confirmer. Comme une forte expression de cathepsine D est associée avec le pronostic défavorable et que des études biologiques suggèrent que ce pronostic défavorable est dû à son activité protéolytique, on peut prévoir que la recherche clinique sur la cathepsine D pourrait amener le développement d'inhibiteurs potentiels de cette activité protéolytique (Baldwin *et al.*, 1993), et ainsi contribuer au contrôle des métastases de cancer mammaire et d'autres cancers.

## **7. De nouvelles voies thérapeutiques.**

La bonne corrélation entre l'expression de protéases stromales et le faible rendement thérapeutique confirme le rôle de l'hôte dans la progression tumorale et signifie que le blocage des protéases auraient un effet important sur la croissance tumorale ainsi que la dissémination de la maladie. Tel que discuté par Dano (Dano *et al.*, 1999), la participation des cellules stromales dans le processus d'invasion tumorale démontre leur importance pour comprendre la progression tumorale et pour envisager de nouvelles approches thérapeutiques. De plus, étant des cellules normales de l'hôte et donc génétiquement plus stables, il est à prévoir que ces cellules stromales seront moins susceptibles de développer des résistances au traitement.

Si la plupart des tentatives thérapeutiques sont actuellement dirigées contre les cellules cancéreuses, d'autres alternatives sont possibles. Une connaissance précise des gènes activés par les cellules néoplasiques sur les cellules de l'hôte permettra probablement de définir de nouvelles stratégies thérapeutiques rationnelles et mieux tolérées. De tels traitements auront comme objectif non seulement de détruire les cellules néoplasiques métastatiques, mais aussi de prévenir leur croissance en interférant avec le milieu environnant et en perturbant l'écosystème tumoral complexe. Des résultats encourageants ont été rapportés dans des essais thérapeutiques.

### **7.1. Des inhibiteurs de protéases.**

La voie qui semble la plus facile est de trouver des inhibiteurs des protéases associés à l'invasion tumorale. En particulier, il est possible que l'inhibition des métalloprotéases et des sérines protéases permette de prévenir l'invasion cancéreuse.

Par des modèles animaux, des études ont aussi démontré que des inhibiteurs de protéases comme l'aprotinine pourraient contrôler le développement et la formation de métastases de carcinome pulmonaire à petites cellules et de tumeur de Lewis (Clark *et al.*, 1993; Uetsuji *et al.*, 1992).

Le groupe de Rosenberg *et al.* a produit un antagoniste spécifique de l'uPAR pour inhiber l'interaction uPA-uPAR (Tressler *et al.*, 1999). Dans le modèle de souris nues/nues, cet antagoniste maintient les cellules tumorales dans un état quiescent et la croissance tumorale peut être complètement inhibée avec des doses importantes. Cependant, la croissance tumorale peut recommencer quand cet antagoniste est retiré. Le résultat est très encourageant et très peu de toxicité est observée à ce jour. Cet effet cytostatique n'est pas simplement dû à l'inhibition de l'interaction uPA-uPAR, car l'utilisation d'un anticorps contre l'uPAR ne donne pas cet effet cytostatique. Les chercheurs ont rapporté que l'antagoniste améliorait l'adhésion cellulaire à la vitronectine et donc bloque la motilité par augmentation de l'adhésion cellulaire. On croit que l'effet combiné des inhibiteurs synthétiques de l'uPA et des MMPs serait synergique.

Des inhibiteurs de MMPs ont été également appliqués à la thérapie anticancéreuse dans des expériences cliniques. Les Batimastat (BB-94) et le Marimastat (BB2516) sont des inhibiteurs synthétiques des MMPs qui ont permis *in vitro* de contrôler l'invasion des cellules du gliome (Tonn *et al.*, 1999). Dans un modèle animal, des cellules de carcinome mammaire expérimental HOSP.1P, qui présentent un niveau élevé de MMP-2 et MMP-9 ont été injectées par voie veineuse. L'administration de Batimastat a permis de contrôler 80% du développement de colonies tumorales dans les poumons et inhiber de façon significative le développement de métastases par voie sanguine (Eccles *et al.*, 1996). La première génération d'inhibiteurs synthétiques de MMPs a été utilisée dans des expériences cliniques de phase I et II pour des carcinomes pancréatiques, colorectaux, gastriques, ovariens et prostatiques (Primrose *et al.*, 1999; Rosemurgy *et al.*, 1999; Steward, 1999). Les résultats sont encourageants : chez des patients avec cancer gastrique inopérable, le traitement avec le Marimastat a réussi à diminuer la taille de la tumeur et l'hémorragie. La survie a été

améliorée chez ces patients avec les carcinomes avancés de l'estomac et du pancréas (Jones *et al.*, 1999). Une expérience de phase III est en cours avec la combinaison de Marimastat et d'agents de chimiothérapie pour le cancer du poumon, du pancréas, et de l'estomac (Steward, 1999).

Des études sont aussi effectuées pour la stromélysine-3, même si son substrat spécifique n'est pas encore élucidé. Parmi 140,000 produits synthétiques, un inhibiteur de ST3 a été récemment identifié qui appartenait à la famille de l'antibiotique céphalosporine (Kannan *et al.*, 1999). Cette découverte aidera à développer de nouvelles expériences pour élucider le rôle de la ST3.

## **7.2. Des molécules d'adhésion.**

Des études suggèrent que la présence de macromolécules d'adhésion dans l'ECM aurait une valeur pronostique (Hand *et al.*, 1985). Cioce *et al.* ont trouvé une surexpression du récepteur de laminine de 67 kDa dans le carcinome de côlon (Cioce *et al.*, 1991). L'anticorps contre le récepteur de laminine a été utilisé pour l'immunodétection du cancer mammaire sur les spécimens obtenus par biopsie aspiration à l'aiguille (Castronovo *et al.*, 1990). La surexpression des récepteurs de laminine a été observée dans 71% des frottis de lésion cancéreuse. Les intégrines ont été largement étudiées. L'expression d'une intégrine,  $\alpha^6\beta^4$ , a été recherchée chez des patients porteurs des carcinomes de la sphère ORL, et pouvait prédire les patients avec pronostic défavorable (Wolf *et al.*, 1990).

L'administration de liposomes contenant de l'adriamycine couplée à l'anticorps anti-récepteur de laminine a été testée en culture de cellules cancéreuses mammaires *in vitro*, et ceci augmentait significativement la destruction des cellules épithéliales cancéreuses par rapport aux cellules épithéliales normales (Rahman *et al.*, 1989). On observe une prise sélective d'adriamycine et la destruction de cellules en culture avec le liposome couplée à l'anticorps.

## **7.3. Anti-angiogénèse.**

Des études d'angiogénèse ont mené au développement de nouvelles approches diagnostiques et de nouveaux agents anti-métastatiques. Le nombre et la densité des microvaisseaux

corrélaient avec le taux de métastase chez les patientes, suggérant que la quantité de néovascularisation était un marqueur potentiel de progression tumorale (Weidner *et al.*, 1991).

L'angiogenèse est maintenant une nouvelle cible thérapeutique. Ingber *et al.* ont démontré que l'analogue synthétique de la fumagilline inhibait l'angiogenèse et supprimait la croissance tumorale (Ingber *et al.*, 1990). La fumagilline est un produit naturel, un antibiotique dérivé de l'*Aspergillus fumigatus fresenius*. La fumagilline et ses analogues ont inhibé la croissance tumorale *in vitro* et dans des souris porteuses de tumeurs. Maione *et al.* en 1990 ont démontré que le facteur-4 recombinant de plaquette humaine serait un inhibiteur potentiel de l'angiogenèse (Maione *et al.*, 1990). Le produit inhibe la croissance des cellules endothéliales *in vitro* et inhibe la prolifération des vaisseaux dans la membrane chorioallantoïque de poulet, et cet effet de l'inhibition est dose-dépendent. Cette famille de produits fournit une alternative aux agents anticancéreux traditionnels.

Au total, l'invasion tumorale peut être considérée comme un processus de remodelage tissulaire qui implique une dégradation protéolytique de la matrice extracellulaire dans le tissu normal/hôte du voisinage. La dégradation protéolytique péricellulaire est le résultat d'une interaction entre différentes molécules. Différentes populations cellulaires synthétisent des constituants individuels impliqués dans la dégradation de la matrice.

Si les protéases de dégradation protéolytique favorisent la progression tumorale, l'effet protéolytique est indépendant de la source stromale ou cancéreuse. La confirmation des valeurs pronostiques de la cathepsine D, la stromélysine-3 et l'uPA d'origine stromale telle que présentée dans cette thèse supporte l'hypothèse selon laquelle ces protéases stromales participent à la progression tumorale mammaire.

Les cellules tumorales interagissent avec diverses composantes de la matrice extracellulaire et avec les cellules hôtes (cellules immunitaires, fibroblastes, cellules épithéliales et endothéliales). De telles interactions conditionnent l'angiogenèse, la croissance tumorale, le remodelage et la destruction des tissus hôtes, la migration locale des cellules cancéreuses et leur dissémination métastatique.

## Conclusion

En conclusion, de nombreux arguments nous amènent à penser que les protéinases, et leur équilibre avec leurs inhibiteurs, jouent un rôle important dans l'invasion tumorale. Il semble que plusieurs protéinases dont les protéases stromales appartenant à des groupes différents puissent être impliquées dans le processus.

L'invasion tumorale est comme un remodelage tissulaire incontrôlable mais associé à la tumeur. Les interactions qui s'établissent entre les cellules tumorales et les cellules normales activées de l'hôte avec la matrice extracellulaires modulent l'agressivité des cellules malignes. Les tumeurs représentent donc un écosystème particulier caractérisé par de nombreuses interactions cellules normales-cellules tumorales ou cellules tumorales-matrice extracellulaire, ou cellules normales-matrice extracellulaire modifiée.

Le fait que les cellules stromales non-néoplasiques jouent un rôle crucial en biologie tumorale peut avoir des implications profondes pour nous aider à mieux comprendre certains phénomènes fondamentaux du cancer. L'implication nécessaire de certaines cellules stromales à la progression tumorale peut expliquer partiellement la longue période de latence caractéristique de la carcinogenèse, et le délai à l'établissement de foyers métastatiques. De la même façon, la prédilection des métastases de certains cancers pour certains organes démontre le besoin spécifique de certaines cellules pour l'établissement des métastases à des sites précis.

Pour élucider ce processus non-néoplasique et associé à la tumeur, l'analyse *in situ* et le carcinome expérimental dans le modèle de souris nous aident à simuler la voie d'expression et la relation avec les éléments cancéreux dans des tumeurs humaines (Johnsen *et al.*, 1998).

La conséquence logique de ces résultats est le développement de méthodes pour bloquer l'invasion tumorale, soit avec des inhibiteurs de protéinases, soit avec des agents interférant sur les récepteurs de protéinases.

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