UNIVERSITÉ DU QUÉBEC À MONTRÉAL

IDENTIFICATION DU RÔLE DE LA MÉLANOTRANSFERRINE DANS L'ACTIVATION DU PLASMINOGÈNE : IMPLICATION DANS LA DÉGRADATION DE LA MATRICE EXTRACELLULAIRE ET L'INVASION TUMORALE

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ABRÉVIATIONS

α_2-AP	Alpha2-antiplasmine
α_2MR	Alpha2-macroglobuline
ARNm	acide ribonucléique messager
ATF	fragment aminoterminal d'uPA
BBB	barrière hemato-encéphalique
Beas2B	cellule épithéliale de poumon
βFGF	facteur de croissance basique des fibroblastes
C-terminale	segment terminant par l'acide carboxylique d'une protéine
EGF	facteur de croissance épidermique
ERK	kinase régulée par les signaux de régulation extracellulaire
FXIIIa	facteur de coagulation activé XIII
FN	fibronectine
gp130	glycoprotéine130
GPI	glycosylphosphatidylinositol
HGF	facteur de croissance hépatocytaire
HMWK	Kininogène à poids moléculaire élevé
IGF_2	facteur de croissance insuline 2
IL-8	interleukine-8
kDa	kilodalton
LDL	lipoprotéine à basse densité
LMW-uPA	activateur urokinase du plasminogène de poids moléculaire bas
LRP	récepteur de la protéine apparenté à la lipoprotéine à basse densité
Man6Pr	récepteur mannose-6-phosphate
MEC	matrice extracellulaire
MMP	métalloprotéase
MTf	mélanotransferrine
mMTf	mélanotransferrine membranaire

sMTF	mélanotransferrine soluble humain recombinante
NFκb	facteur nécrotique kappa b
NGFγ	facteur de croissance neuronal-γ
N-terminale	extrémité amine d'une séquence protéique
PAI-1	inhibiteur des activateurs du plasminogène 1
PAI-2	inhibiteur des activateurs du plasminogène 2
Plg	plasminogène
Pln	plasmine
Pro-uPA ou Sc-uPA	précurseur de l'activateur urokinase du plasminogène
RAP	protéine associée à l'uPAr
SK-Mel-28	lignée cellulaire de mélanomes
TAFI	inhibiteur carboxypeptidase de la thrombine
TF	facteur tissulaire
Tf	transferrine
TGFβ	facteur de croissance de transformation bêta
TR	récepteur de la transferrine
tPA	activateur tissulaire du plasminogène
uPA	activateur urokinase du plasminogène
uPAr	récepteur de l'activateur urokinase du plasminogène
VEGF	facteur de croissance endothéliale vasculaire
VEGFr-2	récepteur du facteur de croissance endothéliale vasculaire-2
VN	vitronectine

RÉSUMÉ

L'activation du plasminogène est importante dans les phénomènes liés au cancer. Ma thèse démontre que la mélanotransferrine agit comme accélérateur dans l'activation du plasminogène avec ses deux activateurs : l'uPA, lié au cancer et le tPA, lié à la fibrinolyse. Son action ambivalente, à la fois protumorale et antitumorale, est caractérisée par son site d'action au niveau cellulaire. La mélanotransferrine membranaire agit comme un catalyseur pour amplifier l'activation du plasminogène à la membrane, tandis que la forme soluble de la mélanotransferrine entre en compétition avec la mélanotransferrine membranaire pour inhiber son effet.

Dans la présente étude, nous avons découvert que deux composantes du système plasminolytique : le pro-uPA, précurseur de l'uPA et le plasminogène interagissent avec la mélanotransferrine. L'interaction de la mélanotransferrine avec le système plasminolytique stimule l'activation du plasminogène par ses deux activateurs. Nous démontrons également avec un anticorps dirigé contre la mélanotransferrine ou par l'inhibition de son expression par un siRNA que la mélanotransferrine intervient dans le processus menant aux métastases par l'inhibition de la migration cellulaire. De plus, nous démontrons que la mélanotransferrine module la dissolution de caillots de fibrine dépendante du tPA ou de l'uPA, une composante importante de la matrice provisoire extracellulaire impliquée dans la dispersion des métastases. Dans un modèle où le dépôt de fibrine provoqué par le facteur tissulaire (TF) stimule l'invasion des cellules de mélanome (SK-Mel-28), nous démontrons une inhibition de l'invasion induite par le TF dans le poumon lorsque l'expression de la mélanotransferrine est inhibée par un siRNA.

Ces résultats suggèrent que la mélanotransferrine est directement impliquée dans la propagation des métastases des mélanomes qui l'expriment fortement.

Mots clés : activation du plasminogène, invasion tumorale, migration, fibrinolyse, uPA, tPA.

CHAPITRE I

1. INTRODUCTION

1.1 La mélanotransferrine (MTf)

1.1.1 L'historique

La MTf, initialement appelée la protéine p97, a été identifiée au départ comme un marqueur extérieur des cellules malignes de mélanomes (Brown et coll., 1981; Brown et coll., 1982). La protéine p97 s'est ensuite appelée la MTf en raison de son homologie élevée (37–39 %) avec la transferrine humaine et la lactoferrine (Brown et coll., 1982). Par la suite, son expression a été détectée de façon endogène dans plusieurs lignées cellulaires en culture telles que les cellules de mélanomes, les cellules épithéliales et les cellules intestinales ainsi que dans divers tissus non cancéreux, dont les glandes salivaires, les cellules endothéliales du foie, les chondrocytes et l'endothélium cérébral (Brown et coll., 1981; Alemany et coll., 1993). De plus, la MTf est fortement exprimée au niveau des tissus fœtaux et dans les cellules néoplasiques (Rose et coll., 1986).

La MTf appartient à une famille des protéines impliquées dans le transport du fer. En effet, il est à noter que les gènes codant pour la transferrine (Tf), son récepteur (TR) ainsi que pour la MTf se retrouvent sur la même région chromosomique, soit le chromosome 3q21 (Food et coll., 1994). Le fer est un élément essentiel comme source d'énergie cellulaire puisqu'il intervient dans de nombreuses réactions métaboliques majeures telles que les synthèses de l'ATP et de l'ADN (Sekyere et Richardson, 2000). Les membres de cette famille possèdent une caractéristique majeure et commune consistant à pouvoir lier de façon réversible deux ions Fe^{3+} avec deux ions CO_3^{2-} par molécule. L'homologie

implication de la MTf dans le transport du fer (Yang et coll., 2004). Toutefois, une modification de quatre acides aminés dans la portion C-terminale Asp-395, Asp-465, Thr-461 et Thr-466 entraîne une perte de la capacité à lier un des deux fers. Cette modification laisse présager un rôle moins important de la MTf dans le transport du fer. La Tf libère le fer aux cellules par le biais de l'internalisation par endocytose du complexe formé avec son récepteur. Le Tf lie le fer et son récepteur TR. Le récepteur est internalisé dans les endosomes qui en s'acidifiant permettent le relâchement de Tf de son récepteur. Le TR est recyclé par la suite à la surface de la cellule, tandis que le Tf est dirigé vers les lysosomes. Le fer est ainsi relâché à l'intérieur des lysosomes par la digestion de Tf. Il est ensuite utilisé directement par la cellule ou emmagasiné sous forme de complexe avec la ferritine. Bien que la MTf lie le fer, elle s'avère peu efficace en ce qui a trait à l'apport de fer aux cellules (Brown et coll., 1982). Afin d'expliquer la faible efficacité de la MTf dans le transport de fer, il a été suggéré que la MTf libérait ce dernier aux cellules par un mécanisme infructueux impliquant une internalisation non spécifique suivie de pinocytose (Food et Richardson, 2002).

Plusieurs rôles ont été proposés pour la MTf comme la peroxydation des lipides ou une activité catalytique puisqu'une étude de modélisation a identifié une séquence consensus au zinc identique à celle présente chez les métalloprotéases (MMPs), dont la thermolysine (Sekyere et Richardson, 2000). Ces rôles, purement théoriques basés sur la structure de la MTf demeurent hautement spéculatifs et nécessiteraient des études approfondies pour être validés. Par contre, l'expression de la MTf a été associée à diverses situations pathologiques, dont la croissance des mélanomes humains. Bien que de faibles niveaux de MTf aient déjà été détectés dans les capillaires cérébraux humains, des taux élevés ont été localisés dans les cellules de la microglie au niveau des plaques séniles de patients atteints de la maladie d'Alzheimer (Yamada et coll., 1999). Chez des individus normaux, la MTf ne représente qu'une partie de la

composition du sérum normal, soit de 1 à 3 ng/mL (Brown et coll., 1981), tandis qu'elle aurait tendance à être 5 à 6 fois plus élevée dans le sérum de personnes atteintes de la maladie d'Alzheimer, selon un test Elisa (Rothenberger et coll., 1996). Toutefois, une étude plus récente montre un niveau stable et similaire de MTf dans le sérum de patients sains et atteints de la maladie d'Alzheimer (Desrosiers et coll., 2003).

Cependant, des études plus approfondies sont nécessaires afin d'éclaircir les fonctions physiologiques réelles de la MTf. De plus, il a été récemment démontré que la MTf traversait la barrière hémato-encéphalique (BBB) avec des taux élevés de transcytose, suggérant ainsi une approche novatrice dans la libération de médicaments au cerveau (Demeule et coll., 2002). La recherche d'un récepteur pour le passage de la MTf à travers la BBB a démontré que le récepteur de la protéine apparenté à la lipoprotéine à basse densité (LRP) est impliqué dans le processus (Demeule et coll., 2002). LRP étant un récepteur éboueur pouvant lier une vaste panoplie de ligands seuls ou complexés, une identification des ligands de LRP pouvant interagir avec la MTf est préconisée. Les composantes du système plasminolytique sont identifiées dans cette thèse comme les éléments les plus prometteurs. D'ailleurs, une étude a démontré que le traitement par la MTf réduit la régénération de l'uPAr à la surface des cellules, lesquelles réduisent son expression et augmentent l'internalisation par son récepteur LRP (Michaud-Lévesque et coll., 2005).

1.1.2 La structure

La MTf est une glycoprotéine monomérique de 97 kDa (Rose et coll., 1986). Cette protéine glycolysée possède deux domaines symétriques contenant plusieurs ponts disulfures (Rose et coll., 1986). Deux formes de MTf ont été identifiées. La première est une forme membranaire qui est attachée à la surface des cellules par une ancre de glycosylphosphatidylinositol (GPI). La deuxième

forme est activement sécrétée et provient d'une modification de l'ARN messager (ARNm) (Food et coll., 1994). L'extrémité C-terminale de la MTf (résidus 712 à 738) est formée de 27 résidus chargés et lipophiles nécessaires pour l'ancre GPI (Alemany et coll., 1993). Ce type d'attachement protéique à la membrane peut être hydrolysé par certaines enzymes. En ce sens, l'action de la phospholipase C sur les cellules de mélanomes provoque le relâchement de plus de 95 % de la MTf sous forme soluble, confirmant ainsi le rôle de l'ancre GPI pour la forme membranaire de la MTf (Alemany et coll., 1993). La présence de l'ancre GPI comme moyen d'attachement cellulaire pour la MTf lui confère diverses propriétés avantageuses, dont la possibilité d'être relarguée rapidement sous une forme soluble ou même d'être internalisée afin d'en réguler facilement la présence à la surface cellulaire (Food et coll., 1994). La relation entre la MTf ancrée à la membrane et la forme soluble de la protéine a été mise en évidence par l'équipe de Jefferies (Yang et coll., 2004). Leurs études, basées sur des essais de délétion d'acides aminés et de mutation dirigée, démontrent qu'un minimum de 13 acides aminés lypophiles à l'extrémité C-terminale de la MTf est nécessaire pour former une ancre GPI fonctionnelle. La délétion de ces 13 acides aminés permet la sécrétion de la MTf. La MTf soluble est donc obtenue à partir du même gène que la forme membranaire, mais subit un épissage supplémentaire. Cette dernière est traduite à partir d'un ARNm de 3916 bases nucléotidiques, où seules 2211 bases représentent la séquence codante. Suite à la traduction, le précurseur de 738 acides aminés subit un clivage afin de générer la forme mature de 694 résidus qui possède trois sites potentiels de glycosylation et 14 ponts disulfures conservés à travers l'homologie avec la Tf (Yang et coll., 2004).

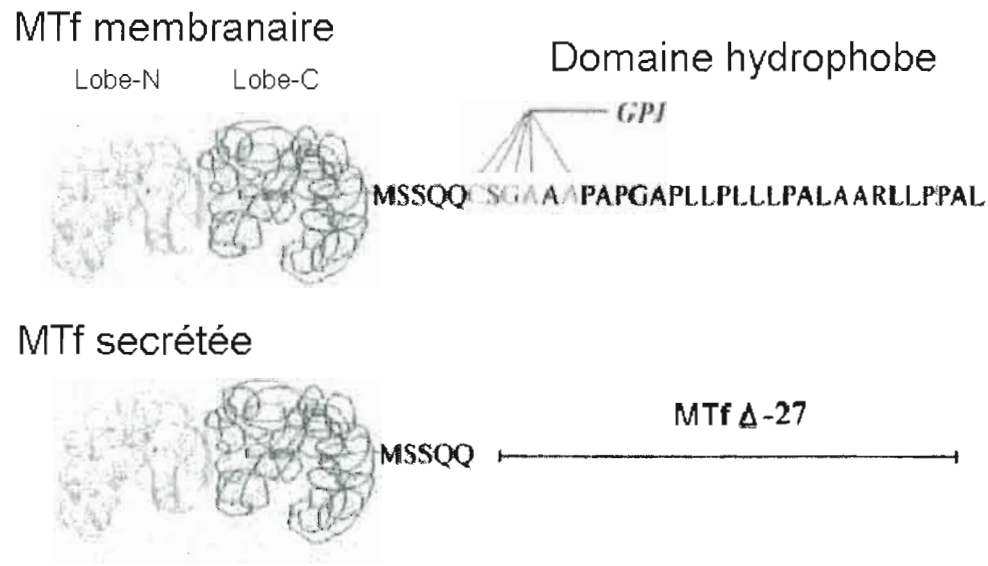

Figure 1: Schéma de la MTf membranaire et de la MTf sécrétée

La séquence du domaine d'acides aminés de la MTf est responsable de l'attachement à une ancre GPI qui encode le type sauvage (membranaire) et par la délétion (sécrétée) dans le domaine hydrophobe de la région C-terminale (Yang et coll., 2004).

1.1.3 Les études précédentes sur son rôle

Des fonctions de la forme tronquée de la MTf ont été proposées. Une première étude a prouvé que la MTf tronquée pouvait lier le fer des complexes citrate-Fe (Brown et coll., 1982). Cette observation suggère que la MTf peut être impliquée dans le transport du fer. Cependant, des études sur les cellules humaines de mélanomes (SK-Mel-28) exprimant des niveaux élevés de MTf démontrent que cette protéine ne joue pas un rôle important dans la prise du fer (Richardson et Baker 1991). Toutefois, dans une autre étude portant sur des cellules ovariennes de hamster (CHO) déficientes en TR et transfectées avec la MTf humaine, la prise du fer est doublée. Les CHO transfectées expriment 2 à 3 x 10^6 molécules de MTf par cellule (Kennard et coll., 1995), comparativement à 0,34 × 10^6 molécules de MTf par cellule de SK-Mel-28 (Richardson et Baker, 1990). Des

études démontrent que la diminution de MTf dans les cellules de mélanomes (Richardson, 2000) et dans d'autres types de cellules (Kriegerbeckova et Kovar, 2000) n'a pas d'effet marqué sur la prise du fer. Une récente étude indique que la MTf soluble ne se lie pas au TR et par conséquent ne peut délivrer du fer aux cellules par ce mécanisme (Food et coll., 2002).

Récemment, nous avons rapporté que la MTf humaine s'accumule significativement dans le cerveau de souris à la suite d'une injection intraveineuse (Demeule et coll., 2002). Dans un modèle de BBB *in vitro,* nous avons également démontré que la MTf possède une plus grande capacité de transcytose comparativement à d'autres protéines de taille comparable comme la Tf et l'albumine (Demeule et coll., 2002). Dans le cerveau humain, la MTf a été détectée dans l'endothélium capillaire (Rothenberger et coll., 1996). Elle a été également localisée dans les cellules gliales liées aux plaques amyloïdes trouvées par autopsie dans le cerveau des patients atteints de la maladie d'Alzheimer (Yamada et coll., 1999). Malgré le degré élevé de similarité entre la MTf et la transferrine, un récepteur différent est impliqué dans le transport transendothélial. Le récepteur LRP, appartenant la famille des protéines à basse densité, semble être impliqué dans le transport transendothélial de la MTf. L'accumulation de la MTf au cerveau, son taux élevé de passage et son niveau très bas dans le sang n'interférant pas avec la MTf exogène suggèrent qu'elle pourrait être avantageusement utilisée comme nouveau moyen d'acheminer des médicaments directement au cerveau (Demeule et coll., 2002).

L'expression de l'ARNm de la MTf est répandue dans les tissus humains normaux et est généralement plus grande chez l'adulte que dans les tissus fœtaux. Le niveau d'expression le plus élevé est retrouvé dans la glande salivaire (Richardson, 2000). D'autre part, une étude rapporte que la protéine de la MTf est davantage exprimée dans les cellules néoplasiques et dans les tissus foetaux

que dans les tissus normaux (Brown et coll., 1981). Une explication de cette différence entre l'ARNm de la MTf et l'expression de la protéine est que les techniques d'analyses employées évaluent des épissages différents.

La MTf affecte l'expression du récepteur de facteur de croissance endothélial vasculaire-2 (VEGFr-2). Par contre, la MTf ne se lie pas directement à l'intégrine $\alpha_v\beta_3$ qui influence le facteur de croissance endothélial vasculaire (VEGF) ou VEGFr-2 lui-même (Sala et coll., 2002). Une corrélation possible entre la vascularisation et la progression du mélanome impliquerait l'expression de la MTf et du VEGF. Cette possibilité a été établie par l'immunolocalisation des deux protéines dans des sections de mélanomes à différentes étapes cliniques de progression. Cette corrélation signifie donc que la MTf peut participer à la vascularisation des tumeurs (Sala et coll., 2002). De plus, le VEGF perméabilise les vaisseaux sanguins libérant son contenu, le sang, dans la région extravasculaire. Le sang contient les composants du système plasminolytique.

Une étude dans notre laboratoire a démontré que la transcytose de la MTF à travers la BBB est diminé par un ligand de LRP (Demeule et al. 2002). Aussi, le LRP a comme autres ligands des composantes du système plasminolytique et que le système plasminolytique joue un rôle crucial dans la progression tumorale (Andreasen et coll., 2000), nous avons émis les hypothèses d'interactions potentielles entre la MTf et le système plasminolytique. De plus nous soupçonnons une implication de la MTf dans la progession tumorale au niveau de l'activité plasminolytique.

1.2 Le système plasminolytique

Le système plasminolytique comporte une proenzyme inactive (plasminogène) qui peut être convertie en enzyme active (plasmine). Deux activateurs physiologiques du plasminogène (PA) ont été identifiés celui du type tissulaire

(tPA) et celui du type urokinase (uPA). L'activateur uPA se lie à un récepteur uPA cellulaire (uPAr). L'inhibition du système plasminogène se produit au niveau des PA par les inhibiteurs (PAI) ou au niveau de la plasmine, principalement par l'α_2-antiplasmine (α_2-AP). Le double rôle du système plasminogène est bien établi : 1) la voie tPA est principalement impliquée dans l'homéostasie de la fibrine et représente le système fibrinolytique (Plow et coll., 1991) et 2) la voie uPA est principalement impliquée dans les phénomènes tels que la migration des cellules et la transformation des tissus et représente le système plasminolytique tumoral (Mignatti et Rifkin, 1993).

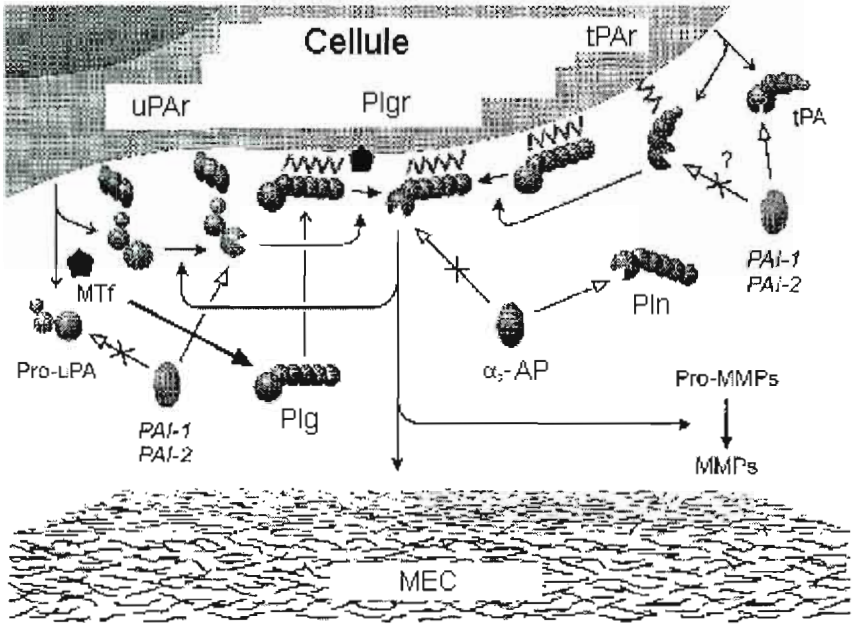

Figure 2 : Les deux voies indépendantes pour la génération péricellulaire de la plasmine (Pln)

La liaison du pro-uPA sécrété à son récepteur cellulaire, l'uPAr, mène à un système réciproque d'activation du zymogène plasminogène (Plg) dans lequel l'uPA s'active préférentiellement à la membrane de la cellule et la Pln liée à la membrane peut efficacement activer le pro-uPA lié à son récepteur. De même, la liaison du tPA sécrété à son ou à ses récepteurs putatifs mène à l'activation efficace du Plg associé aux cellules (R. Bass et V. Ellis, 2002). Les deux voies

augmentent ainsi considérablement la génération de la Pln. La MTf pourrait intervenir dans le mécanisme en étant un récepteur pour le Plg et le pro-uPA.

L'activation du Plg joue un rôle important dans l'invasion tumorale et la dispersion des métastases. Les PA relâchés par les cellules cancéreuses catalysent le Plg en plasmine (protéase active, Pln). La Pln dégrade alors les protéines de la membrane basale et de la matrice extracellulaire. Ce processus facilite l'invasion des cellules cancéreuses dans les tissus environnants (Werb, 1997). Une fois produite, la Pln peut dégrader beaucoup de protéines non fibrillaires de la matrice extracellulaire (MEC) et peut activer certaines métalloprotéases matricielles (MMPs) (collagénase interstitielle, stromelysine-1, gélatinase B) et active ou libère des facteurs de croissance tels que la facteur de croissance de transformation bêta (TGFβ) et le facteur de croissance basique des fibroblastes (β-FGF). Les voies d'activation du Plg sont également modulées par des inhibiteurs physiologiques. La Pln à la membrane est protégée de l'α_2-antiplasmine (α_2-AP) (présente dans le plasma). Les inhibiteurs de la Pln (PAIs), en particulier PAI-1, semblent avoir des fonctions distinctes dans la régulation de l'uPA et dans l'activité du tPA. La pro-uPA n'est pas inhibée par les PAIs, mais une fois qu'elle est convertie en uPA active liée à son récepteur, l'enzyme est entièrement disponible pour l'inhibition. En revanche, le tPA secrété est susceptible d'être inhibé par les PAIs. Toutefois, la liaison à son récepteur cellulaire protège partiellement le tPA contre cette inhibition. Ces étapes seraient également présentes dans d'autres processus associés au remodelage tissulaire tels que l'angiogenèse (Dvorak et coll., 1987). Même si elle est liée à l'invasion et la migration de cellules non cancéreuses, l'angiogenèse influence grandement la croissance, l'invasion tumorale et les métastases.

1.2.1 Les structures des composantes du système plasminolytique

Les enzymes du système plasminogène sont des protéinases à sérine. Leur site actif se compose d'une « triade catalytique » composée de sérine, d'acide

aspartique et d'histidine. Ce site actif est localisé dans la région C-terminale des molécules (protéinases à sérine), tandis que la région N-terminale contient un ou plusieurs domaines structuraux (modules) illustrés à la figure 3.

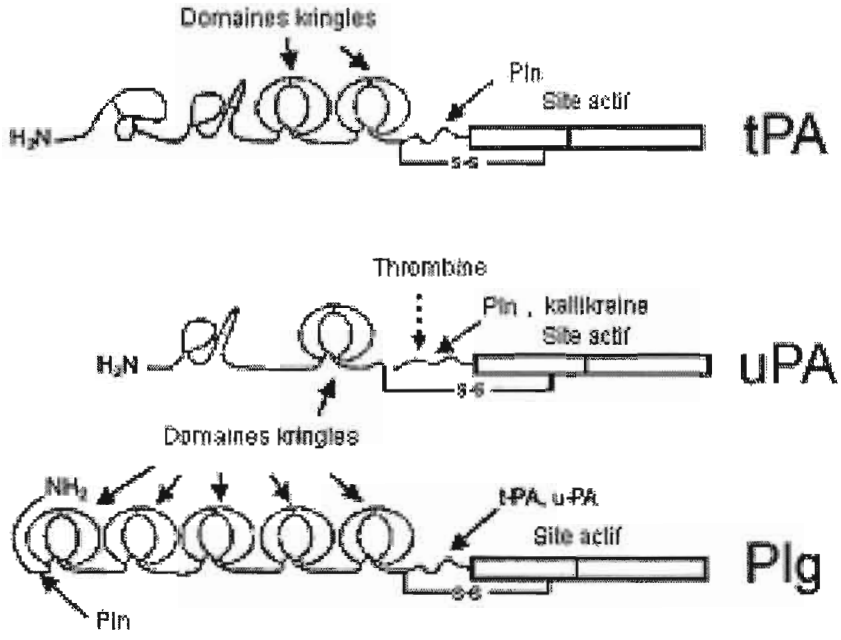

Figure 3 : La structure des domaines du tPA, de l'uPA et du Plg

Le schéma des protéines avec leurs domaines kringles et le site de clivage nécessaire pour l'activation de la protéine par ses activateurs.

Le tPA est composé d'un domaine de reconnaissance pour la fibronectine (FN), ainsi que pour le facteur de croissance épidermique (EGF), de deux domaines kringles et du domaine catalytique. La liaison du tPA à la fibrine est inhibée par le domaine de la FN et les domaines kringle-2. Le lien à PAI-1 est inhibé par le domaine kringle-2 et les résidus 296-304 de la région N-terminale dans le domaine catalytique. La simple chaîne uPA (pro-uPA) comprend le domaine épidermique EGF, un domaine kringle et un domaine catalytique. La Pln et la kallikréine coupent la pro-PA à la Lys^{158} avec la formation de l'enzyme

entièrement active de deux chaînes (tc-uPA ou uPA). La thrombine peut couper la pro-uPA à l'Arg^{156} avec la formation d'un dérivé de deux chaînes qui n'active pas le Plg libre, mais peut être lentement activé après s'être reclivée. Le Plg se compose de cinq domaines kringles, qui peuvent se lier avec la fibrine, l'α_2-AP, des récepteurs cellulaires et à son domaine catalytique. L'activation du Plg se produit après son clivage à la position Arg^{561}. La Pln peut cliver après l'Arg^{67} ou la Lys [76,77] produisant des formes tronquées du Plg qui ont une affinité plus élevée pour la fibrine. Ces formes tronquées sont également activées par le tPA ou l'uPA plus rapidement que la molécule mère (Collen et Lijnen, 1995).

1.2.2 Le plasminogène (Plg)

Le Plg est une glycoprotéine de 92 kDa formée d'une seule chaîne de 791 acides aminés. Le Plg contient sept domaines structuraux : un « peptide de préactivation » (résidus 1 à 77), cinq domaines homologues séquentiels kringles (structures triples adjacentes d'environ 80 résidus chacun constitué d'une boucle liée par un pont disulfure) et un domaine de protéinase du côté C-terminal (résidus 562 à 791). Les domaines kringles contiennent des sites de liaison dépendant de lysine qui jouent un rôle crucial dans la liaison spécifique de la fibrine à la surface de la cellule et à l'α_2-AP. La concentration plasmatique du Plg est d'environ 2 μM (Collen, 1980; Collen et coll., 1998).

1.2.2.1 La plasmine (Pln)

La Pln est une protéase à sérine dont le poids moléculaire est de 90 kDa. Elle est composée de deux chaînes reliées par des ponts disulfures. La chaîne A, du côté N-terminal, contient 5 domaines kringles. La chaîne B, du côté C-terminal, contient le domaine catalytique responsable de l'activité de la Pln : celle-ci catalyse l'hydrolyse des protéines du côté C-terminal au niveau des résidus lysine ou arginine.

Le Plg est le précurseur de la Pln, mais l'activité du Plg est jusqu'à 10^6 fois plus faible que celle de la Pln. La conversion du Plg en Pln est effectuée par le clivage entre l'arginine à la position 561 et la valine à la position 562 par les PA (uPA et tPA). Une fois coupée les deux unités sont retenues par un pont disulfure (Jovin et Muller-Berghaus, 2004).

La Pln possède un large spectre d'activité et peut dégrader plusieurs glycoprotéines (laminine, fibronectine), protéoglycans et la fibrine. La Pln active les métalloprotéases (pro-MMPs) et active ou libère des facteurs de croissance de la matrice extracellulaire : le TGFβ, le βFGF et le VEGF (Rifkin et coll., 1999).

1.2.2.2 L'angiostatine

L'angiostatine est un inhibiteur de l'angiogenèse. L'angiostatine est un fragment de Pln composé des domaines kringles 1 à 3 ou 1 à 4. Elle peut être produite *in vitro* grâce à l'élastase pancréatique, plusieurs métalloprotéases matricielles (MMP) et l'autolyse de la Pln ce qui permet autorégulation de l'activité plasminolytique (O'Reilly et coll., 1994). Les domaines kringles sont liés par des ponts disulfures essentiels pour l'activité anti-angiogenique de l'angiostatine. De récents travaux ont prouvé que les domaines kringles de plusieurs autres protéines empêchaient également l'angiogenèse. Ainsi, les domaines kringles peuvent fournir une base structurale pour l'identification d'inhibiteurs d'angiogenèse. Étonnamment, la plupart des domaines kringles inhibent l'angiogenèse seulement une fois qu'ils sont séparés de la protéine mère, qui elle-même n'a pas d'activité anti-angiogenique au départ. Ces résultats suggèrent que les domaines kringles sont des fragments cachés dans la protéine (Cao et coll., 2002).

1.2.3 L'α2-antiplasmine (α2-AP)

L'α_2-antiplasmine (α_2-AP ou α_2-inhibiteur de la plasmine) est une glycoprotéine à chaîne simple de 464 acides aminés et pesant 70 kDa. Elle est unique parmi les serpines, car elle possède une prolongation de 51 résidus d'acides aminés du côté C-terminal. Cette prolongation contient un site de liaison qui réagit avec le Plg et la Pln.

Les résidus glycines en N-terminal de l'α_2-AP peuvent être réticulés aux chaînes α de la fibrine par un processus qui exige des ions calcium et qui est catalysé par le facteur de coagulation activé XIII (FXIIIa). Dans une première étape, l'α_2-AP réagit avec de la Pln par formation d'un complexe 1:1-molaire réversible. Par la suite, un lien covalent s'établit entre les deux protéines menant à la formation d'un complexe irréversible. Par contre, la Pln est protégée contre l'action de l'α_2–AP quand elle est liée à son récepteur à la surface des cellules. Son inactivation devient de 100 à 1000 fois plus lente qu'en circulation libre (Collen et Lijnen, 1991).

1.2.4 L'activateur de plasminogène de type urokinase (uPA)

L'uPA se compose du domaine épidermique de facteur de croissance, d'un domaine kringle et d'un domaine peptidique. L'uPA activé consiste en deux chaînes liées par des ponts disulfures entre la cystéine 148 et la cystéine 279. La chaîne A, du côté N-terminal, contient le domaine de facteur de croissance et le domaine kringle. La chaîne B possède le domaine de protéase à sérine. Le poids moléculaire de l'uPA est de 55 kDa. Contrairement à la plupart des protéases de dégradation de la matrice extracellulaire, l'uPA limite son action à un substrat spécifique. Son action protéolytique est la conversion du plasminogène inactif en plasmine active.

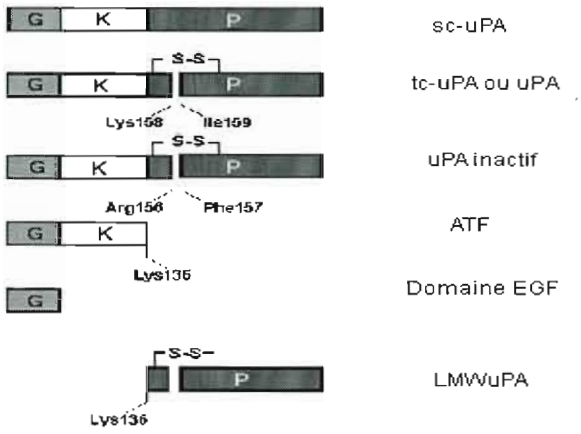

Figure 4 : Les produits dérivés de l'activation et de la dégradation de la pro-uPA

Les fragments de l'uPA produits par le traitement protéolytique de l'urokinase à la surface de cellules. G: le domaine EGF; K : le domaine kringle; P : le domaine de protéolytique (Stepanova et Tkachuk, 2001).

Dans la figure 4, le précurseur de l'uPA (pro-uPA) est 250 fois moins actif dans sa forme en une seule chaîne que sa forme à deux chaînes. La conversion du pro-uPA en uPA s'obtient par clivage entre le 158^{e} AA (lysine) et le 159^{e} AA (isoleucine). Cette conversion peut être effectuée par la Pln et plusieurs autres protéases (Andreasen et coll., 1997). Elle entraîne la formation de 2 fragments principaux : l'ATF et l' uPA de bas poids moléculaire (LMW-uPA). L'ATF est enzymatiquement inactif, mais augmente l'expression de l'uPA (Shetty et Idell, 2001). Le LMW-uPA convertit aussi le Plg en Pln. Le domaine EGF est responsable de la liaison de l'uPA à son récepteur (uPAr) présent sur la surface de la cellule. Bien que l'activateur physiologique soit inconnu, différentes protéases, dont la Pln, la cathepsine B, la cathepsine L, le facteur de croissance neuronal — (NGFγ), la tryptase des cellules pancréatiques et la kallikréine sont reconnues pour catalyser l'activation de l'uPA *in vitro* (Andreasen et coll., 1997).

1.2.5 Les inhibiteurs des activateurs du Plg

Les principaux inhibiteurs des PA (PAI) sont le PAI-1 et le PAI-2. Les PAI-1 et PAI-2 appartiennent à la famille des serpines. Leur mécanisme d'inhibition est produit par l'internalisation du complexe entre l'uPA et le PAI-1 par les cellules. Le complexe est par la suite dirigé vers les lysosomes pour leur destruction (Silverman et coll., 2001).

Le PAI-1 est une glycoprotéine de 52 kDa à simple chaîne exprimé chez une variété de cellules telles que les cellules endothéliales, les cellules musculaires lisses, les plaquettes et les hépatocytes. Le PAI-1 peut s'attacher à la vitronectine, une protéine matricielle qui peut potentiellement moduler l'adhérence et la migration cellulaires (Duffy, 2004).

Le PAI-1 est un inhibiteur rapide du tPA. Le modèle de réaction est semblable à celui de l'inhibition de la plasmine par l'α_2–AP. Le tPA lié à la fibrine est protégé contre l'inhibition par un mécanisme d'encombrement allostérique. Le PAI-1 serait également l'inhibiteur physiologique primaire de l'uPA, mais il lie aussi la pro-uPA. Il réagit rapidement avec l'uPA en formant un complexe stable avec une stoechiométrie de 1:1.

Une propriété importante du PAI-1 est sa capacité d'induire l'internalisation et la dégradation de l'uPA lié à son récepteur (uPAr). L'internalisation du complexe uPA :uPAr :PAI-1 exige l'interaction avec le récepteur apparenté à la lipoprotéine de basse densité (LRP). Tandis que le complexe uPA/PAI-1 est dégradé, l'uPAr est recyclé de nouveau à la surface des cellules (Nykjaer et coll., 1992).

Le PAI-2 existe sous deux formes ayant des propriétés cinétiques comparables : une forme intracellulaire non glycosylée de 47 kDa et une forme extracellulaire glycosylée de 60 kDa (Kruithof et coll., 1995). Les deux formes résultent de la

traduction du même ARNm et possèdent une activité antiprotéolytique semblable. Comme le PAI-1, le PAI-2 forme également un complexe stoechiométrique de 1:1 avec l'uPA. Il a été rapporté que le niveau d'expression élevé du PAI-2 empêche l'apoptose et favorise le développement de cancers (Zhou et coll., 2001). Ces effets ne semblent pas impliquer son interaction avec l'uPA. Bien que le PAI-2 soit un inhibiteur efficace de l'uPA, il agit plus lentement que le PAI-1 (Kruithof et coll., 1995). Contrairement à PAI-1, le PAI-2 ne semble pas implique dans l'adhérence ou la migration des cellules. Cependant, des études récentes suggèrent que le PAI-2 joue un rôle significatif dans la prolifération et la différenciation des cellules embryonnaires (Kruithof et coll., 1995) et dans les mécanismes de défense cellulaire contre des infections virales (Shafren et coll., 1999).

1.2.6 Le récepteur d'uPA (uPAr)

Le récepteur spécifique de l'uPA (uPAr), également connu sous le nom de CD87, est synthétisé initialement en polypeptide de 313 acides aminés. Il se compose de trois domaines structuraux homologues, dont le N–terminal qui lie l'uPA. L'uPAr est clivé du côté C-terminal en une protéine de 283 acides aminés ancrée à la membrane plasmatique par une ancre GPI (Blasi et Carmeliet, 2002). L'uPAr lie avec une affinité élevée toutes les formes d'uPA contenant un domaine intact de facteur de croissance.

La liaison de l'uPA à l'uPAr renforce non seulement la protéolyse, mais mène également à la signalisation de la transduction (Blasi et Carmeliet, 2002). La signalisation exige l'interaction entre le récepteur et une protéine associée, car l'uPAr ne possède pas de domaine transmembranaire. Les protéines associées à l'uPAr incluent les intégrines, les récepteurs des protéines G et la cavéoline (Blasi et Carmeliet, 2002). La liaison de l'uPA à l'uPAr permet au complexe de stimuler la prolifération cellulaire et d'augmenter la migration cellulaire ainsi que

de moduler l'adhérence de la cellule à la matrice extracellulaire (Sidenius et coll., 2002).

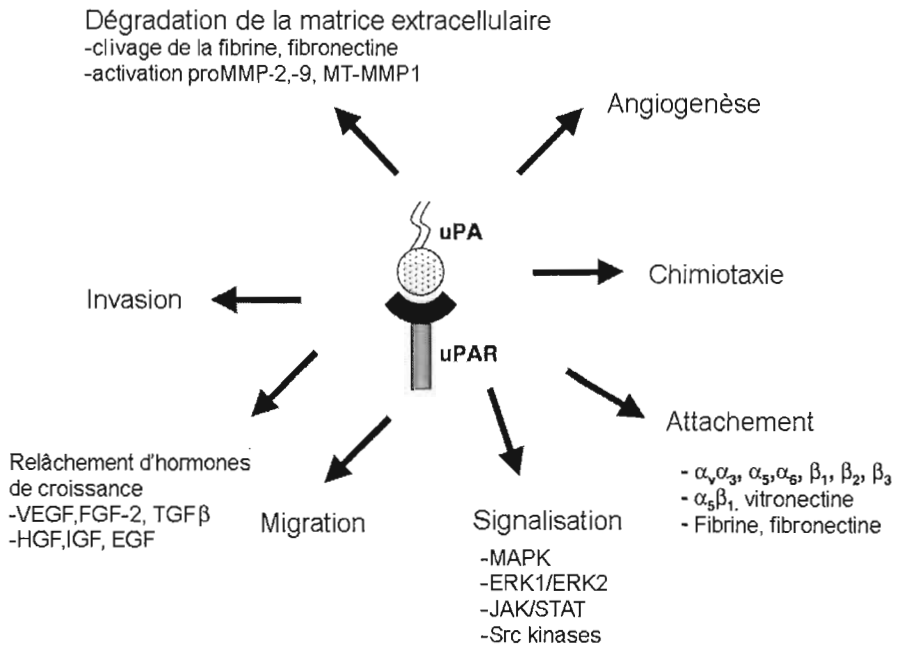

Figure 5 : Les effets du système plasminolytique sur les mécanismes extracellulaires et intracellulaires

La Pln convertit le pro-uPA plus efficacement en forme active, quand le complexe d'uPA-uPAr est lié à la surface des cellules, que lorsqu'elle est en solution (Chapman, 1997). *In vivo*, l'uPA catalyse la conversion du Plg en Pln lorsqu'il est fixé à son récepteur membranaire l'uPAr. Le Plg peut également se lier à la surface de la cellule par un récepteur comme l'annexine II et l'énolase, renforçant ainsi la protéolyse à l'extérieur des cellules (Miles et coll., 1988). L'uPAr est souvent polarisé du côté apical sur la surface des cellules et semble avoir un rôle critique pour influencer la direction migratoire des cellules envahissaqntes.

1.2.6.1 Les mécanismes extracellulaires

L'uPA lie l'uPAr sur la surface des cellules (Mondino et coll., 1999). L'uPAr peut également se lier à la vitronectine (VN) sur un domaine autre que le domaine de liaison à l'uPA (Kanse et coll., 1996). Par contre, une modification au niveau du domaine 3 de l'uPAr affecte le lien avec la VN, suggérant ainsi que le lien se fait seulement avec un récepteur intact (Hoyer-Hansen et coll., 1997). La formation d'un complexe entre la VN, l'uPA et l'uPAr augmente la surface du foyer d'attachement entre la cellule et la matrice extracellulaire, ce qui entraîne l'activation accrue de l'uPA local, un phénomène exigé pour la migration et la transformation des tissus (Chavakis et coll., 1998).

Dans une réaction semblable, une interaction dépendante du zinc entre l'uPAr et le kininogène est responsable de l'activation du kininogène en kinine sur les cellules endothéliales humaines. Tout comme dans le cas de l'interaction avec la VN, la formation d'un tel complexe peut augmenter l'efficacité catalytique du système plasminogène à la surface des cellules (Colman et coll., 1997).

La protéine GP130 forme un complexe avec l'uPAr sur les cellules épithéliales humaines. Cette protéine transmembranaire lie divers récepteurs activant les noies de signalisation JAK et STAT. Cette voie est impliquée dans la migration cellulaire (Koshelnick et coll., 1997). La combinaison de la GP130 avec l'uPAr entraîne l'activation de la voie JAK/STAT lors de la signalisation intracellulaire.

Une étude suggère que la transduction et la migration dépendante du signal d'uPAr seraient modulées par des intégrines (Koshelnick et coll., 1997). Une autre étude décrit le lien de l'uPAr avec les intégrines β_1, β_2 et β_3, comme un lien réversible qui module l'affinité des intégrines avec leurs ligands de la matrice extracellulaire. (Chapman, 1997). Une autre étude suggère que l'adhérence

dépendante des intégrines facilitée par l'uPAr accentue le rôle d'amplification de l'uPAr (Yebra et coll., 1996).

1.2.6.2 Les mécanismes intracellulaires

Le récepteur uPAr forme également des complexes stables avec la cavéoline-1, une protéine qui relie le cytosol du côté intérieur de la membrane cellulaire (Stahl et Mueller, 1995). Ce processus fournit un lien transmembranaire lorsque la cavéoline se lie à la chaîne α des intégrines. Cette association par ancrage module la croissance cellulaire dépendante des intégrines (Wary et coll., 1998). La liaison d'ATF à l'uPAr entraîne la phosphorylation des protéines focales d'adhérence et l'activation de la kinase (Chen et coll., 2001). L'inhibition de la signalisation de la kinase peut mener à une inhibition de la prolifération, de la migration et de l'invasion des cellules (Chetrasekar et coll., 2003). Les autres voies de signalisation importantes (voir figure 6) incluent la voie des kinases dans la migration de l'uPAr, l'activité dans la migration du ERK1/2 de cellules de carcinomes du sein, ainsi que la voie de JAK/STAT impliquée dans la migration dépendante d'uPAr dans les cellules musclaires lisses vasculaires (Nguyen et coll., 2000). De plus, Src, une tyrosine kinase, peut également avoir un rôle comme activateur de cette famille des kinases et mener à la migration (Chiaradonna et coll., 1999).

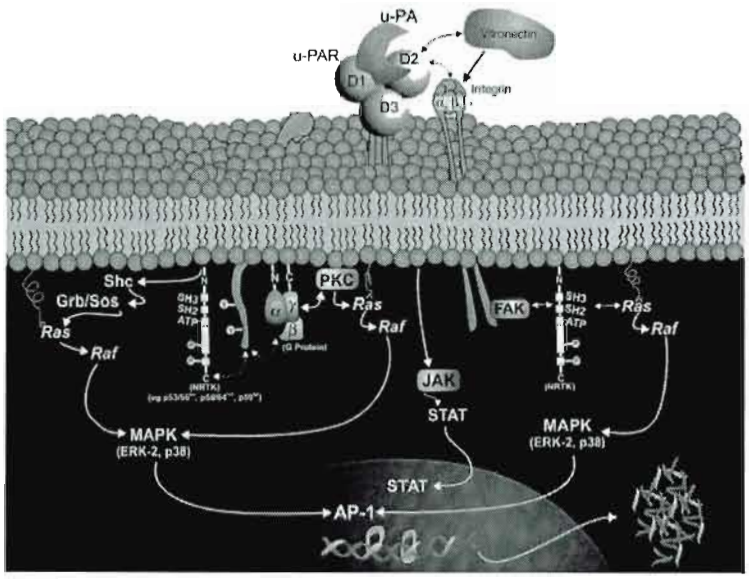

Figure 6 : Les voies postulées de la signalisation par l'uPAr

Les principales voies de signalisation associées au complexe uPA et son récepteur uPAr. FAK : kinase d'adhérence focale; AP : protéine d'activateur de transcription; JAK : kinase de Janus; STAT : signal capteur et activateur de transcription; ERK-2 : kinase de régulation extracellulaire 2 (Dear et Medcalf 1998).

1.2.7 L'activité des ligands de l'uPAr

Trois ligands extracellulaires impliqués dans la dégradation de la matrice extracellulaire et l'adhérence des cellules ont été identifiés pour l'uPAr : l'uPA, la VN et le kininogène. La fonction principale de l'uPAr est de lier la partie N-terminale du pro-uPA. Cette liaison déclenche la cascade classique de dégradation de la matrice extracellulaire par l'activation des MMPs.

1.2.7.1 L'activateur plasminogène physiologique du type urokinase (uPA)

Le premier substrat identifié pour l'uPAr est l'uPA. L'activité de l'uPA favorise la propagation du cancer quand il interagit avec son récepteur. En activant le Plg, l'uPA initie l'activation de plusieurs cascades dont une amenant à la dégradation

de la matrice extracellulaire, ce qui permet un déplacement des cellules cancéreuses. De plus, des études immunohistochimiques et une étude d'hybridation *in situ* ont indiqué que la majorité des tumeurs peuvent co-exprimer l'uPA et l'uPAr (Choong et coll., 1996). Par contre, dans quelques tumeurs agressives, comme les adénocarcinomes métastatiques, les cellules cancéreuses ne produisent pas d'uPA. L'uPA est fourni aux cellules tumorales par les fibroblastes ou par les cellules inflammatoires (Andreasen et coll., 1997). Par conséquent, les changements du microenvironnement de la cellule cancéreuse peuvent modifier la quantité de liaisons uPAr-uPA et le potentiel invasif des cellules cancéreuses. De plus, la dégradation de la matrice extracellulaire associée au cancer s'apparente à la fibrinolyse considérant l'infiltration du contenu sanguin dans l'environnement immédiat des cellules cancéreuses pendant ce phénomène. La fibrinolyse excessive pourrait altérer l'invasion de cellules endothéliales et des tumeurs par la destruction complète des emplacements d'attachement sur la matrice extracellulaire. Ces considérations ont incité Reijerkerk et ses collaborateurs (Reijerkerk et coll., 2000) à proposer l'hypothèse de l'hyperfibrinolyse dans le traitement du cancer, selon l'idée de « *No Grip and No Go* » (pas d'attache, aucune croissance). Ceci implique que les facteurs pro-envahissants reconnus, tel que l'uPA ou la PIn peuvent devenir anti-envahissant une fois administrée à des doses très élevées. En effet, bien que la littérature scientifique ait mis en évidence le lien entre l'invasion des cellules et le degré de production de l'uPA, il y a également des études qui relient inversement la production de l'uPA avec l'efficacité métastatique des sous-clones de cancers (Odekon et coll., 1992). De ces informations, il a été proposé que les cellules cancéreuses orchestrent un environnement équilibré pour leur dispersion en alternant d'un mécanisme d'appuis à celui d'une dégradation des attaches (Reijerkerk et coll., 2000).

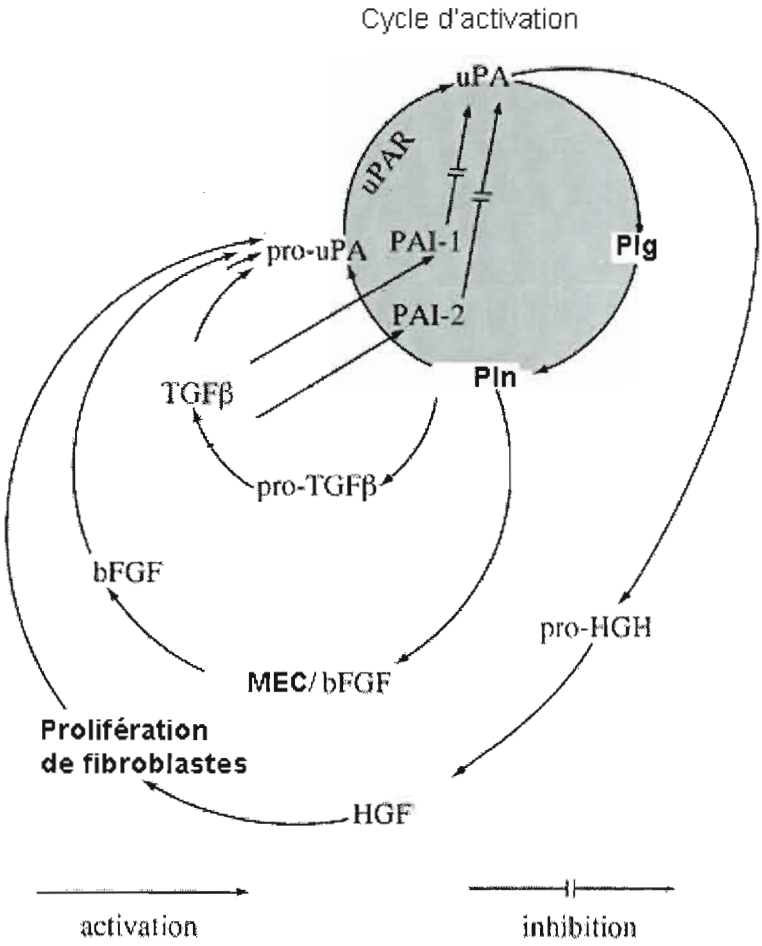

Figure 7 : Le cycle d'activation des facteurs de croissance par l'uPA

L'uPA, de par son action directe ou son action sur la Pln, mène au dégagement ou à l'activation de divers facteurs de croissance, dont plusieurs sont pro-angiogeniques. Ceux-ci incluent le βFGF, le VEGF, hépatocytaire (HGF) (Park et coll., 1993), insulinique (IGF) (Naldini et coll., 1995), épidermique (EGF) (Remacle-Bonnet et coll., 1997), ainsi que le TGFβ. Une flèche indique l'activation de l'élément suivant alors qu'une ligne discontinue l'inhibition.

Les expériences *in vivo* effectuées avec le modèle de membrane allantoïque de poussins ont prouvé que le βFGF est libéré par l'action de l'uPA (Ribatti et coll., 1999). L'uPA peut également cliver le VEGF en l'une de ses formes actives, le VEGF 189 (Naldini et coll., 1995). L'uPA est connu pour activer directement le

HGF, qui réagit alors avec son récepteur. Il est démontré que l'inhibition du récepteur de HGF empêche l'expression de l'uPA et de l'uPAr sur des mélanomes (Webb et coll., 2000). De plus, la régulation à la hausse de ces facteurs de croissance accroît l'expression du système plasminogène par un mode de régulation paracrine.

1.2.7.2 La vitronectine (VN)

La VN est une glycoprotéine de 75 kDa. L'uPAr (par un lien qui serait situé dans ses domaines 2 et 3) est un ligand efficace pour la forme matricielle de la VN. L'interaction entre l'uPA et l'uPAr augmente l'affinité de l'uPAr pour la VN multimérique soluble et pour la VN immobilisée (Kanse et coll., 1996). Puisque la VN est une molécule structurale de la matrice extracellulaire, ce genre d'attache confère à l'uPAr les propriétés nécessaires pour la migration des cellules. Le PAI-1 se lie également à la VN avec une affinité élevée. La VN stabilise l'inhibiteur dans sa configuration active et module la liaison de l'inhibiteur aux caillots de fibrine qui sont protégés contre la fibrinolyse (Podor et coll., 2000). La liaison du PAI-1 à la VN élargit également la spécificité de l'inhibiteur en le convertissant en inhibiteur de la thrombine (Stoop et coll., 2000) ou en inhibiteur efficace de la protéine activée C (Rezaie, 2001). De plus, le complexe VN-PAI-1 actif circule dans le sang. Ces observations laissent croire que la VN est un coenzyme du PAI-1. De plus, une étude histochimique localise la VN avec le PAI-1 et la thrombine (Stoop et coll., 2000). La VN est essentielle pour la stabilisation des thrombus artériels (Konstantinides et coll., 2001). Puisque les deux liens pour l'uPAr et le PAI-1 sur la VN sont près du domaine N-terminal, le PAI-1 peut agir en tant que facteur antiadhésif sur le caillot (Deng et coll., 1996). Ces activités de l'uPAr et du PAI-1 sont indépendantes de l'activité protéolytique et antiprotéolytique et contribuent à la dualité du système d'uPA-uPAr dans l'adhérence des cellules et dans la dégradation de la matrice extracellulaire.

1.2.7.3 Le kininogène à poids moléculaire élevé (HMWK)

Le HMWK est une α-globuline dont le poids moléculaire varie entre 88 et 120 kDa. La concentration du kininogène dans le plasma varie entre 70 et 90 µg/ml (Adam et coll., 1985).

Le HMWK est une glycoprotéine multifonctionnelle composée de six domaines ayant chacun des fonctions distinctes. Le domaine 1 a un emplacement liant le calcium avec une faible affinité (Higashiyama et coll., 1987). Les domaines 2 et 3 ont des séquences d'AA (Gln-Val-Val-Ala-Gly) qui empêchent l'activité des protéases à cystéine (Salvesen et coll., 1986). De plus, le domaine 3 lie les cellules endothéliales et les plaquettes (Jiang et coll., 1992). Le domaine 5 lie les cellules et l'héparine et a une propriété antiangiogenique (Colman et coll., 2000). Le HMWK peut également se lier aux surfaces négativement chargées par la région histidine du domaine 5. Le domaine 6, quant à lui, lie le facteur XI (Tait et Fujikawa, 1987).

La chaîne lourde du HMWK (64 kDa) contient les domaines 1, 2 et 3. La chaîne légère (45 à 58 kDa) est composée des domaines 5 et 6. Ces deux chaînes sont reliées par le domaine 4, qui contient le domaine actif (BK). Chez la plupart des mammifères, la kinine réfère au domaine BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (Sakamoto et coll., 1987).

Le HMWK est activé en kinine par la Pln. La kinine lance alors l'activation de la voie intrinsèque de la coagulation. La kinine est impliquée dans beaucoup de processus physiologiques et pathologiques. En vertu de sa capacité à activer les cellules endothéliales, la kinine contribue à la vasodilatation des vaisseaux, à l'augmentation de la perméabilité vasculaire, au relâchement de tPA par les cellules endothéliales, à la production de monoxyde d'azote (NO) ou encore à la

mobilisation de l'acide arachidonique. La kinine aide également le cœur et les reins à réguler la tension artérielle, et elle participe à des processus pathologiques comme l'inflammation (Moreau et coll., 2005).

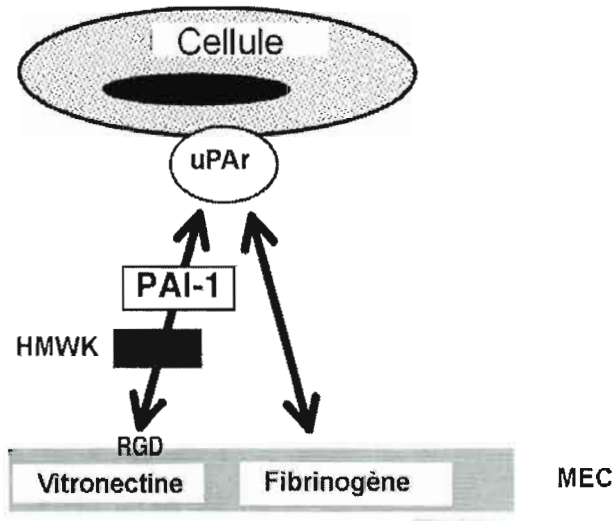

Figure 8 : Schéma représentant le rôle du PAI-1 et du HMWK dans la régulation de l'uPAr et dans l'adhésion cellulaire (Chavakis et coll., 1998)

Le complexe HMWK/PAI-1 avec le complexe uPA/uPAr permet à la cellule de s'attacher à la matrice extracellulaire *via* la VN.

Tel que mentionné précédemment, le HMWK peut se lier aux surfaces négativement chargées. Cette nouvelle forme lie l'uPAr avec l'affinité élevée d'un mode dépendant du zinc (Colman et coll., 1997) et déplace la VN de l'uPAr, agissant de ce fait en tant que facteur antiadhésif. Normalement, le HMWK fait la navette pour la kallikréine, une protéase à sérine qui peut activer le Plg en Pln. Une fois sur l'uPAr, le HMWK concentre l'activité enzymatique de la kallikréine à la surface des cellules (Colman et coll., 1997).

1.2.7.4 L'activité des corécepteurs d'uPAr

Plusieurs autres molécules ont été décrites comme pouvant interagir avec les domaines 2 et/ou 3 d'uPAr. Ces corécepteurs de l'uPAr interagissent pour potentialiser ou réguler l'activation des corécepteurs ou de l'uPAr. Ces corécepteurs incluent le récepteur de lipoprotéine de densité faible et de l'α_2-macroglobuline (α_2MR/LRP), le récepteur de mannose-6-phosphate (Man6PR), le facteur de croissance de l'insuline 2 (IGF_2), la glycoprotéine 130 (GP130), la protéine RAP et les intégrines.

Le récepteur α2MR/LRP est un membre de la famille des récepteurs de lipoprotéine de basse densité qui a comme fonction l'endocytose des apolipoprotéines et des complexes de protéinases/inhibiteurs (complexe uPA/PAI-1) pour les transformer en complexe tripartite : uPAr/uPA/PAI-1 (Andreasen et coll., 1997). Les récepteurs mannose-6-phosphate (Man6Pr) et IGF_2, associés à l'uPAr, lient la proforme latente du TGFβ aussi bien que le Plg. Ce corécepteur permet d'optimiser l'activation du Plg par l'uPA sur l'uPAr, qui en retour peut se lier à la Pln. La Pln dans ce complexe active le dégagement du TGFβ (quand le complexe Man6Pr/IGF_2 est associé à l'uPAr). Ainsi, l'interaction uPAr/Man6Pr/IGF_2 représente un mécanisme possible pour l'activation du TGFβ par les cellules (Godar et coll., 1999).

Après le regroupement de l'uPA/uPAr, la GP130, une composante critique de la voie JAK1/STAT1, s'associe à l'uPAr. La transduction du signal mène à la croissance, à la migration et à l'adhérence dans une ligne de cellules épithéliales tumorales de reins humains (Koshelnick et coll., 1997). Des études ont récemment démontré la formation d'un complexe trimoléculaire spécifique lors de l'addition de la pro-uPA aux cellules U937 humaines. Ce complexe inclut l'uPAr, la pro-uPA et la protéine RAP. La protéine RAP fait partie de la famille des

récepteurs de mannose de macrophage et elle contient un domaine putatif liant le collagène (type de fibronectine de domaine II), ainsi que huit domaines d'hydrates de carbone. Le RAP se lie fortement à un type simple de collagène, réaction obligatoire menant à la formation du collagène de type V. Le collagène se retrouve à l'emplacement exact de l'activation du plasminogène sur la cellule, favorisant ainsi des fonctions adhésives avant sa dégradation. Le collagène, par son élasticité, contribue à la dégradation cellulaire de la matrice. Par ces deux phénomènes, le collagène est doté d'un rôle putatif dans l'invasion cellulaire (Behrendt et coll., 2000).

1.2.8 Les liens entre les systèmes plasminolytique et fibrinolytique

Considérant le large spectre d'activité de la Pln décrit précédemment, il est possible que la fibrine soit une cible biologiquement appropriée lors de processus physiologiques et pathologiques et que la MTf y participe. Des études portant sur des souris déficientes en Plg ont permis de mettre en évidence des manifestations pathologiques, pouvant être évitées si la déficience en fibrinogène est génétiquement superposée (Bugge et coll., 1996). Dans le processus d'invasion métastatique, le système plasminolytique agit sur la dissolution de la matrice extracellulaire provisoire produite par le système fibrinogène (Chamber et coll., 1995). La Pln joue un rôle essentiel dans la migration des cellules et la transformation des tissus. Lors de la migration, ces cellules dégradent les composantes extracellulaires de la matrice, un préalable à l'inflammation des muscles lisses, des cellules endothéliales et des métastases. La Pln active les cytokines ou libère des facteurs de croissance séquestrés. Le transfert de gènes chez la souris confirme le rôle du système plasminolytique dans l'angiogenèse, la croissance des tumeurs et le développement de métastases (Carmeliet et Collen, 1998).

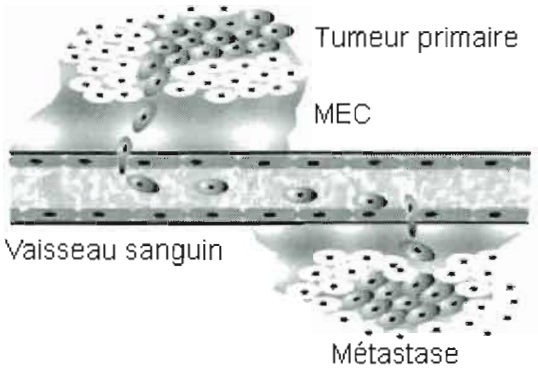

Figure 9 : Invasion métastatique

Dans la cascade métastatique, la diffusion de la tumeur débute avec la prolifération cellulaire et la croissance locale de la tumeur. Le mouvement des cellules exige un équilibre entre l'adhérence, la migration à travers la matrice extracellulaire et sa dégradation subséquente. L'induction de l'angiogenèse supporte la croissance de la tumeur et fournit les moyens par lesquels les cellules tumorales peuvent laisser la tumeur primaire et gagner l'entrée dans la circulation systémique. Les mêmes mécanismes qui ont facilité la libération des cellules tumorales dans la circulation permettent à ces cellules de s'implanter à un emplacement éloigné où leur croissance locale provoque une tumeur secondaire.

1.3 Le système fibrinolytique

L'équilibre hémostatique est contrôlé par le système fibrinolytique. Ce système joue un rôle important dans le maintien de la viscosité du sang dans des conditions normales et dans l'hémostasie lors d'hémorragies (Sidelmann et coll., 2000). Le système fibrinolytique est activé par diverses enzymes agissant en opposition : la thrombine est responsable de la formation de la fibrine insoluble, tandis que la plasmine est une enzyme fibrinolytique et thrombolytique.

La thrombine est formée pendant l'activation des mécanismes vasculaire, plaquettaire et plasmatique lors de la coagulation sanguine. L'activation du Plg en Pln se produit préférentiellement à la surface d'un caillot de fibrine, ce qui permet sa dissolution (Rijken, 2001).

La transglutaminase (FXIIIa), une enzyme responsable de la stabilisation de la fibrine, a une fonction indépendante. Elle est activée par la thrombine qui convertit le facteur inactif XIII du précurseur en forme active. En présence des ions de calcium, la FXIIIa forme un lien εglucine-lysine entre les monomères adjacents à la fibrine. La FXIIIa règle donc les propriétés hémostatiques de la fibrine. Le FXIIIa stimule également la formation de liens similaires entre le fibrinogène et la fibrine, la fibrine et l'α_2-AP, ainsi que la fibrine et la VN (Lorand, 2000).

L'activité de la Pln et de ses activateurs dans le sang est régulée par des inhibiteurs (l'α_2-AP, le PAI-1 et le PAI-2) produits par des monocytes et les cellules endothéliales. L'inhibiteur carboxypeptidase de la thrombine (TAFI) empêche la fibrinolyse en séparant des résidus Lys et/ou Arg du côté C-terminal des molécules de fibrine impliquées dans la liaison de la Pln et des activateurs du Plg (Wang et coll., 1998). Autrement, la Pln causera la dégradation protéolytique du fibrinogène et d'autres protéines impliquées dans la coagulation du sang. Les plaquettes jouent un rôle important dans la régulation de ce système (Devine et Carter, 1995).

1.3.1 L'activateur tissulaire du Plg (tPA)

Le tPA est une chaîne unique de 530 acides aminés de 68 kDa. Il se compose de plusieurs domaines homologues à d'autres protéines : un domaine en doigt de zinc (résidus 4 à 50), un domaine de facteur de croissance (résidus 50 à 87), deux domaines kringles d'environ 80 résidus, ainsi que le domaine protéolytique

(résidus 276 à 527), comportant le site catalytique. Le tPA est synthétisé par les cellules endothéliales et libéré dans le sang (Van Hinsbergh,1988).
L'activation du tPA chez l'humain s'effectue par le clivage du précurseur entre le 275^{e} AA (arginine) et le 276^{e} AA (isoleucine). Les deux chaînes sont retenues ensemble par des ponts disulfures. La chaîne N-terminale A contient un domaine fibronectine de type II, un domaine facteur de croissance et deux domaines kringles. La chaîne C-terminale B contient le domaine protéase à sérine. Le précurseur tPA, en une chaîne, possède une activité de 10 à 50 fois moindre que le tPa activé (Wu, 1995).

Le tPA est une enzyme fibrinolytique (Rijken, 1995). Il a été proposé que le lien entre le tPA et la fibrine se fait par l'intermédiaire des deuxièmes domaines kringles. L'activation du Plg par le tPA se produit à la surface de la fibrine et à la surface des cellules endothéliales. Ce processus tient compte de l'activation localisée du Plg, puisque l'activité catalytique du tPA en surface est beaucoup plus forte qu'en solution (Plow et coll., 1991). Le tPA a une faible affinité pour le Plg en l'absence de la fibrine (Km = 65 µM) et a une affinité beaucoup plus élevée en sa présence (Km entre 0,15 et 1,5 µM). La formation du complexe tPA/fibrine augmente son activité. Dans cette réaction, le tPA se lie par l'intermédiaire du domaine kringle 2 à la fibrine et accroche le plasminogène par l'intermédiaire des lysines dans le kringle 1. Ainsi, la régulation de la fibrinolyse se produit au moment de l'activation du plasminogène localisé à la surface de la fibrine (Collen, 2001). Dans une recherche, l'impact de la forme tronquée de mélanotransferrine soluble (sMTf) sur l'activation du Plg a été évalué. De façon générale, ces données suggèrent que la surstimulation de l'activation du Plg par le tPA en présence de MTf mène à la dégradation de la matrice de fibronectine (FN) et au détachement de la MEC (Rolland et coll., 2006).

1.3.2 Le facteur tissulaire (TF)

Le TF est une glycoprotéine transmembranaire de 47 kDa dont la fonction est crucial pour la régulation de l'homéostasie. La cascade extrinsèque se déroule comme suit : le facteur sanguin VII (FVII) se lie au TF et s'active en FVIIa. Ce complexe active le facteur X qui initie l'activation de la prothrombine (FII) en thrombine. La thrombine générée convertit le fibrinogène soluble en fibrine non soluble (Morrissey et coll., 1993).

Le TF est produit par plusieurs types de cellules (Morrissey et coll., 1987). Cette large distribution assure l'intégrité des vaisseaux sanguins. En cas d'hémorragie, le TF active la cascade de coagulation pour éviter les pertes sanguines. Le TF est en circulation dans le sang (100 à 150 pg/ml) et il est associé à des microparticules subcellulaires (Koyama et coll., 1994). Ce TF en circulation s'accumule dans un caillot en formation et participe à la génération de fibrine.

Le TF sanguin provient à l'origine des petites particules membranaires et des leucocytes (Giesen et coll., 1999). L'absence du gène TF est létale au 10^{e} jour de développement chez les souris. Un embryon déficient en TF présente des défauts dans l'intégrité des vaisseaux sanguins (Carmeliet et coll., 1996). Des études récentes suggèrent que l'expression du TF pourrait changer le phénotype des cellules cancéreuses. L'expression anormale de TF a été détectée dans diverses tumeurs humaines, dont celles des ovaires (Hanahan et Folkman, 1996; Lwaleed et Cooper 2000; Ueda et coll., 2001), du sein (Fernetez et Rickles, 2002), du poumon (Zhang et coll., 1994) et du pancréas (Sorense et coll., 1999). L'expression élevée du TF dans les tumeurs est associée à certains indicateurs de pronostics défavorables tels que l'angiogenèse, les métastases, les phases avancées de la maladie et la résistance aux médicaments (Camerer et coll., 2000).

L'expression accrue du TF peut également être stimulée par une variété de cytokines, renforçant de ce fait la formation de fibrine sur les tissus endommagés (Rowland et coll., 1984). Les produits de la dégradation de fibrine peuvent provoquer des réponses de cellules endothéliales et augmenter la perméabilité vasculaire (Dang et coll., 1985).

1.3.3 La matrice provisoire de fibrine extracellulaire

La fibrine est une composante majeure des caillots sanguins. Lors d'une blessure vasculaire, le TF initie la coagulation sanguine, amenant la génération de la thrombine par la voie extrinsèque qui produit la fibrine à partir du fibrinogène (Morrissey, 2001). La matrice extracellulaire (MEC) fournit, quant à elle, un microenvironnement dynamique pour l'adhérence, la différenciation, la migration et la prolifération des cellules (Boudreau et Jones, 1999). Le caillot fournit également un échafaudage de matrices pour le recrutement des cellules à un emplacement blessé. Spécifiquement, la fibrine et la fibronectine agissent comme une matrice temporaire pour l'afflux des monocytes, des fibroblastes et des cellules endothéliales (Clark et coll., 1996). Les récepteurs d'intégrine présents sur les cellules en migration lient la fibrine, la fibronectine et la VN pour interagir avec la matrice du caillot (Gailit et coll., 1997).

Puisque les molécules de la matrice extracellulaire peuvent fournir des signaux pour l'expression de gènes par des récepteurs d'intégrine, l'interaction de ces cellules avec la matrice temporaire pourrait être utilisée pour modifier le phénotype et la fonction des cellules. En fait, il est prouvé qu'une matrice extracellulaire riche en fibronectine peut augmenter l'expression de la collagénase (MMP-1). De plus, la matrice de fibronectine (FN) et de fibrine peut moduler la réponse des fibroblastes aux cytokines et l'expression des intégrines sur les cellules endothéliales (Huhtala et coll., 1995).

1.3.3.1 La fibronectine (FN)

Une composante abondante et omniprésente dans la MEC est la FN. La FN est une protéine organisée en réseau fibrillaire par des interactions directes avec des récepteurs de surface des cellules. La FN peut être synthétisée par différents types de cellules; elles la sécrètent sous forme de dimères de 230 ou de 270 kDa reliés par un pont disulfure. Chaque sous-unité contient trois types de modules (types I, II et III).

La matrice de FN est importante pour l'adhérence et la croissance normale des cellules *via* l'intégrine $\alpha_v\beta_1$. L'expression diminuée et la dégradation élevée de la FN sont responsables de certains des changements morphologiques observés au niveau des tumeurs (Ahmed et coll., 2005). La FN joue également un rôle critique dans le développement embryonnaire. Les souris déficientes en FN ont un phénotype embryonnaire non viable résultant de défauts dans la migration des cellules et la formation du mésoderme.

En tant que protéine importante dans le sang et composante de la matrice temporaire lors de blessures, la FN contribue à la réparation des tissus et à la survie neuronale suite à une ischémie cérébrale (Sakai et coll., 2001). La FN est donc une composante-clé dans beaucoup de processus qui dépendent de la MEC.

1.3.3.2 La fibrine

La fibrine est constituée par l'action de la thrombine sur les fibrinopeptides A et B du fibrinogène. Le clivage de la fibrine par la thrombine expose des domaines cachés qui en provoquent la polymérisation formant un caillot de fibrine. Le caillot est stabilisé par le facteur actif XIII, une transglutaminase. La fibrine réticule de façon covalente les chaînes α de fibrine aux chaînes γ. La fibrine réticulée a été trouvée dans différentes tumeurs malignes humaines. De plus, les liens du

facteur XIII à l'intégrine $\alpha v\beta 3$ pourraient également exercer des effets antiapoptotiques sur les cellules endothéliales (Xi et coll., 2004).

La fibrine est présente dans l'endothélium des vaisseaux angiogeniques dans des spécimens de cancers envahissants, mais pas dans les vaisseaux des tumeurs bénignes (Bootle-Wilbraham et coll., 2000). La vascularisation induite par la fibrine est basée sur des mécanismes de coagulation connexes qui comportent l'activation des plaquettes et le dépôt de caillots. Tout d'abord, la fibrine se lie aux cellules inflammatoires ou aux macrophages. La matrice de fibrine qui se développe alors autour des tumeurs fournit un environnement pro-angiogenique temporaire qui soutient l'infiltration et stimule la prolifération et la migration des cellules endothéliales (Clark et coll., 1996) en augmentant l'expression du récepteur d'intégrine $\alpha_v\beta_3$ pour faciliter la migration et la formation des capillaires (Maekawa et coll., 2000). C'est l'intégrine $\alpha_v\beta_3$ qui fournit les signaux de survie aux cellules endothéliales pendant leur interaction avec la fibrine. La matrice de fibrine stimule également la production de VEGF, de βFGF, d'IGF1 et d'Il-8 pour favoriser une boucle pro-coagulante autocrine en induisant l'expression du TF dans les cellules endothéliales (Nakasaki et coll., 2002).

Un fragment de fibrinogène et de fibrine est appelé DD-dimère. Le DD-dimère est une protéine de 190 kDa contenant des fragments de chacune des trois chaînes (α, β et γ) du monomère de fibrine stabilisée. Le DD-dimère contient deux liens d'isopeptides formés par Lys405 et Gln397 dans des régions de C-terminal de la chaîne γ (Doolittle, 1984). Les études en laboratoire confirment que le DD-dimère désorganise les monocouches des cellules vasculaires (Dang et coll., 1985). La présence des DD-dimères est associée à un mauvais pronostique chez les patients atteints de cancers (Van Hinsbergh et coll., 2001).

Le fragment E ((DD)E) est un produit soluble important de dégradation de la fibrine réticulée. Il stimule l'angiogenèse *in vitro* et *in vivo* (Dirix et coll., 2002). Aussi, le (DD)E stimule efficacement le tPA. De plus, le (DD)E protège la plasmine contre l'inhibition de cette dernière par l'α_2-AP, favorisant du même coup la dégradation de la Pln par le tPA. Le (DD)E et la fibrine réduisent le taux d'inhibition de la Pln par l'α_2-AP respectivement de 5 et de 10 fois. La liaison dépendante du domaine kringle de la Pln au (DD)E et à la fibrine, avec des valeurs de Kd respectivement de 52 et de 410 nM, contribue à l'effet protecteur. Quand le (DD)E est dégradé par la Pln, le fragment E se sépare du DD et la protection de la plasmine contre l'inhibition par l'α_2-AP est atténuée. La Pln peut se lier au (DD)E et le dégrader. Ceci fournit un mécanisme pour limiter la concentration de (DD)E et maintenir la spécificité de la fibrine au tPA (Lee et coll., 2001).

1.3.4 Les effets du système plasminolytique dans le cancer

Une forte corrélation a été observée entre le cancer et les composantes du système Plg. Ce système est impliqué dans l'angiogenèse et l'hypercoagulopathie observées dans la dispersion des métastases.

1.3.4.1 Les types de cancers et le système plasminolytique

Une corrélation entre l'expression des composantes du système plasminolytique et des résultats cliniques a été rapportée chez les patients atteints de cancer du sein, du système reproducteur, du système gastro-intestinal, du poumon, du cerveau et du rein. Dans tous les cas, une expression au-dessus de la normale de l'uPA et de l'uPAr a été associée au phénotype malin. Selon une analyse de tumeurs du sein, les niveaux de l'uPA, de l'uPAr et de PAI-1 sont sensiblement plus haut que dans le carcinome *in situ* ou le carcinome envahissant que dans un tissu normal (Fisher et coll., 2000). L'importance du niveau d'expression de

l'uPA, de l'uPAr et du PAI-1 dans le développement des tumeurs, a été mise en évidence pour la première fois dans le carcinome du sein (Duffy et coll., 1988) et depuis, d'autres preuves substantielles ont été accumulées. L'expression de ces facteurs est étroitement liée au développement des métastases et une étude a démontré que le niveau d'expression de ces marqueurs peut être utilisé comme dianostique (Janicke et coll., 1989). Fait à noter, l'expression du PAI-1 est le seul marqueur indépendant de survie des patients qui ont le cancer (Schmitt et coll., 1997).

Pour les tumeurs des systèmes reproducteur et urinaire, chez la femme, l'expression élevée de l'uPA et du PAI-1 dans le carcinome ovarien primaire est corrélée avec un pronostique de survie plus faible et cela reste vrai après stratification par la taille de la tumeur (Kuhn et coll., 1994). L'expression du PAI-2 est associée à une plus grande probabilité de récurrence pour les endométriomes (Gleeson et coll., 1993), tandis que l'uPA et le PAI-1 sont des marqueurs de la maladie récurrente et d'un pronostique de survie plus faibles dans le carcinome cervical de stade 2. Chez l'homme, l'expression accrue d'uPA dans la prostate est associée à un taux plus élevé d'invasion extracapsulaire de la maladie (Wary et coll., 1998). Finalement, l'expression stromale élevée de l'uPA et l'expression primaire de PAI-1 dans la tumeur sont associées aux métastases nodales (Kobayashi et coll., 1994). L'expression de l'uPA est le marqueur indépendant le plus fort du pronostique dans le cancer de la vessie (Hasui et coll., 1996). Dans une étude portant sur des patients ayant un carcinome rénal, les niveaux élevés de l'expression de l'uPA, de son récepteur et de son inhibiteur dans la tumeur primaire étaient prédictifs de la répétition locale et systémique de la maladie (Deng et coll., 1996).

Pour les tumeurs gastro-intestinales, les niveaux élevés de l'uPA et du PAI-1 dans le tissu tumoral sont les indicateurs pronostiques indépendants d'un taux de

survie plus faible chez les patients présentant un carcinome gastrique (Nekarda et coll., 1994). La présence de l'uPAr dans ces tumeurs est un marqueur significatif pour les métastases (Heiss et coll., 1995). Les patients présentant les niveaux d'uPA ou de PAI-2 les plus élevés ont survécu en moins grand nombre. L'expression d'uPA, d'uPAr et de PAI-1 et 2 est corrélée avec un taux de survie globale plus faible et le développement des métastases de foie chez les patients souffrant d'un carcinome colorectal. En outre, les niveaux d'expression plus élevés de l'uPA ou du PAI-1 sont liés à l'agressivité de la tumeur (Buo et coll., 1995).

Pour les tumeurs de la peau, le système plasminolytique joue un rôle dans l'invasion et dans la diffusion métastatique des cellules tumorales (de Vries et coll., 1996). Indépendamment de l'expression de l'uPA, les cellules de mélanomes diffèrent des cellules dérivées d'autres tumeurs dans l'expression élevée du tPA. De plus, le système plasminolytique est démontré comme un facteur important dans la progression des mélanomes et la migration des cellules de mélanomes (de Vries et coll., 1996). De plus, les mélanomes sont des tumeurs agressives et expriment la MTf (Shoemaker et coll., 1991).

Pour les autres tumeurs, l'expression de l'uPA est corrélée avec l'ADN diploïde, les métastases et les sarcomes (Choong et coll., 1996). Dans un modèle *in vivo* d'ostéosarcome, l'expression de l'uPA, de l'uPAr et du PAI-1 s'est avérée élevée dans le tissu. L'expression maximale coïncide avec l'infraction corticale et la prolongation extra-osseuse de la tumeur (Fisher et coll., 2000). Un niveau élevé d'expression de l'uPA, par rapport aux tumeurs bénignes, a été rapporté dans les gliomes malins. La progression des tumeurs et le taux de survie des patients sont corrélés avec l'expression de l'uPA. L'une est proportionnelement et l'autre est inversement proportionnellement (Hsu et coll., 1995). Un niveau élevé de PAI-1, mais sans l'expression d'uPA, est associé à une diminution du taux de

survie chez les patients présentant un adénocarcinome du poumon (Pedersen et coll., 1994).

Donc, le système plasminolytique agit sur plusieurs types de cancers et la régulation de celui-ci peut amener un contrôle de cette maladie.

1.4 L'angiogenèse

La formation de nouveaux vaisseaux sanguins est essentielle pour la croissance soutenue des tumeurs au-delà d'une taille critique de 2 mm^3. L'angiogenèse accrue favorise la pénétration des cellules tumorales dans la circulation et la diffusion des métastases (Carmeliet et Jain, 2000). Les enzymes protéolytiques, dont les protéases à sérine et les MMP, sont impliquées dans l'angiogenèse (Pepper, 2001). L'implication des protéases à sérine et des MMP pendant la progression du cancer s'effectuerait lors de la dégradation des composantes de la matrice extracellulaire. Cette dégradation contribuerait à différents événements tels que la transformation de la matrice extracellulaire, ainsi que la migration et l'invasion cellulaires. En plus de dégrader les composantes extracellulaires de la matrice, les protéases sont également impliquées dans l'activation des cytokines et le dégagement des facteurs de croissance séquestrés dans la matrice extracellulaire (McQuibban et coll., 2002). Des études démontrent un lien entre la mélanotransferrine et l'angiogenèse (Sala et coll., 2002; Michaud-Levesque et coll., 2006).

1.4.1 Le système plasminolytique et l'angiogenèse

L'expression de l'uPAr sur la surface des macrophages en coculture augmente l'invasion des carcinomes du sein (Hidenbrand et coll., 1995). Cependant, les macrophages cultivés d'un tissu normal de seins expriment peu l'uPAr (Hidenbrand et coll., 1999). Le rôle des macrophages dans la tumorigénèse n'est

pas clair, mais des spécimens provenant de biopsies de carcinome du sein ont montré une association entre les macrophages et la néovascularisation tumorale. L'hypothèse est que la réponse inflammatoire autour des tumeurs pourrait être provoquée par des macrophages recrutés par le système de l'uPA et que les macrophages sont les stimulateurs initiaux de l'angiogenèse. En plus de son effet chimiotactique, l'action de l'uPA et de l'uPAr sur des macrophages peut être importante pour l'angiogenèse parce que les macrophages sécrètent des facteurs angiogeniques tels que le VEGF, le FGF-2 et l'Il-8 (Hidenbrand et coll., 1999).

Les niveaux élevés de l'uPA et de ses composantes, l'uPAr et les PAI auraient un rôle précurseur pour l'invasion tumorale. Ce rôle est renforcé par l'observation de l'expression élevée de l'uPAr dans des vaisseaux sanguins de seins, de métastases et de carcinomes rénaux, ce qui suggère que la régulation de l'uPA dans la tumorigénèse est liée à l'angiogenèse (Xu et coll., 1997). Le système d'uPA est également important pour la migration et l'invasion des cellules endothéliales pendant l'angiogenèse. Le VEGF est sécrété par des macrophages et des plaquettes à l'emplacement de l'inflammation, causant une augmentation de la perméabilité de la vascularisation locale, ce qui mène à la diffusion du fibrinogène et d'autres protéines de plasma dans l'espace extracellulaire. Le fibrinogène, par l'action de la thrombine, est converti en fibrine, qui forme une matrice transitoire sur laquelle les cellules endothéliales peuvent migrer pendant l'angiogenèse. Les expériences *in vitro* ont montré l'importance des facteurs pro-angiogeniques tels que le βFGF et le VEGF qui induisent la migration des cellules endothéliales (Ribatti et coll., 1999). Ces mêmes facteurs de croissance sont libérés par l'action protéolytique du système plasminolytique associé au cancer. De plus, l'uPAr, qui est important pour la migration des cellules endothéliales, est localisé sur les cellules endothéliales envahissantes dans la matrice de fibrine *in vitro* (Mignatti et Rifkin 1996). De plus, le βFGF et le VEGF

sont augmentés. Les antagonistes et les inhibiteurs de l'expression d'uPA empêchent également la formation de tube des cellules endothéliales (Lansink et coll., 1998). Une étude provenant de notre laboratoire a rapporté que la stimulation excessive du système plasminolytique par le sMTf mène au détachement principal des cellules endothéliales (Rolland et coll., 2006). Puisque l'expression du tPA par les cellules endothéliales est induite par des facteurs angiogeniques, y compris le facteur βFGF et le VEGF, le détachement de la cellule endothéliale et la mort subséquente de cellules devraient jouer un rôle important dans le processus angiogenique. Ces données montrent que la sMTf porrait stimuler l'activation du plasminogène par tPA produit dans la tumeur et interférer dans la prolifération et la survie de la cellule endothéliale (Rolland et coll., 2006).

1.5 La coagulation et le développement du cancer

Jusqu'à présent, plusieurs études ont démontré que les tumeurs activent la coagulation sanguine. Cependant, le mécanisme fondamental par lequel les facteurs de coagulation favorisent la croissance des cellules tumorales, l'invasion, les métastases et l'angiogenèse, reste à être démontré.

Plusieurs études portant sur l'interaction entre diverses protéines et les cancers font mention du TF (Rickles et coll., 1992). Deux cascades, l'une intrinsèque et l'autre extrinsèque, mènent à la formation d'un caillot de fibrine. Bien qu'elles soient amorcées par des événements distincts, les deux cascades convergent sur une voie commune qui inclut la thrombine, LRP et la fibrine. La cascade intrinsèque, qui est lancée quand le contact est fait entre le sang et la surface exposée des cellules endothéliales, exige les facteurs de coagulation VIII, IX, XI et XII, la prékallikréine, le kininogène, les ions calcium, ainsi que les phospholipides à poids moléculaires élevés sécrétés par les plaquettes. La

cascade extrinsèque de coagulation est lancée à l'emplacement des dommages tissulaires en réponse au dégagement du facteur activé par le TF. Le facteur X est l'élément de convergence des cascades intrinsèque et extrinsèque de coagulation convergent. Le TF et le facteur VIIa contribuent à la cascade extrinsèque et sont probablement impliqués dans le développement du cancer. Jusqu'à 50 % de tous les patients atteints de cancers et 90 % de ceux avec des métastases ont des anomalies hémostatiques (Yousef et coll., 2003). Ces anomalies sont reflétées dans la dominance de la voie procoagulante associée aux cellules tumorales, qui mène à la génération et de l'hypercoagulation. L'activation de la coagulation dans les tumeurs malignes peut être déclenchée par des mécanismes directs ou indirects. L'activation directe de la coagulation du sang par l'induction de la thrombine peut se produire par l'activité procoagulante des cellules tumorales, tandis que l'activation indirecte peut se produire par la production des cytokines des macrophages. La production de TF est déclenchée par des macrophages ou des cellules endothéliales. Les composantes de voie de coagulation peuvent contribuer à la prolifération, à l'invasion et aux métastases de cellules tumorales (Sampson et Kakkar, 2002), bien que ces changements puissent également être une conséquence du stade avancé de la maladie (Diamandis et coll., 2003).

1.6 Objectifs

L'objectif principal porte sur la caractérisation du rôle de la MTf dans le cancer. Pour répondre à cet objectif, nous déterminerons le rôle de la MTf dans la régulation du plasminogène sur la migration cellulaire et de la dégradation de la matrice extracellulaire. De plus, nous explorerons l'invasion tumorale liée au système plasminolytique et l'applicabilité de l'utilisation de la MTf comme agent thérapeutique.

Les articles

2.1 1er article

2.1.1 Résumé :

Nous avons récemment rapporté que la mélanotransferrine humaine recombinante soluble (sMTf) possède un taux élevé de transfert à travers la barrière sang-cerveau impliquant possiblement le récepteur de basse densité relié à la lipoprotéine (LRP). Nous démontrons de nouvelles interactions entre la MTf et des ligands de LRP. Par l'utilisation d'une analyse des interactions biospécifiques, le précurseur de l'uPA (pro-uPA) et le plasminogène (Plg) interagissent avec la MTf immobilisée. De plus, l'activation du Plg par la pro-uPA est augmentée en présence de la MTf soluble. En prenant en considération le rôle crucial dans la migration cellulaire sur le système d'activation du Plg, dans le cancer et l'angiogenèse, nous avons mesuré l'impact de la MTf soluble et de la MTf membranaire sur la migration cellulaire. L'anticorps monoclonal L235 qui reconnaît un épitope conformationnel de MTf inhibe la migration cellulaire dans une lignée vasculaire (HMECs-1) et une lignée de mélanomes (SK-Mel-28). Ces données indiquent que la MTf membranaire est associée dans ce processus. En outre, une faible concentration de MTf exogène soluble (10 et 100 nM) inhibe la migrastion de ces lignées par plus de 50 %. Ces résultats indiquent que la MTf soluble et membranaire affecte la capacité migratoire des cellules endothéliales et de mélanomes suggérant une implication de la MTf dans la régulation de l'activation du Plg par la pro-uPA et le Plg.

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HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

2.1.2 Titre: Regulation of plasminogen activation: a role for melanotransferrin (p97) in cell migration

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La participation du Dr Demeule comme permier co-auteur avec moi-même, a permis de rehausser la qualité de l'article étant mon premier article dans une revue qui possède un très bon facteur d'impact. La rédaction a été produite conjointement. J'ai produit les figures 2, 3, 4, 5, 6b, 8 et 9. Le Dr Demeule a produit la figure 1 et les tableaux 1 et 2. Jonathan Michaud-Levesque a corrigé une partie du texte. Yannève Rolland a contribué à la figure 6 et 7.

2.1.3 Abstract

We recently reported that human recombinant melanotransferrin (p97) presents a high transport rate across the blood-brain barrier that might involve the low-density lipoprotein receptor–related protein (LRP). We now report new interactions between p97 and another LRP ligand, the urokinase plasminogen activator (uPA) complex. By using biospecific interaction analysis, both pro-uPA and plasminogen are shown to interact with immobilized p97. Moreover, the activation of plasminogen by pro-uPA is increased by soluble p97. Because the uPA system plays a crucial role in cell migration, both in cancer and in angiogenesis, we also measured the impact of both endogenous membrane-bound and exogenous p97 on cell migration. The monoclonal antibody L235 (which recognizes a conformational epitope on p97) inhibited the migration of human microvascular endothelial cells (HMECs-1) and of human melanoma SK-Mel-28 cells, indicating that endogenous membrane-bound p97 could be associated with this process. In addition, low concentrations of exogenous p97 (10 and 100 nM) inhibited HMEC-1 and SK-MEL28 cell migration by more than 50%. These results indicate that membrane-bound and soluble p97 affect the migration capacity of endothelial and melanoma cells and suggest that p97 could be involved in the regulation of plasminogen activation by interacting with pro-uPA and plasminogen.

2.1.4Introduction

Melanotransferrin (p97) possesses a high level of homology (37%-39%) with human serum transferrin, human lactoferrin, and chicken transferrin.[1,2] It is a glycosylated protein that reversibly binds iron and was first found at high levels in malignant melanoma cells.[1,3] Two forms of p97 have been reported, one of which is bound to cell membranes by a glycosylphosphatidylinositol anchor while the other form is both soluble and actively secreted.[4-6] The exact physiologic role of either membrane-bound p97 or secreted p97 is largely unexplored.[7]

In the early 1980s, p97 was found to be expressed in much larger amounts in neoplastic cells and fetal tissues than in normal tissues.[3,8,9] More recently, it was reported that p97 mRNA is widespread in normal human tissues.[10] p97 is also expressed in reactive microglia associated with amyloid plaques in Alzheimer disease.[11] Normal serum contains very low levels of p97,[3] which were reported to increase by 5- to 6-fold in patients with Alzheimer disease.[12]

We previously demonstrated that recombinant human melanotransferrin (p97) is transported at high rate into the brain, using both an in vitro model of the blood-brain barrier (BBB) and in situ mouse brain perfusion.[13] We also showed that p97 transcytosis might involve the low-density liproprotein receptor–related protein (LRP). This receptor is also known to mediate the internalization of the urokinase:plasminogen activator inhibitor:urokinase receptor complex (uPA:PAI-1:uPAR). Briefly, single-chain proenzyme-uPA is activated upon binding to its cell surface receptor uPAR,[14] which is a glycosylphosphatidylinositol (GPI)–anchored membrane protein.[15,16] After its activation, uPA (which catalyzes the conversion of plasminogen to plasmin) is quickly inhibited by the plasminogen activator inhibitor type-1 (PAI-1). The inactive uPA:PAI-1 complex binds to uPAR and then is rapidly internalized by LRP.[17,18] The uPA:PAI-1 complex is degraded in lysosomes, whereas the uPAR is recycled at the cell surface.[19] Other LRP

ligands include pro-uPA, PAI-1, receptor-associated protein (RAP), and a diverse spectrum of structurally unrelated proteins.[20]

Because uPA plays a crucial role in enhancing cell migration and invasion during embryogenesis, wound healing, and metastasis,[21-24] and since p97 and the uPA/uPAR complex may share the same pathway for endocytosis, we investigated potential interactions between p97 and components of the uPA/uPAR complex. We used the BIAcore apparatus (BIAcore, Piscataway, NJ) for examining potential protein-protein interactions in real time. We demonstrate here that pro-uPA and plasminogen interact in vitro with immobilized p97. In addition, we report that p97 stimulates the activation of plasminogen by pro-uPA. We also show that either an antibody directed against endogenous p97 or application of low concentrations of exogenous p97 inhibited cell migration of human endothelial and melanoma cells. These data suggest that the balance between membrane-bound p97 and soluble p97 could be important in cell migration, which is crucial for angiogenesis and tumor growth.

2.1.5Materials and methods

2.1.5.1Materials

Soluble human recombinant p97, which is produced by introducing a stop codon following the glycine residue at position 711, and monoclonal antibodies (mAbs) directed against p97 were kindly provided by Biomarin Pharmaceutical (Novato, CA). Tissue plasminogen activator (tPA), PAI-1, and plasmin were from Calbiochem (La Jolla, CA). Pro-uPA and plasminogen were from American Diagnostica (Greenwich, CT). Angiostatin was purchased from Angiogenesis Laboratories (Tucson, AZ), and uPA was from Roche Biochemicals (Laval, QC, Canada). CM5 sensor chips were from BIAcore (Piscataway, NJ). The plasmin substrate (D-Val-Leu-Lys-*P*-nitraniline, or VLK-pNA) and other biochemical reagents were from Sigma (Oakville, ON).

2.1.5.2Blood-brain barrier model and transcytosis experiments

The in vitro model of the blood-brain barrier (BBB) was established by using a coculture of bovine brain capillary endothelial cells (BBCECs) and newborn rat astrocytes as previously described.[25] p97 was radioiodinated with standard procedures using an iodo-beads kit and D-Salt Dextran desalting columns from Pierce (Rockford, IL), as previously described.[13] Transcytosis experiments were performed as follows: one insert covered with BBCECs was set into a 6-well microplate with 2 mL Ringer–HEPES (N-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) and was preincubated for 2 hours at 37°C. [^{125}I]-p97 (0.5-1.5 μCi [18-55 kBq] per assay), at a final concentration of 25 nM, was then added to the upper side of the insert. At various times, the insert was sequentially transferred into a fresh well to avoid possible reendocytosis of p97 by the abluminal side of the BBCECs. At the end of the experiment, [^{125}I]-p97 was assayed in 500 μL of the lower chamber of each well following trichloroacetic acid (TCA) precipitation.

2.1.5.3Cell culture

Cells were cultured under 5% CO_2/95% air atmosphere. Human microvascular endothelial cells (HMECs-1) were from the Centers for Disease Control and Prevention (Atlanta, GA) and were cultured in MCDB-131 medium (Sigma) supplemented with 10 mM L-glutamine, 10 ng/mL epidermal growth factor (EGF), 1 µg/mL hydrocortisone, and 10% inactivated fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) and SK-Mel-28 cells were obtained from American Type Culture Collection (Manassas, VA). HUVECs were cultured in an EGM-2 bullet kit from BioWhittaker (Walkersville, MD). Melanoma SK-Mel-28 cells were grown in modified Eagle medium (MEM) supplemented with 1 mM Na-pyruvate, 100 U/mL penicillin-streptomycin, 1.5 g/L Nabicarbonate, and 10% FBS.

2.1.5.4BIAcore analysis

p97, PAI-1, and plasminogen were covalently coupled to a CM5 sensor chip via primary amine groups using the *N*-hydroxysuccinimide (NHS)/*N*-ethyl-*N*'-(dimethylaminopropyl)carbodiimide (EDC) coupling agents as previously described.[26] Briefly, the carboxymethylated dextran was first activated with 50 µL NHS/EDC (50 mM/200 mM) at a flow rate of 5 µL/min. p97, PAI-1, or plasminogen (5 µg) in 20 mM acetate buffer, pH 4.0, was then injected and the unreacted NHS esters were deactivated with 35 µL 1 M ethanolamine hydrochloride, pH 8.5. Approximately 8000 to 10 000 relative units of p97, PAI-1, or plasminogen were immobilized on the sensor chip surface. Ringer solution or a 50 mM Tris (tris(hydroxymethyl)aminomethane)/HCl buffer (pH 7.5) containing 150 mM NaCl and 50 mM $CaCl_2$ was used as the eluent buffer. Proteins were diluted in the corresponding eluent buffer and injected onto the sensor chip surface. Protein interactions were analyzed using both the Langmuir binding model, which is the simplest model for 1:1 interaction between analyte and

immobilized ligand, and a 2-state conformational change model that describes a 1:1 binding of analyte to immobilized ligand followed by a conformational change.

2.1.5.5Enzymatic assay and cell treatment with soluble p97

The enzymatic activity of pro-uPA was measured using a colorimetric assay. The reaction was performed in a final volume of 200 µL in an incubation medium consisting of 50 mM Tris/HCl buffer (pH 7.5), 150 mM NaCl, and 50 mM $CaCl_2$. This incubation medium also contained 15 µg/mL VLK-pNA with or without plasminogen. Enzymatic activity was assessed in the absence or presence of p97. The reaction was started by the addition of pro-uPA. In this assay, the cleavage of VLK-pNA results in a *P*-nitraniline molecule that absorbs at 405 nm. The reaction product was monitored at 405 nm using a Microplate Thermomax Autoreader (Molecular Devices, Sunnyvale, CA).

HMECs-1 were grown to 85% confluency in 6-well plates and were incubated for 18 hours under 5% CO_2/95% air atmosphere in cell culture medium with or without p97 (100 nM). Endothelial cells were washed twice with Ringer solution and mechanically scraped from the wells. Cells were counted and frozen at –80°C until used. A volume corresponding to 100 000 cells was incubated in the plasmin assay as above and plasmin activity was monitored at 405 nm for 60 minutes. HMECs-1 were also individualized by phosphate-buffered saline (PBS) citrate solution (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4–$7H_2O$, 15 mM Na citrate pH 6.8) for 15 minutes. Cells were washed twice in Ringer-HEPES solution (150 nM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$–$6H_2O$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM glucose, pH 7.4) and counted. A volume corresponding to 100 000 cells was incubated in the plasmin assay with mAb L235 (325 nM) or immunoglobulin G (IgG) control. Plasmin activity was monitored at 405 nm for 480 minutes.

2.1.5.6Cell migration assay

HMEC-1, HUVEC, and SK-Mel-28 cell migration was performed using Transwell filters (Costar, Corning, NY; 8 µm pore size) precoated with 0.15% gelatin for 2 hours at 37°C. The Transwell filters were assembled in 24-well plates (Falcon 3097, Fischer Scientific, Montreal, QC, Canada) and the lower chambers filled with 500 µL cell culture medium. To study the effect of p97, mAb L235, or mouse IgG on cell migration, HMEC-1, HUVEC, and SK-Mel-28 cells were harvested by trypsinization and centrifuged. Approximately 10 000 cells were resuspended in 100 µL fresh Dulbecco MEM (DMEM) with or without p97, mAb L235, or mouse IgG and added into the upper chamber of each transwell (lower chamber of the transwell also contained p97, mAb L235, or nonspecific mouse IgG). The plates were than placed at 37°C in 5% CO_2/95% air for 18 hours. Cells that had migrated to the lower surface of the filters were fixed with 3.7% formaldehyde in PBS, stained with 0.1% crystal violet/20% MeOH, and counted (4 fields per filter). Photomicrographs at 100 x magnification were taken using a digital Nikon Coolpix 5000 camera (Nikon Canada, Mississauga, ON, Canada) attached to a Nikon TMS-F microscope (Nikon Canada).

2.1.5.7Data analysis

Statistical analyses were made with the Student paired *t* test using GraphPad Prism (San Diego, CA). Significant difference was accepted for *P* values less than .05.

2.1.6Results

2.1.6.1Transcytosis of p97 through BBCEC monolayers

We first evaluated the transcytosis of p97 across an in vitro model of the BBB at 37°C (Figure 10A). A significant (> 50%) reduction in the transport of [^{125}I]-p97 (25 nM) from the apical (blood) side to the basolateral (brain) side of BBCEC

monolayers was observed in the presence of 640 nM RAP. Transcytosis of [^{125}I]-p97 was unaffected by a 200-fold molar excess of bovine serum albumin (BSA). The permeability coefficent for sucrose is similar in the absence or presence of RAP, indicating that the integrity of the BBCEC monolayers was unaffected by this protein (data not shown). The results with RAP also suggest that LRP might be involved in p97 transcytosis, since it has been reported to be an LRP ligand, whereas BSA was shown to bind to megalin, another member of the low-density lipoprotein (LDL) receptor family, probably via cubilin.[27] To determine whether protein interaction could occur between p97 and RAP, leading to a reduction in p97 transcytosis, protein interactions were investigated by using biologic interaction analysis in real time (Figure 10B). For this analytical approach, p97 was first immobilized on the surface of a sensor chip. Using standard NHS/EDC coupling procedures, about 8 to 10 ng/mm^2 of p97 were immobilized. RAP or BSA (0.05 µg/µL) was then injected over immobilized p97. No interactions could be observed between these proteins and p97, suggesting that the inhibition of [^{125}I]-p97 transcytosis is not related to protein interactions between p97 and RAP.

2.1.6.2Pro-uPA and p97 interaction

To evaluate the impact of immobilization procedures on the structural integrity of p97, different mAbs directed against various conformational epitopes of p97 were injected over p97 (Figure 11). The surface plasmon resonance (SPR) signal generated by the interaction between p97 and various mAbs varied from 250 relative units (RU) to 2500 RU. These data show that the mAbs could still recognize p97, indicating that the protein is intact following its immobilization on the sensor chip surface. Table 1 shows the kinetic parameters estimated by the BIAevaluation software (BIAcore) for antibody interactions with p97. From these values, the affinity constant ($K_A = k_a/K_d$) of these mAbs for immobilized p97 ranged from 0.08 to 2.7 nM^{-1}, and the relative affinities are HybE < L235 < 9B6 < 2C7, HybC < HybF.

When pro-uPA and tPA (0.05 µg/µL) were injected over immobilized p97, protein interaction occurred between pro-uPA and p97 but not between tPA and p97 (Figure 12A). Around 8 to 10 ng/mm^2 of PAI-1 was also immobilized onto another well of a sensor chip surface using NHS/EDC coupling conditions. No interaction between p97 and immobilized PAI-1 could be detected (Figure 12A). However, a strong interaction could be observed when tPA was injected over PAI-1, indicating that PAI-1 can still interact with tPA following immobilization (data not shown). In addition, plasminogen, plasmin, and angiostatin (0.05 µg/µL) were injected over immobilized p97 (Figure 12B). According to the SPR, plasminogen also interacts with immobilized p97, whereas plasmin and angiostatin, 2 plasminogen fragments, do not. The kinetic data obtained from binding of pro-uPA or plasminogen to immobilized p97 biosensor surface were evaluated using both the 1:1 Langmuir binding model and the 2-state conformational change model. Interestingly, the 2-state conformational change model was a better fit than the 1:1 Langmuir binding model when comparing a single concentration of either pro-uPA and plasminogen over p97 biosensor surface. Kinetic data obtained with the 2-state conformational model are presented in Table 2. Kinetic data for the interaction between pro-uPA and p97 show an association constant (k_{a1}) of 0.6 x 10^4 $M^{-1}s^{-1}$ and a dissociation rate constant (k_{d1}) of 1.7 x $10^{-3}s^{-1}$. Furthermore, the forward rate constant (k_{a2} = 3.2 x $10^{-3}s^{-1}$) and backward rate constant (k_{d2} = 7.1 x $10^{-4}s^{-1}$) for the conformational change provide an apparent equilibrium dissociation constant [(K_D = k_{d1}/k_{a1})/(k_{d2}/k_{a2})] of 65 nM. The kinetic analysis of plasminogen interaction with p97 shows an association constant (k_{a1}) of 2.1 x 10^4 $M^{-1}s^{-1}$. The dissociation rate constant (k_{d1} = 43.0 x $10^{-3}s^{-1}$), as well as the forward rate constant (k_{a2}) of 6.0 x $10^{-3}s^{-1}$ and backward rate constant (k_{d2}) of 1.1 x $10^{-3}s^{-1}$, are different from those seen for the pro-uPA interaction with p97. However, the apparent equilibrium dissociation constant (K_D) between p97 and plasminogen is 350 nM, which is different from that observed for the interaction of pro-uPA with immobilized p97.

To evaluate the effect of p97 interaction on pro-uPA, we measured the serine activity (VLK-pNA hydrolysis) of pro-uPA and tPA using a colorimetric assay both with and without p97 (Figure 13). In the absence of p97, only a slight activity was measured for both pro-uPA and tPA. However, after 180 minutes, the VLK-pNA hydrolysis by pro-uPA goes from less than 0.02 absorbance unit (AU) in the absence of p97 to more than 0.18 AU when p97 is added into the incubation (Figure 13A). Addition of p97 to tPA elicits no observable effect and p97 alone had no proteolytic activity (Figure 13B). The results from both SPR and enzymatic activity indicate that the change in pro-uPA conformation induced by p97 increased its ability to degrade the plasmin substrate. To determine whether interaction with p97 leads to a cleavage of pro-uPA, the proteins were coincubated for 5 minutes at 37°C (Figure 13C). They were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using a 12.5% acrylamide gel, and stained with standard Coomassie blue. Under these conditions, p97 and uPA migrated as 97-kDa and 33-kDa bands, respectively, whereas pro-uPA migrated as a single band at 55 kDa. No major degradation of either protein could be detected, indicating that the incubation of pro-uPA with p97 under the conditions used to perform the VLK-pNA hydrolysis did not cleave either protein. Even after 6 hours' incubation at 37°C, both proteins were stable (data not shown). In the presence of plasminogen, pro-uPA was cleaved after an incubation of 5 minutes at 37°C and 2 major fragments of 33 kDa and 29 kDa could be observed. When p97 was added to the incubation medium, the generation of these fragments did not change. We further estimated the impact of p97 on plasminogen fragmentation by pro-uPA using 6 hours' incubation at 37°C (Figure 13D). As controls, the proteins used for this experiment (p97, Glu-plasminogen, or Lys-plasminogen), in the same amount (3 µg), were incubated for the same length of time at 37°C. Proteins were separated on a 7.5% acrylamide gel under nonreducing conditions and stained with Coomassie blue. When p97 was added to Glu-plasminogen no

apparent fragment was generated. In contrast, the addition of a low amount (10 ng) of pro-uPA, which could not be detected using standard Coomassie blue staining, induced degradation of Glu-plasminogen with the appearance of fragments that migrated at the same molecular weight as Lys-plasminogen. Moreover, when p97 is added to Glu-plasminogen and pro-uPA, the degradation profile of Glu-plasminogen is changed. In the presence of p97 with Glu-plasminogen and pro-uPA, higher levels of bands migrating at the same molecular weight as Lys-plasminogen were observed and 2 other fragments appeared at 50 and 30 kDa. These fragments do not seem to be related to angiostatin, since they migrated at a different molecular weight than did the control angiostatin at 42 kDa. These results suggest that p97 alters the cleavage of Glu-plasminogen by pro-uPA.

2.1.6.3Plasminogen activation by p97

We futher characterized the interaction of p97 with pro-uPA by measuring the activation of plasminogen by pro-uPA in the presence of p97 (Figure 14). When p97 is added to pro-uPA and plasminogen, the VLK-pNA hydrolysis is 4-fold higher after 180 minutes (Figure 14A). Control experiments performed with p97 indicated that this protein alone does not generate plasmin when it is added to plasminogen. We also measured the plasmin activity in the presence of various concentrations of p97 (Figure 14B). Since the generation of plasmin proceeds at a constant rate under the assay conditions used, plotting the experimental data as a function of time $(t)^2$ allowed us to determine the initial rate of plasmin formation (data not shown). From these linear curves, the initial plasmin activity measured in the absence of p97 was subtracted from the activities obtained in the presence of various p97 concentrations. Thus, the data represent the initial rates of plasmin activity (corresponding to the slopes) in the presence of various p97 concentrations. p97 stimulates the plasminogen cleavage by pro-uPA in a dose-dependent manner, with half-maximal stimulation occurring at 25 ± 6 nM. We

further measured the effect of p97 on plasmin activity in the presence of various concentrations of plasminogen (Figure 14C). Initial rates of plasmin activity calculated at several plasminogen concentrations were plotted as a function of plasminogen concentrations. The resulting experimental data were fitted using nonlinear regression analysis. p97 decreased the apparent Michaelis constant (K_m) of pro-uPA for plasminogen from 188 ± 22 to 102 ± 17 nM and increased the maximal velocity (V_{max}) from 6.9 ± 0.4 to 8.9 ± 0.6 mAU/min. These results indicate that p97 positively affects the activation of plasminogen by pro-uPA by increasing the catalytic efficiency by a factor of 2.4.

To determine whether the induction of plasmin formation by p97 was specific, we measured the formation of plasmin by pro-uPA in the presence of either the mAb L235 (directed against p97) or a nonspecific IgG (Figure 14D). MAb L235 (50 nM) inhibited the pro-uPA activation induced by p97 by 50%. These results suggest that the effect of p97 upon pro-uPA's activation of plasminogen is specific and may involve the epitope recognized by the mAb L235.

2.1.6.4Inhibition of cell migration by mAb L235

Since p97 affects the activation of plasminogen in vitro and since the uPA/uPAR system is important in cell migration, we further investigated whether endogenous p97 might be associated with this process. Migration of HMECs-1 and HUVECs was evaluated in the presence of either mAb L235 or nonspecific mouse IgG by using modified Boyden chambers (Figure 15A). In addition, because p97 was first identified in melanoma cells,[3] we also measured the impact of the mAb L235 on the migration of human melanoma (SK-Mel-28) cells (Figure 15A). In the presence of mAb L235 (50 nM), the migration of HMEC-1 and SK-Mel-28 cells was inhibited by 54% and 48%, respectively. However, cell migration of HUVECs was unaffected by this concentration of mAb L235. Endogenous p97 was immunodetected in lysates from HMEC-1, SK-Mel-28, and HUVEC cells as well

as in their conditioned culture media (Figure 15B). p97 migrated under unreduced conditions at 73 and 60 kDa, as previously observed.[28] It was highly expressed in lysates from HMEC-1 and SK-Mel-28 cells and at lower levels in their respective conditioned culture media. In HUVECs, p97 was, however, almost undetectable. In fact, the exposure time was at least 30 times greater to detect a much lower level of p97 in HUVECs compared with HMEC-1 and SK-Mel-28 cells. These results indicate that mAb L235, by interacting with endogenous p97, inhibits the migration of HMEC-1 and SK-Mel-28 cells. This also suggests that the endogenous p97 in these cells is involved in cell migration.

2.1.6.5Effect of exogenous p97 on cell migration

In addition, we estimated whether exogenous p97 could affect the migration of HMEC-1 and SK-Mel-28 cells (Figure 16A-B). Exogenous p97, at 10 nM and 100 nM, inhibited the migration of HMEC-1 cells by 34% and 50% (Figure 16C). The migration of SK-Mel-28 cells was inhibited by 44% and 70% in the presence of 10 and 100 nM p97. Migration of HUVECs was unaffected by these concentrations of p97 (data not shown). Moreover, this inhibition of cell migration is not related to a reduction of endothelial or melanoma cell adhesion, since the same concentrations of p97 did not affect adhesion on gelatin of either HMEC-1 or SK-Mel-28 cells (data not shown).

2.1.6.6Inhibition of plasminolytic activity at the cell surface by soluble p97 and mAb L235

HMECs-1 were incubated for 18 hours with or without p97. Following this treatment, the plasmin activity at the surface of control and treated cells was measured. When cells were treated with p97 (100 nM), plasminogen activation was inhibited by 95% (Figure 17A). This marked reduction in the plasminolytic capacity of these cells by soluble p97 could explain the inhibition of HMEC-1 migration. Moreover, when HMECs-1 were treated with the mAb L235, the

plasminolytic activity was inhibited by more than 50% compared with nonspecific mouse IgG (Figure 17B). This inhibition by the mAb L235 suggests that endogenous, membrane-bound p97 could participate in plasminogen activation in HMECs-1.

2.1.7Discussion

Using an in vitro model of the BBB, we have previously shown that RAP, which is a folding chaperone for LRP,[29] inhibits transcytosis of p97.[13] In this previous study we also observed that p97 inhibited the transcytosis of lactoferrin, which, in an earlier study, had been reported to be LRP-dependent.[30] From these results, we suggested that LRP is involved in the transport of p97.[13] However, because the members of the LDL-receptor family (which includes LRP) transport similar substrates,[29] we cannot exclude the possibility that other receptors of this family could also bind and transport p97. In the present study, the BIAcore technology shows no interaction between p97 and RAP, indicating that the transport inhibition caused by this LRP ligand is not caused through its interaction with p97. Because LRP recognizes a large variety of ligands and is associated with the homeostasis of proteinases and proteinase inhibitors,[31] we further investigated the potential interactions between other LRP ligands and p97. Our data clearly show that both pro-uPA and plasminogen interact with p97 and that these interactions are specific, since no interaction between p97 and other proteins, including tPA, PAI-1, plasmin, angiostatin, BSA, and ovalbumin, could be measured. These results are the first to describe potential interactions between p97 and proteins of the uPA system.

In addition to its interaction with pro-uPA and plasminogen, p97 stimulates plasminogen activation by decreasing the K_m of pro-uPA for plasminogen and by increasing the V_{max} of the reaction. The conversion of pro-uPA to Tc-uPA occurs by proteolytic cleavage of a single peptide bond (Lys158-Ile159 in human uPA).[32]

This conversion can be catalyzed by plasmin or several other proteases, such as plasma kallikrein, blood coagulation factor XIIa, cathepsin B, cathepsin L, and prostate-specific antigen.[33] In the present work the SPR assay, the enzymatic assay, and electrophoresis experiments all suggest that p97 induces a conformational change that increases pro-uPA activity without any apparent cleavage of pro-uPA. The 2-state conformational model gave the best fits for the interactions of both pro-uPA and plasminogen with immobilized p97 on the BIAcore. Such good fits of experimental data to a multistate model of interaction are an indication that a conformational change may be taking place. Other approaches, such as limited proteolysis, circular dichroism spectrometry, or magnetic nuclear resonance (MNR) technology, would be helpful in confirming and providing additional information on the interaction between p97 and either pro-uPA or plasminogen. Interestingly, the fragments of plasminogen generated by adding p97 were different from the plasminogen degradation by pro-uPA alone. These biochemical analyses further suggest that p97 could also be seen as a cofactor in uPA-dependent plasminogen activation.

The uPA/uPAR system has been involved in several pathologic and physiologic processes thsat require cell migration, such as tumor cell invasion and metastasis. Several reports have shown that the uPA/uPAR system plays a key role in signal transduction as well as in regulation of melanoma cell migration and angiogenesis.[34-37] A recent study showed that p97 promotes in vivo angiogenesis and HMEC-1 migration when used as a chemoattractant.[38] In our conditions, when p97 is added to both compartments of the Boyden chamber, migration of HMECs-1 is inhibited by more than 50%. Thus, given the important role of plasmin,[39] a protein like p97 that targets the formation of plasmin and acts on the migration of endothelial cells as well as of SK-Mel-28 cells might be expected to affect angiogenesis and cancer progression. We also observed that the basal capacity for plasminogen activation by HMECs-1 decreased following p97

treatment. A recent study demonstrated that the expression of LDL receptor–related protein 1B (LRP1B), a new member of the LDL receptor family, leads to an accumulation of uPAR on the cell surface, which event inhibits the migration of Chinese hamster ovary (CHO) cells.[40,41] From these results, it was proposed that LRP1B negatively regulates uPAR regeneration and function, whereas the net results of uPAR regeneration seems to depend on the relative expression of the 2 receptors. Since p97 transcytosis may involve LRP and p97 interacts with pro-uPA, further experiments are required to determine the effects of p97 treatment on both LRPs.

Recently, it was shown that when Glu-plasminogen is bound to cell surfaces, plasmin generation by plasminogen activators is markedly stimulated compared with the reaction in solution.[42] This is a key element for cell migration, where the process of "grip and go" would play an important role.[39,42] The process of plasminogen activation system is regulated by 2 different mechanisms: (1) cell surface–binding sites that facilitate the productive catalytic interactions with plasminogen and thereby increase plasmin generation, and (2) protein inhibitors such as serpin inhibitors, which restrict the activities of the proteases.[43] In light of this, soluble p97 could participate in the activation of plasminogen without being in the pericellular environment (Figure 18A). Our data also indicate that the migration and the plasminolytic activity of cells expressing p97 are inhibited by mAb L235, suggesting that endogenous, membrane-bound p97 may be involved in these processes, which are associated with cancer and angiogenesis (Figure 18B). Moreover, both the migration of HMECs-1 and the plasminolytic activity are diminished when exogenous p97 is added, suggesting that soluble p97 affects the regulation of plasminogen activation at the cell surface (Figure 18C). Thus, by breaking the equilibrium between soluble p97 and membrane-bound p97, it is possible to affect cell migration of HMEC-1 and SK-Mel-28 cells. Further studies are required to establish the impact of p97 treatment on components of the uPA

system at the cellular membrane in order to elucidate the molecular events by which both L235 and exogenous p97 affect cell migration.

In conclusion, these are the first results indicating that p97 can potentially interact with pro-uPA as well as with plasminogen and regulate the activation of plasminogen by pro-uPA. We are also reporting that migration of HMEC-1 and SK-Mel-28 cells is inhibited by mAb L235 and soluble p97, indicating that active and functional p97 participates in this process. Collectively, our results thus suggest that the balance between membrane-bound and soluble p97 could affect cell migration. Further studies are now under way to establish the molecular events characterizing both the soluble and membrane-bound p97 inhibitory actions and to determine the potential involvement of p97 in angiogenesis.

2.1.8Acknowledgements
We are very thankful to Dr Kennard for providing us with the human recombinant p97 and the different mAbs directed against p97 and to Dr Bu for providing us with RAP. We also thank Julie Poirier for her technical support. Many thanks to Ben Sulsky for his enlightened vision of science.

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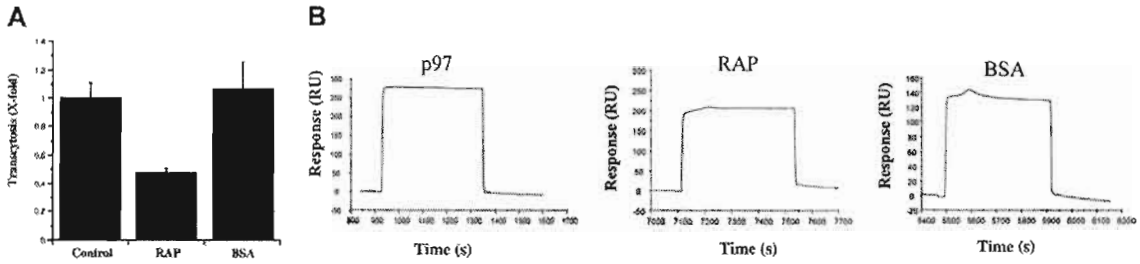

Figure 10 Transcytosis of p97 across BBCEC monolayers.

(A) Transcytosis experiments were performed at 37°C for 2 hours. [^{125}I]-p97 (25 nM) was added to the upper side of the cell-covered filter in the absence or presence of RAP (650 nM) or BSA (5 μM). At the end of the experiment, radiolabeled proteins were measured in the lower chamber of each well by TCA precipitation. Results represent means ± SE (n = 6). (B) p97 was immobilized on a sensor chip surface (CM5) as described in "Materials and methods." p97, RAP, and BSA (5 μg/100 μL) were injected over immobilized p97. One representative experiment is shown (n = 3).

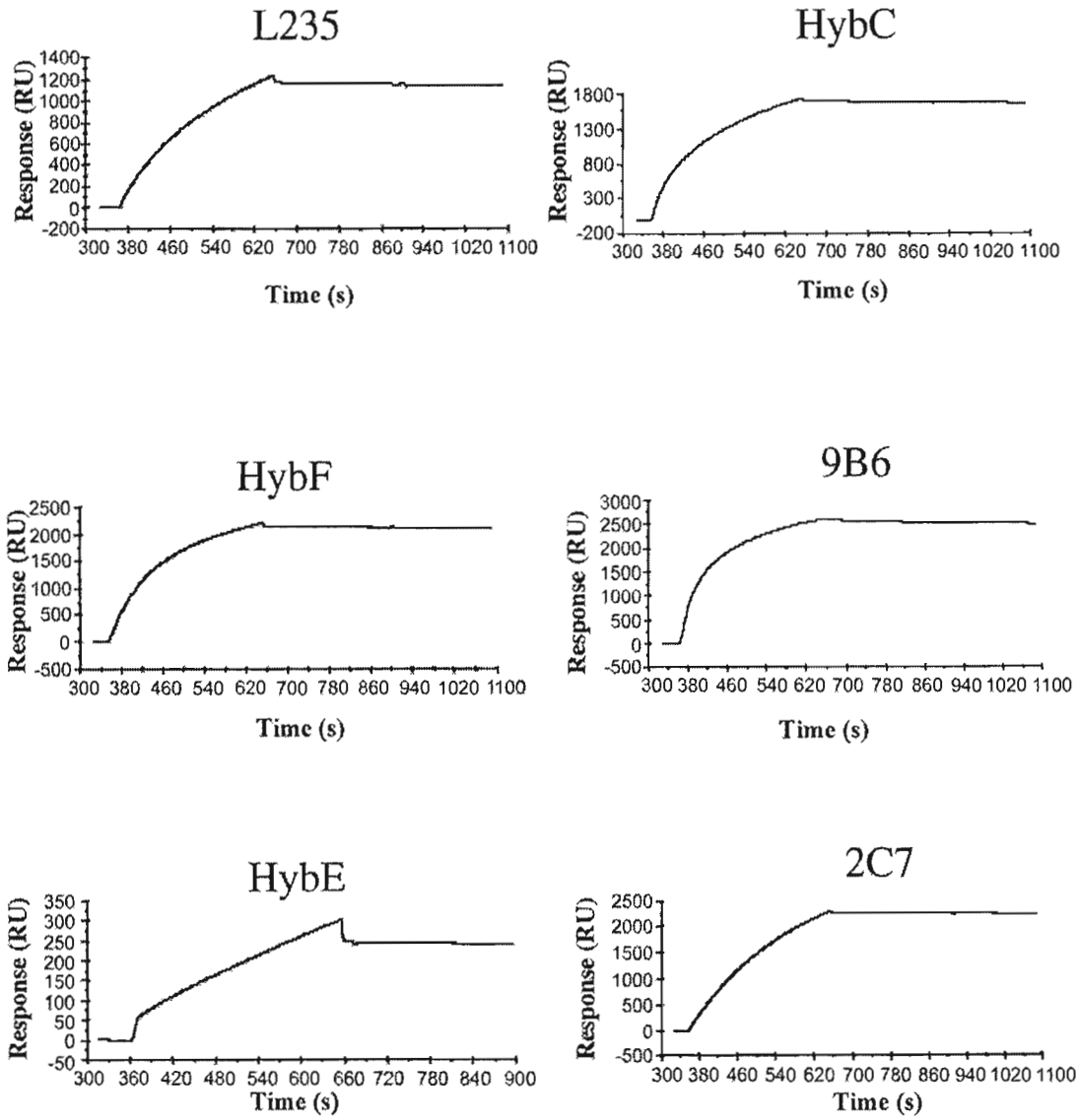

Figure 11 Biospecific interaction analysis in real time between p97 and various anti-p97 mAbs.

P97 was immobilized on a sensor chip (CM5) using standard coupling procedures incorporating NHS, EDC, and ethanolamine. Different mAbs directed against p97 (HybC, HybE, HybF, L235, 2C7, 9B6), diluted to 0.05 µg/µL in Ringer-HEPES, were injected into the BIAcore at a flow rate of 5 µL/min. The surface plasmon resonance response obtained for these mAbs was plotted (in relative units [RU]) as a function of time. After each injection, immobilized p97 was regenerated with 0.2M glycine at pH 2 for 2 minutes followed by a 2-minute injection of ferric ammonium citrate (1 mM) (n = 4).

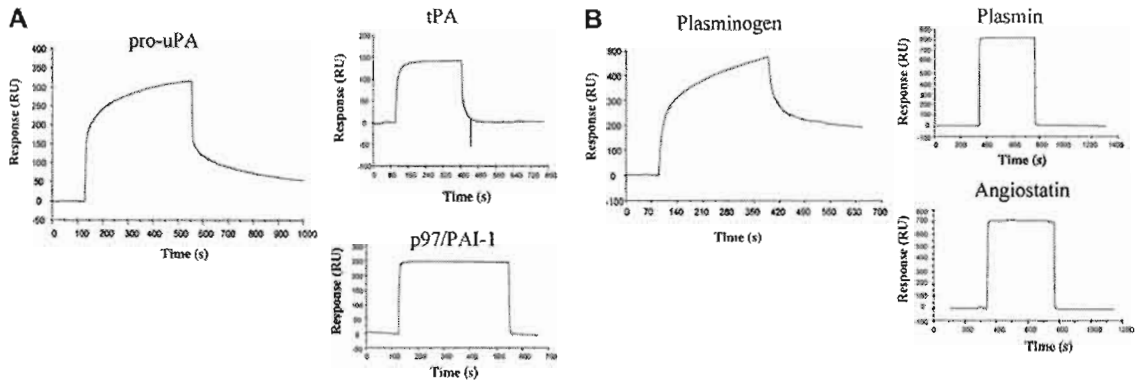

Figure 12 Molecular interactions of p97 and various components of the PA:plasmin system.

(A) Pro-uPA and tPA (0.05 µg/µL), diluted in Ringer-HEPES, were injected onto immobilized p97 on a sensor chip at a flow rate of 5 µL/min. The SPR response for these proteins was plotted in RU as a function of time. p97 (0.05 µg/µL) was also injected over immobilized PAI-1 (p97/PAI-1). (B) Plasminogen, plasmin, or angiostatin (0.05 µg/µL) was injected onto immobilized p97. The SPR response for these proteins was plotted in RU as a function of time. The results indicate that pro-uPA and plasminogen interact with p97. After each injection the sensor chip surface with immobilized p97 was regenerated by injecting 10 mM glycine, pH 2.2, for 2 minutes.

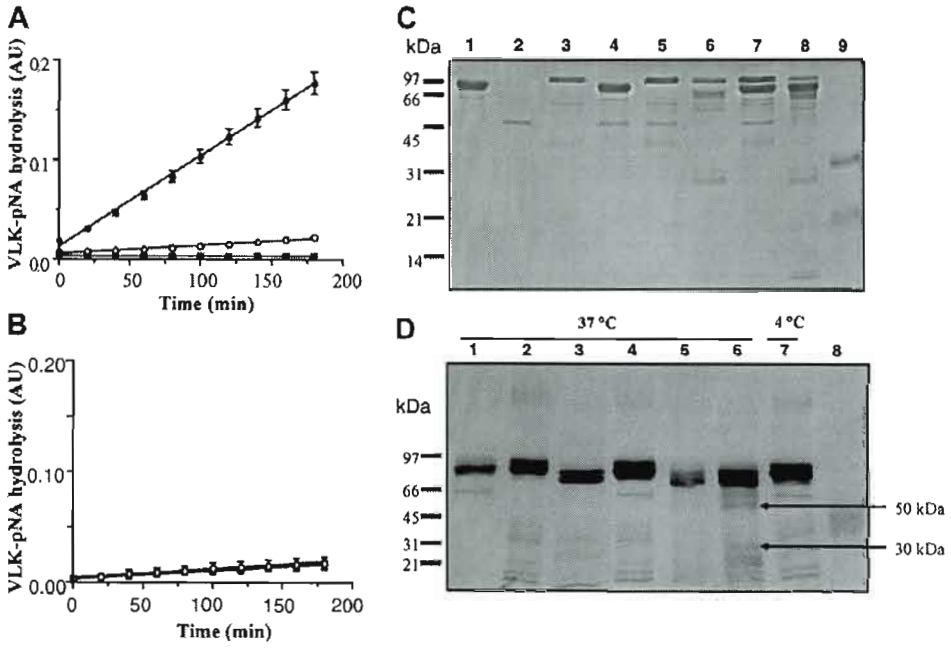

Figure 13 Effect of p97 on pro-uPA, tPA, and plasminogen.

The serine activity of 90 nM pro-uPA (A) and 75 nM tPA (B) were measured in the absence (○) or presence (●) of 70 nM p97 without plasminogen. The reaction was performed in a final volume of 200 µL as described in "Materials and methods." In both panels A and B, controls were also performed with p97 (■) but without pro-uPA or tPA (n = 9 for pro-uPA; n = 6 for tPA). (C) The effect of p97 on pro-uPA was evaluated after incubation in the presence or absence of plasminogen. 2 µg p97 (lane 1), 1 µg pro-uPA (lane 2), and 2 µg plasminogen (lane 3) were incubated for 5 minutes at 37°C alone as controls. Pro-uPA (2 µg) was incubated at 37°C for 5 minutes with 2 µg p97 (lane 4). Plasminogen and pro-uPA were added without incubation (lane 5) and with 5 minutes' incubation at 37°C (lane 6). Pro-uPA with 2 µg of both p97 and plasminogen was added without incubation (lane 7) or with 5 minutes' incubation at 37°C (lane 8). Tc-uPA (2 µg) was also loaded as a control (lane 9). Proteins were then separated by SDS-PAGE, using a 12.5% acrylamide gel under reducing conditions. After electrophoresis, proteins were visualized by staining the gel with Coomassie blue. (D) Effect of p97 on plasminogen degradation by pro-uPA. Quantities (3 µg) of p97 (lane 1), Glu-plasminogen (lane 2), and Lys-plasminogen (lane 3) were incubated alone for 6 hours at 37°C. In lane 4, 3 µg of both glu-plasminogen and p97 was also incubated for 6 hours at 37°C. Pro-uPA (20 ng) was added to plasminogen for the same period of incubation at 37°C (lane 5). p97 was added to pro-uPA and plasminogen for 6 hours at 37°C (lane 6) or 4°C (lane 7). In lane 8, 3 µg angiostatin was also added as a control. Proteins were then separated by SDS-PAGE, using a 7.5% acrylamide gel under nonreducing conditions. After electrophoresis, proteins were visualized by staining the gel with Coomassie blue.

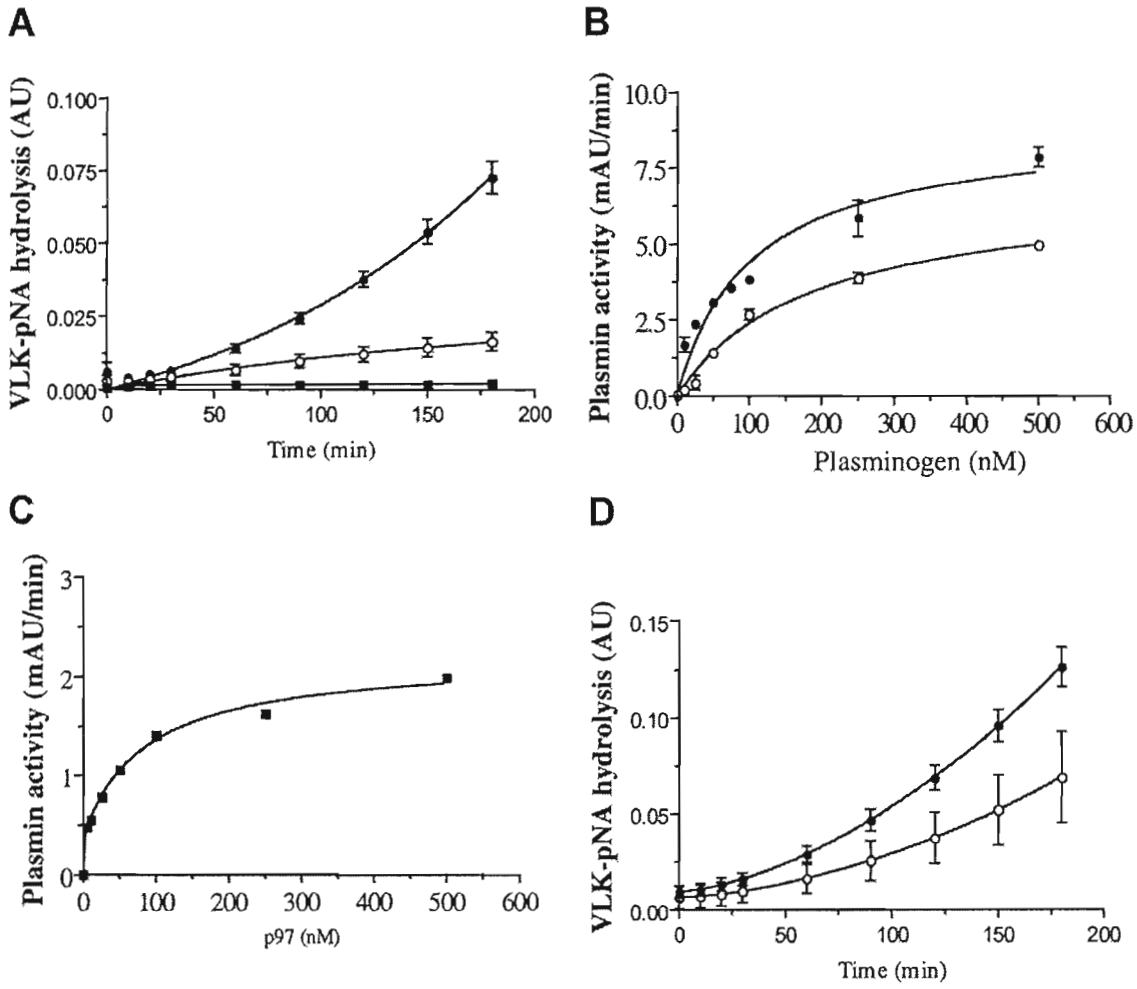

Figure 14 Effect of p97 on plasminolytic activity induced by pro-uPA.

(A) The plasminolytic activity of 1 nM pro-uPA was measured without (○) or with (●) 70 nM p97 in the presence of 30 nM plasminogen. The reaction was performed in a final volume of 200 µL as described in "Materials and methods." As a control, the enzymatic activity in the presence of p97 alone was also measured (■). (B) Plasmin activity induced by pro-uPA was measured in the presence of various p97 concentrations. (C) Plasmin activity induced by pro-uPA was measured without (○) or with (●) 250 nM p97 and various concentrations of plasminogen. (D) Inhibition by the mAb L235 of the increase in plasminolytic activity induced by p97. The plasminolytic activity of pro-uPA was measured in the presence of 70 nM p97 and 65 nM of either mAb L235 (○) or nonspecific mouse IgG (●). One representative experiment is shown and data represent the means ± SDs (n = 3).

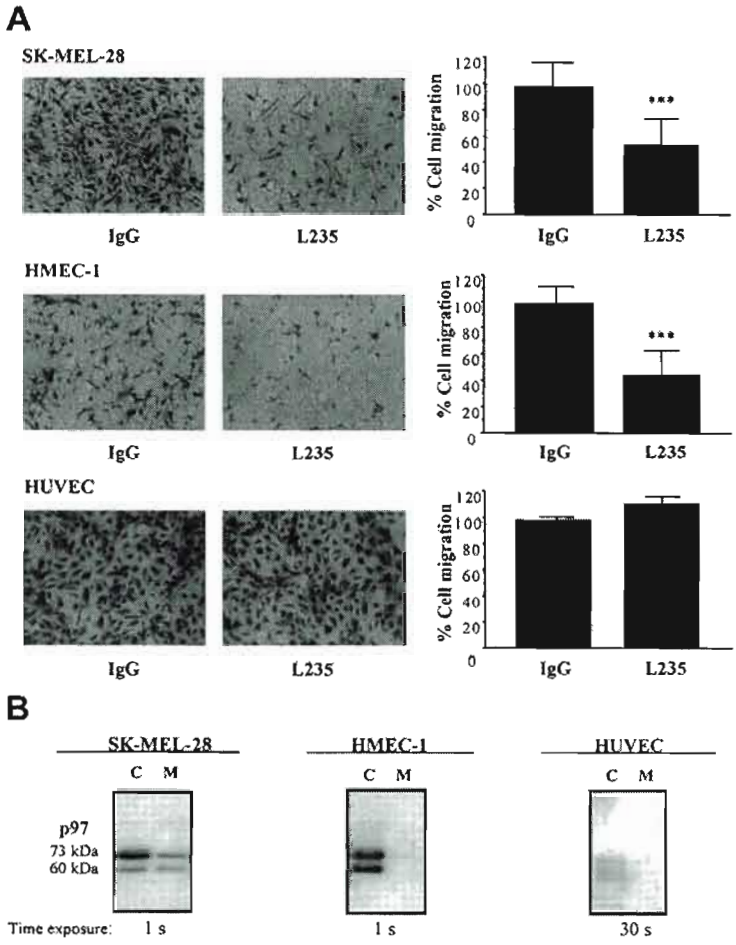

Figure 15 Effect of mAb L235 on cell migration.

(A) Cell migration of HMEC-1, SK-Mel-28, or HUVEC cells was measured using modified Boyden chambers as described in "Materials and methods." Cells that had migrated to the lower surface of the filters were fixed and stained with crystal violet. Images obtained from a representative experiment are shown. Cells that had migrated in the presence of 50 nM mAb L235 or a nonspecific mouse IgG were also counted. The results were expressed as the percentage of the control measured in the presence of a nonspecific mouse IgG and represent means ± SDs (n = 5 for HMEC-1; n = 4 for SK-MEL28; n = 3 for HUVEC). ***Statistically significant differences ($P < .001$; Student t test). (B) Detection of endogenous p97 by Western blot analysis. p97 was immunodetected in lysates or serum-deprived culture media (18 hours) from HMEC-1, SK-MEL28, and HUVEC-1 cells. Proteins were separated by SDS-PAGE and were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. p97 was detected by Western blotting, using mAb L235 and a secondary antimouse IgG linked to peroxidase.

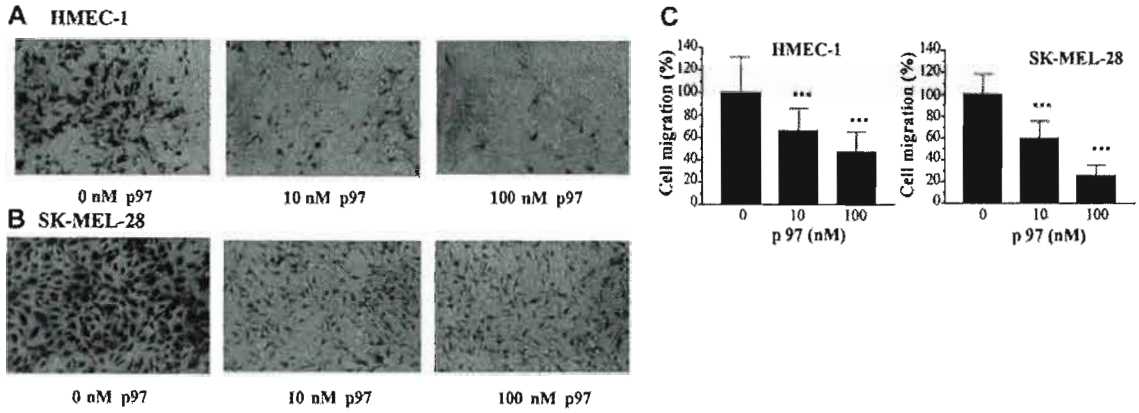

Figure 16 Effect of exogenous p97 on cell migration and plasminolytic activity.

HMEC-1 (A) and SK-Mel-28 (B) cell migration was performed using modified Boyden chambers as described in "Materials and methods." Cells that had migrated in the presence or absence of p97 (100 nM) to the lower surface of the filters were fixed and stained with crystal violet. Photos (orginial magnification, x 100) obtained from a representative experiment are shown. (C) Cells that had migrated were also counted and expressed as a percentage of the control cells, measured in the absence of p97 (n = 4 for HMEC-1; n = 3 for SK-MEL28). Data represent means ± SDs. ***P < .01 (student t test).

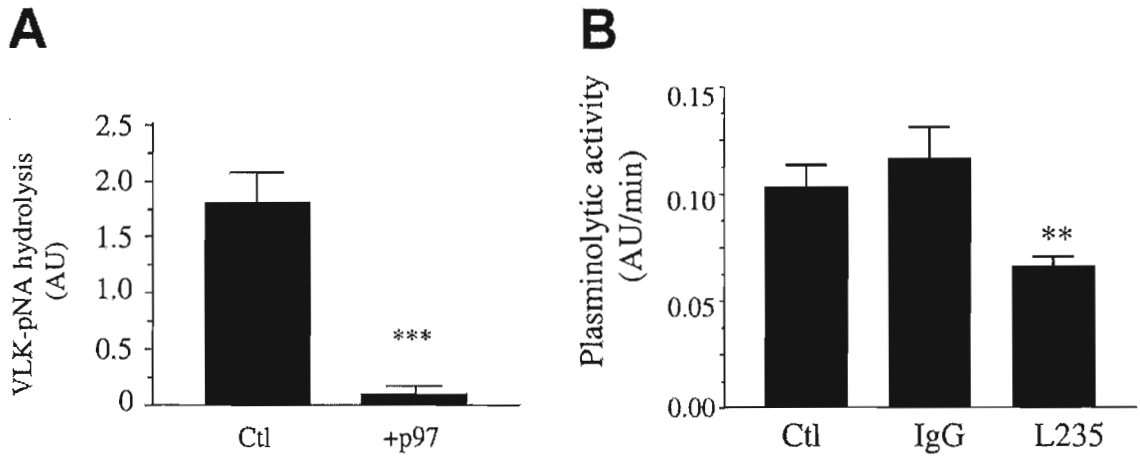

Figure 17 Inhibition of plasminolytic activity at the cell surface of HMECs-1 by soluble p97 and mAb L235.

(A) Effect of p97 on plasminolytic activity. HMECs-1 were treated for 18 hours with 100 nM p97 (+p97) or Ringer solution (Ctl). Following this treatment the plasminolytic activity was measured using standard conditions, as described in "Materials and methods." (B) Effect of mAb L235 on plasminolytic activity of HMECs-1. HMECs-1 (1 x 10^5 cells) were preincubated for 1 hour at 37°C with Ringer solution (Ctl) or with 250 nM mAb L235 or nonspecific mouse IgG. Following this preincubation, the plasminolytic activity was measured for 6 hours by adding pro-uPA (1 nM) and plasminogen (50 nM) using standard conditions, as described in "Materials and methods." The plasminolytic activity of HUVECs was also measured, using 1 x 10^5 cells under the same conditions. Data represent the means ± SDs of 3 independent experiments performed in triplicate. **P < .01, ***P < .001 (Student t test).

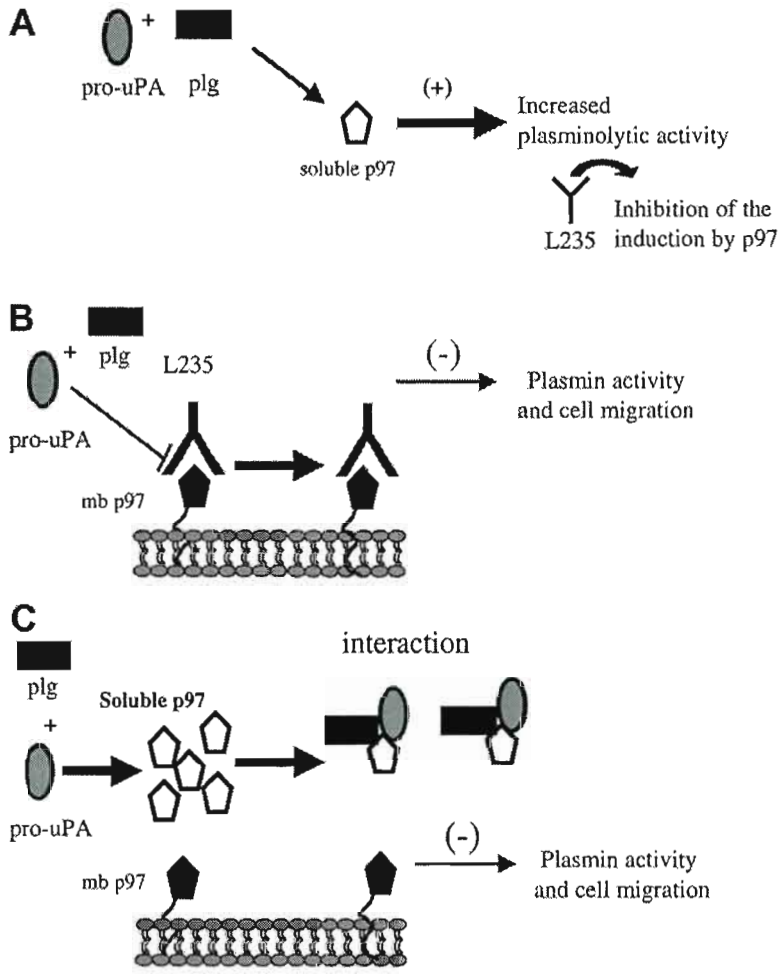

Figure 18 Schematic representation of p97 regulation of plasminogen and cell migration.

This schematic representation summarizes the results obtained for p97 in the present study. (A) The interaction of pro-uPA and plasminogen with soluble activity increases the activation of plasminogen. This induction could be inhibited by the mAb L235, which recognizes a conformational epitope on p97. (B) The addition of mAb L235 reduces the plasminolytic activity on HMEC-1 cell surface and results in an inhibition of cell migration. (C) The interaction of plasminogen and pro-uPA with membrane-bound p97 (mb p97) is diminished when exogenous, competing human recombinant p97 is added. This also caused a decrease in the activation of plasminogen (plg) and leads to an inhibition of cell migration. This representation of the interaction between p97 and pro-uPA indicates that the balance between membrane bound and soluble p97 may be crucial for cell migration.

Table 1 Kinetics of interaction between immobilized p97 and mAbs

Antibody	ΔRU	K_a, M^{-1} s^{-1}	K_d, s^{-1}	$K_A = K_a/K_d$, M^{-1}	$K_D = K_d/K_a$, M
L235	1055 ± 82	4.4×10^4	5.3×10^{-5}	0.9×10^{-9}	0.1×10^{-10}
HybC	1509 ± 184	7.2×10^4	4.5×10^{-5}	1.6×10^{-9}	6.4×10^{-10}
HybE	232 ± 52	0.9×10^4	9.8×10^{-5}	0.08×10^{-9}	0.01×10^{-10}
HybF	2199 ± 150	8.0×10^4	3.0×10^{-5}	2.7×10^{-9}	3.8×10^{-10}
9B6	2440 ± 112	1.2×10^4	9.1×10^{-5}	1.3×10^{-9}	7.9×10^{-10}
2C7	2290 ± 87	5.9×10^4	3.8×10^{-5}	1.6×10^{-9}	6.5×10^{-10}

The difference between the relative units measured after and before injection of mAbs directed against p97 are presented (ΔRU) as well as the apparent association (K_a) and dissociation (K_d) constants. The affinity (K_A) and dissociation (K_D) constants were calculated from the K_a and K_d.

Table 2 Kinetics of interaction between immobilized p97 and pro-uPA or plasminogen using the 2-state conformational model

Immobilized protein	Ligand	k_{a1}, x 10^4 M^{-1} s^{-1}	k_{a2}, x 10^{-3} s^{-1}	k_{d1}, x 10^{-3} s^{-1}	k_{d2}, x 10^{-4} s^{-1}	K_D, x 10^{-9} M
p97	Pro-uPA	0.6	3.2	1.7	7.1	65
p97	Plasminogen	2.1	6.0	43.0	11.2	350

Kinetic parameters were based on a 2-state conformational change binding model using the biosensorgram shown in Figures 1 and 2. This model describes a 1:1 binding of analyte to immobilized ligand followed by a conformational change in the complex. It is assumed that the conformationally changed complex can dissociate only through the reverse of the conformational change: A + B = AB = ABx. The dissociation constants (K_D) were derived using both association (k_a) and dissociation (k_d) rates [$K_D = (k_{d1}/k_{a1})\ (k_{d2}/k_{a2})$]. The parameters are k_{a1}, association rate constant for A + B1 = AB1 (M^{-1} s^{-1}); k_{d1}, dissociation rate constant for AB1 = A + B1 (s^{-1}); k_{a2}, forward rate constant for AB = ABx (s^{-1}); k_{d2}, backward rate constant for AB = ABx (s^{-1}). Mean x^2 values for the sensorgram fits were less than 0.4.

2.22[e] article

2.2.1Résumé :

L'activateur tissulaire du plasminogène (tPA) et son substrat plasminogène (Plg) sont des composantes importantes dans le système fibrinolytique. Nous avons récemment démontré que la forme soluble tronquée de la mélanotransferrine (sMTf) peut stimuler l'activation du Plg par l'activateur urokinase du plasminogène et inhiber l'angiogenèse. Puisqu'une variété d'inhibiteurs d'angiogenèse ont été démontrés pour stimuler l'activation du Plg par le tPA, nous avons examiné l'effet de la sMTf sur la fibrinolyse médiée par le tPA. Cette étude montre que la sMTf augmente de 6 fois l'activation du Plg par le tPA. La sMTf aussi augmente le relâchement des fragments de [^{125}I]-fibrine par la plasmine activée par le tPA. De plus, nous avons observé que l'interaction de la sMTf avec le Plg provoque une modification de la structure du caillot de fibrine en clivant la chaîne α et β. Ces résultats suggèrent que les propriétés de la MTf peuvent inclure l'augmentation de la dissolution de la matrice provisoire extracellulaire.

2.2.2Titre: Stimulation of tPA-dependent provisional extracellular fibrin matrix degradation by human recombinant soluble melanotransferrin

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Ma participation est entière pour cet article, le texte, les figures. Dr Demeule a participé au contenant par les corrections du texte et la reformulation. Le Dr Rivard nous a permis d'utiliser son tromboelastogramme pour produire une figure et un tableau.

2.2.3Abstract

Tissue-type plasminogen activator (tPA) and its substrate plasminogen (Plg) are key components in the fibrinolytic system. We have recently demonstrated, that truncated human recombinant soluble melanotransferrin (sMTf) could stimulate the activation of Plg by urokinase plasminogen activator and inhibit angiogenesis. Since various angiogenesis inhibitors were shown to stimulate tPA-mediated plasminogen activation, we examined the effects of sMTf on tPA-dependent fibrinolysis. This study demonstrated that sMTf enhanced tPA-activation of Plg by 6-fold. sMTf also increased the release of [^{125}I]-fibrin fragments by tPA-activated plasmin. Moreover, we observed that the interaction of sMTf with Plg provoked a change in the fibrin clot structure by cleaving the fibrin α and β chains. Overall, the present study shows that sMTf modulates tPA-dependent fibrinolysis by modifying the clot structure. These results also suggest that sMTf properties could involve enhanced dissolution of the provisional extracellular fibrin matrix.

Keywords: Fibrin matrix; Melanotransferrin; Plasminogen

2.2.3Article Outline

2.2.4Introduction

Plasmin is a serine protease that dissolves fibrin clots. Tissue-type plasminogen activator (tPA) plays a key role in fibrinolysis because tPA converts inactive plasminogen (Plg) into enzymatically active plasmin [1]. Melanotransferrin (MTf) is a 97 kDa glycoprotein that shares substantial sequence similarity with human serum transferrin, human lactoferrin, and chicken transferrin [2]. MTf was first identified, in the early 1980s, on the surface of melanoma cells and this glycoprotein is used as a marker for melanoma cells [3]. More recently, it was reported that MTf mRNA is present in many normal human tissues [4]. MTf exists

as both membrane-bound and soluble forms, depending on whether or not this glycoprotein possesses a glycosylphosphatidylinositol (GPI)-anchor that has been attached to the glycoprotein [5].

Because MTf possesses iron-binding properties, it was first proposed that MTf might be involved in iron transport [5]. However, MTf has been shown to play a minor role in the uptake of iron [6]. Recent studies have suggested that MTf could be involved in pathological and physiological processes, including Alzheimer's disease [7], chondrogenic differentiation [8] and transcytosis across the blood–brain barrier [9]. We have previously shown that membrane-bound MTf could bind and stimulate Plg activation at the cell surface [10]. In addition, we also reported that truncated human recombinant soluble melanotransferrin (sMTf) could catalyze the urokinase type activator uPA-mediated activation of plasmin and affect cell migration [11]. We also found that sMTf inhibited endothelial cell movement and tubulogenesis which are important events in angiogenesis [12].

The formation of a provisional extracellular fibrin matrix (PEFM) is an important step in cell migration. This occurs after vascular injury, during inflammation, and in tumors. These phenomenons induce the expression of tissue factor on the endothelial cells [13]. Tissue factor, which is not only present on stimulated endothelial cells but in the subendothelial matrix and on many tumor cells, triggers the formation of PEFM [14]. Fibrin and the other components of this extracellular matrix are involved in the regulation of cell proliferation and migration through interactions with adhesion molecules on cell surfaces [15].

Since the conversion of Plg to plasmin by tissue-type plasminogen activator (tPA) plays a role in fibrinolysis, we investigated whether sMTf could affect fibrinolysis of PEFM. The generation of plasmin as well as the release of [^{125}I]-fibrin fragments by tPA increased in the presence of sMTf. In addition, sMTf enhanced the tPA-dependent fibrinolysis of both fibrin clots and platelet-rich plasma (PRP)

clots by tPA. Overall, the different experimental approaches indicated that sMTf stimulates Plg activation by tPA leading to an increase in the fibrinolysis of PEFM.

2.2.5Materials and methods

2.2.5.1Materials

Truncated human recombinant MTf (sMTf), which is produced by introducing a stop codon following the glycine residue at position 711 (27 C-terminal amino acids deletion), and L235 monoclonal antibody (mAb) were kindly provided by Biomarin Pharmaceutical (Novato, CA). Fibrinogen, thrombin and tPA were from Calbiochem (La Jolla, CA). The antibody directed against MTf (L235) was from American Type Culture Collection (Manassas, VA). Blood tubes were 3.2% citrate-treated Vacutainers® from Becton Dickinson (Franklin Lakes, NJ). Human factor XIII Fibrogammin® P (FXIII) was from Aventis (Marburg, Germany). Plg was from Technoclone (Vienna, Austria). Other biochemical reagents were from Sigma (Oakville, ON).

2.2.5.2Plasmin activity assay

Human glu-Plg (50 nM) (Technoclone, Vienna, Austria) activation by human tPA (50 pM) with or without sMTf (500 nM) (Calbiochem, La Jolla, CA) was measured using a colorimetric assay as previously described [16]. Briefly, the reaction was performed in a final volume of 200 µl in a suitable medium (buffer A, consisting of 50 mM Tris/HCl pH 7.4, 150 mM NaCl, and 50 mM $CaCl_2$). The reaction was started by the addition of tPA. The plasmin produced cleaved the plasmin substrate (Val-Leu-Lys-*p*-nitroanilide) (Sigma ,Oakville, ON). Absorbance was monitored at 405 nm using a Microplate Thermomax Autoreader (Molecular Devices, Sunnyvale, CA). The L235 mAb (2 µM) (American Type Culture Collection, Manassas, VA) was used to inhibit sMTf activity. Concentration

variation of sMTf (0–10 μM) determined the apparent K_m to enhance tPa activation of Plg. K_m was evaluated using the Prism software (GraphPad Software Inc, San Diego, CA).

2.2.5.3BIAcore analysis

Glu-Plg (3 μg) was covalently coupled to a sensor chip (CM5) via primary amine groups using the coupling reagents (*N*-hydroxysuccinimide (NHS)/*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC)) as previously described [17]. sMTf was produced following an established protocol [18]. Proteins were injected onto the biological sensor chip surface. The surface plasmon resonance (SPR) generated by the protein–protein interaction was monitored in real-time and analyzed with BIAevaluation software (BIAcore, Piscataway, NJ) to determine the kinetic parameters of interaction.

2.2.5.4Platelet-rich plasma (PRP) preparation

Human blood samples were collected by a two syringe technique, during which the first ml was discarded, of blood into 3.2% citrate-treated Vacutainers® (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 300×*g* for 5 min at room temperature. Participating subjects had given informed consent in accordance with the Declaration of Helsinki.

2.2.5.5Fibrin plate assay

To examine the effects of sMTf on fibrinolysis, we used a [^{125}I]-fibrin plate assay as previously described [19]. Briefly, in a 24-well microplate, 20 μl of human, labeled [^{125}I]-fibrinogen (Amersham Biosciences, Bucks, UK) (6 nCi/assay) at 3 mg/ml were mixed with Plg (2 μM) and introduced into the wells. Clotting was achieved by the addition of human thrombin (Sigma, Oakville, ON) (0.4 U/ml) with or without human factor XIII Fibrogammin® P (FXIII) (Aventis, Marburg,

Germany) for 60 min at 37 °C. Clots were carefully washed three times with the buffer A. Next, the buffer with tPA were carefully layered onto the surface of the clot and treated with sMTf (500 μM). The release of [^{125}I]-fibrin fragments into the supernatant (100 μl) during a 15 min incubation at 37 °C was measured by a LKB Wallac 1282 Compugamma counter (LKB Instruments, Inc, Gaithersberg, MD).

2.2.5.6PRP fibrinolysis assay

To examine the influence of sMTf on fibrinolysis *ex vivo*, we used a [^{125}I]-fibrinogen labeled PRP clot assay. Labeled [^{125}I]-fibrinogen (6 nCi/assay) was mixed with 20 μl of PRP. Clotting was achieved by the addition of $CaCl_2$ (20 mM final) for 60 min at 37 °C. Next, 100 μl of buffer A containing tPA and various concentrations of sMTf was layered onto the surface of the clot. The release of [^{125}I]-fibrin fragments into the supernatant during a 15 min incubation at 37 °C was measured.

2.2.5.7Radial clot lysis assay

To visualize the enhanced fibrinolysis due to sMTf , radial clot lysis was performed as previously described by Mosesson [16], with minor modifications. Briefly, fibrin clots were obtained by incubating fibrinogen (8.2 μM), Glu-Plg (2 μM) and 0.4 U/ml thrombin in buffer A at 37 °C for 60 min in a 6-well plate. Clot lysis was initiated by adding 2 μl of tPA (1 nM) with or without sMTf (100 nM). Clots were incubated for 30 min at 37 °C and dyed with Chinese ink. Photomicrographs at 40× magnification were taken using a Nikon Coolpix 5000 digital camera attached to a Nikon TMS-F microscope (Nikon Canada, Mississauga, ON).

2.2.5.8Cleavage of fibrin clot by sMTf

To visualize the influence of hr-sMTf on the fibrin clot, we incubated fibrinogen, Plg and thrombin at 37 °C for one h. After the clot had polymerized, the clot was treated with hr-sMTf for 8 h at 37 °C. The clot was then dissolved under reducing conditions [20]. Electrophoresis was carried out on a 9% acrylamide gel at 100 V for 2 h. The gel was dyed afterwards with Coomassie blue. Protein sequencing was performed by NRC Protein and Peptide Sequencing (Montreal, QC).

2.2.5.9Thromboelastography analysis

Thromboelastography analysis was performed with PRP or with an artificial clot model using a computerized dual-channel thromboelastograph (TEG) analyzer (model 5000; Haemoscope Corp., Niles, IL). For the artificial clot model, fibrinogen (8.2 µM) (Sigma, Oakville, ON), Glu-Plg (3.3 µM) and tPA (1 nM) were diluted in buffer A and transferred into the analyzer cups. Artificial clots were polymerized with thrombin (Sigma, Oakville, ON) (0.4 U/ml). For the PRP clots, 350 µl of PRP were transferred into the analyzer cups with tPA (1 nM). $CaCl_2$ (0.2 M) was added to initiate the coagulation of PRP. Thromboelastography analyses for both artificial clot models and PRP clots were performed in both the presence and absence of 1 µM sMTf at 37 °C.

2.2.5.10Data analysis

Statistical analyses were performed using Student's t-test via GraphPad Prism software (San Diego, CA). Significant difference was assumed for *P* values less than 0.05. The measurement of variation is reported as the mean ± standard deviation, measured in triplicate.

2.2.6Results

2.2.6.1Plasmin activity assay

In order to characterize the effect of sMTf on tPA activation of Plg, the plasmin activity assay was evaluated by the hydrolysis of VLK *p*-nitroanilide, a colorimetric substrate of plasmin (Fig. 19). After 3 h of incubation in the presence of Plg, sMTf increased the tPA-mediated generation of plasmin by 6-fold (●), compared to tPA alone (○). sMTf with Plg had no proteolytic or plasmin-like activity itself (■) (Fig. 19a). The specificity of the effect of sMTf was then determined by measuring the induction of tPA-dependent Plg activation by sMTf in the presence of either mAb L235 (○), an antibody directed against sMTf, or a non-specific IgG used as a control (●). The L235 mAb (2 μM) inhibited the effect of sMTf on tPA-induced plasmin generation by 80% (Fig. 19b). To determine the optimal concentration of sMTf needed to enhance the activation of Plg by tPA, the half-maximal concentration was determined. sMTf stimulated the initial rate of tPA-dependent conversion of Plg into plasmin in a dose-dependent manner with a half-maximal stimulation occurring at 53 ± 22 nM (Fig. 19c). The effect of sMTf on plasmin formation by tPA was further evaluated in the presence of various concentrations of Plg. Initial rates of plasmin activity indicated that sMTf decreased the apparent K_m of tPA for Plg from 280 nM (○) to 52 nM (●) (Fig. 19d).

2.2.6.2BIAcore analysis

The ability of sMTf to interact with Plg was monitored in real-time using a BIAcore apparatus. Standard NHS/EDC coupling procedures [17] permitted immobilization of Plg on the BIAcore sensor chip surface. Injections of sMTf over immobilized Plg generated SPR, which increased in an sMTf concentration-dependent fashion (Fig. 20). Using this approach, the apparent equilibrium

dissociation constant K_D, calculated with the Biaevaluation software, was 260 nM. The best fit used was the two state reaction (conformation change).

2.2.6.3Fibrin plate assay

We next measured the release of [^{125}I]-fibrin fragments from labeled clots after adding with Plg at a physiological concentration to determine the effect of sMTf on fibrinolysis (Fig. 21). sMTf increased the release of [^{125}I]-fibrin fragments by 2.5 fold following Plg activation by tPA (Fig. 21a). The release of [^{125}I]-fibrin fragments was next measured from clots produced in the presence of factor XIII (Fig. 21b) and showed that a higher concentration of tPA was needed to release the same amount of [^{125}I]-fibrin fragments as was released in the absence of factor XIII. sMTf increased the rate of [^{125}I]-fibrin fragment release from PRP clots in the presence of tPA (Fig. 22). In this assay system, the apparent concentration of sMTf needed to support a 50% maximal [^{125}I]-fibrin fragment released by tPA is 96 nM.

2.2.6.4Radial clot lysis assay

To further demonstrate the effect of sMTf on tPA-induced clot lysis, a radial clot lysis assay was used. (Fig.23). The addition of sMTf to tPA enhanced fibrinolysis, leading to increased dissolution of the fibrin clot (Fig. 23a). In the absence of tPA, the addition of sMTf to Plg modified the fibrin clot structure, whereas sMTf in the absence of Plg did not seem to affect it. (Fig. 23b).

2.2.6.5Evaluation of fibrin fragmentation

The fibrin fragmentation pattern was next analyzed in the absence of tPA to further investigate the effects of sMTf on the fibrin clot structure. The fragmentation pattern was obtained by measuring the migration of treated fibrin clots on acrylamide gels by electrophoresis under reducing conditions (Fig. 24).

Electrophoresis performed in the presence of the reducing agent β-mercaptoethanol allowed visualization of the 2 chains of fibrin (α, β). The addition of either sMTf or Plg did not affect the electrophoresis separation profiles. However, the incubation of fibrin with both sMTf and Plg strongly altered the separation profile. Both chains (α and β) of fibrin almost completely disappeared, leaving a fragment of 37 kDa. Peptide sequencing of the 37 kDa fragment N-terminal region produced the sequence DVENV, which represents the region between Asp^{165} and Val^{169} of the fibrin β-chain.

2.2.6.6Evaluation of clot viscoelastic properties

Since MTf potentiates Plg activation by tPA, the impact of MTf on clot formation and lysis was therefore evaluated using TEG analysis (Fig. 25). An artificial fibrin-clot model formed by the action of thrombin on fibrinogen was first used (Fig. 25a). This model allowed examination of the effect of MTf on tPA-fibrinolysis in the absence of inhibitors. Monitoring of the TEG parameters indicated that addition of MTf increased the thrombolytic activity of tPA (Table 3). In particular, when MTf (500 nM) was added to tPA, clot lysis after 30 min (LY30) was 5 times higher than that observed in the absence of MTf while the complete lysis time (CLT) diminished by 30%. The impact of MTf on fibrin-clot dissolution was further evaluated using PRP (Fig. 25b). The TEG parameters obtained for these experiments (Table 3) indicated that the addition of MTf to tPA caused a 30% decrease in the maximum clot strength (MA), doubled the LY30 rate and reduced the CLT by 20%.

2.2.7Discussion

Previous studies showed that various angiogenesis inhibitors stimulate tPA-mediated plasminogen activation [21], [22] and [23]. The plasminogen activation system, which leads to the formation of the serine protease plasmin and to subsequent fibrinolysis, has been shown to play an important role in the

breakdown of the PEFM, one of the hallmark of angiogenesis [24]. We report here that sMTf enhances the formation of plasmin by tPA by interacting with Plg. In haemostasis, tPA is predominantly secreted by endothelial cells and cleaves circulating Plg into plasmin which is the enzyme responsible for proteolytic degradation of fibrin [19]. The apparent K_m for Plg cleavage by tPA was similar to that measured in a previous study [22]. The action of sMTf on Plg reduced the K_m for tPA by 5 fold. Inhibition by the L235 mAb of the ability of sMTf to induce the activation of Plg by tPA suggests that its interaction with Plg is specific and might involve the conformational epitope recognized by this mAb [25]. The ability of the anti-MTf antibody to block this inductive effect confirms that the biologically active factor was sMTf itself rather than a contaminating factor. The interaction of sMTf with immobilized Plg confirmed results from a previous study, where we showed that Plg could interact with immobilized sMTf [11]. The K_D values were similar in both studies. Several reports have established that protein–protein interactions can positively modulate the activity of an enzyme [26]. Thus, sMTf can be viewed as a positive modulator of tPA-dependent Plg activation and dose response studies have revealed that sMTf is active in the nM range. sMTf modulates the tPA-induced release of [^{125}I]-fibrin fragments from artificial clots and from PRP clots. Although the introduction of factor XIII augments resistance to tPA-induced fibrinolysis [27], the addition of sMTf still increased the release of [^{125}I]-fibrin fragments. The clot assay provides another line of evidence that the activation of Plg by tPA is increased by sMTf and leads to enhanced dissolution of the PEFM. Fibrin is not strictly required for tumor angiogenesis: however, the absence of fibrin in a knock-out model strongly diminishes the aggressiveness of the tumor [28].

Previous studies have established a clear link between modification of the fibrin clot structure, allowing better penetration of tPA, and acceleration of tPA-dependent clot dissolution [29]. These observations are supported by the

degradation pattern of fibrin in the presence of Plg and sMTf, which demonstrated a cleavage of the fibrin β chain and the degradation of the α chain in the absence of tPA. γ chain under these conditions forms dimers (≅100 kDa) that migrate in the same region of sMTf and Plg [30]. The cleavage of the β chain in the presence of sMTf would thus increase the accessibility of Plg for tPA. Since a higher rate of clot lysis is observed with the increased access of Plg [31], increased accessibility of Plg by sMTf could greatly facilitate the action of tPA. Moreover, the lysis of composite fibrin fibres initiated by either plasmin or tPA proceeds preferentially by lateral section of fibers, rather than by uniform thinning of the whole fibre [32]. This change in fibrin architecture is also visible by the diminished strength and accelerated fibrinolysis of the clot. The cleavage of the α and β chains would fragment the lateral section released and accelerate the degradation of the fibrin. This phenomenon could prevent the exposition of binding sites for anchor receptors of endothelium cells or tumor cells in the PEFM [33].

In conclusion, these first results clearly demonstrated that sMTf catalyzes the activation of Plg by tPA, leading to enhanced tPA-mediated fibrinolysis. Thus, sMTf affects the activation of Plg by both activators: urokinase [10], [11] and [12] and tPA. Our data demonstrate that sMTf, in combination with Plg in the PEFM, modifies the fibrin clot structure. These are the first results suggesting that administration of sMTf could impair the PEFM needed both by the endothelium to produce new capillary networks and by tumor cells to migrate. Further studies are underway to elucidate whether endogenous MTf could be related to fibrinolysis-associated events like vascular disease.

2.2.8Acknowledgements

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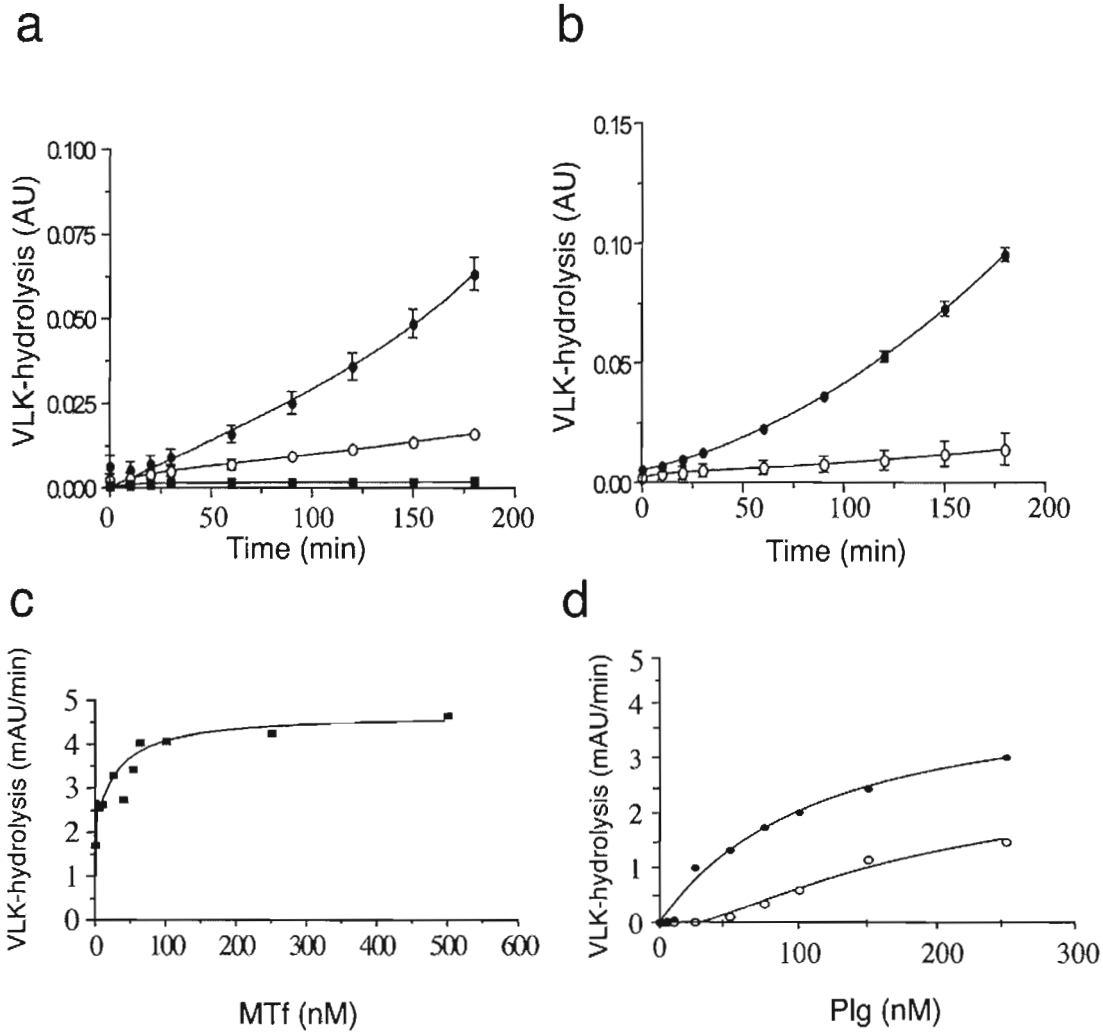

Figure 19 Effects of sMTf on tPA-dependent plasmin activity.

(a) The plasmin activity induced by tPA was measured without (○) or with sMTf (●) in the presence of Plg. The reaction was performed as described in Materials and methods. The plasminolytic activity in the presence of sMTf (■) alone was also measured. Data shown are based upon 3 independent experiments. *P< 0.05 vs. tPA. (b) The plasmin activity induced by tPA was measured in the presence of sMTf with either the mAb L235 (○) or a non-specific mouse IgG (●). (c) Plasmin activity induced by tPA was determined by measuring VLK-hydrolysis in the presence of various sMTf concentrations. (d) Initial rates of VLK-hydrolysis during Plg activation by tPA were measured without (○) or with (●) sMTf in the presence of various concentrations of Plg. Data shown are means ± SD of 3 different experiments.

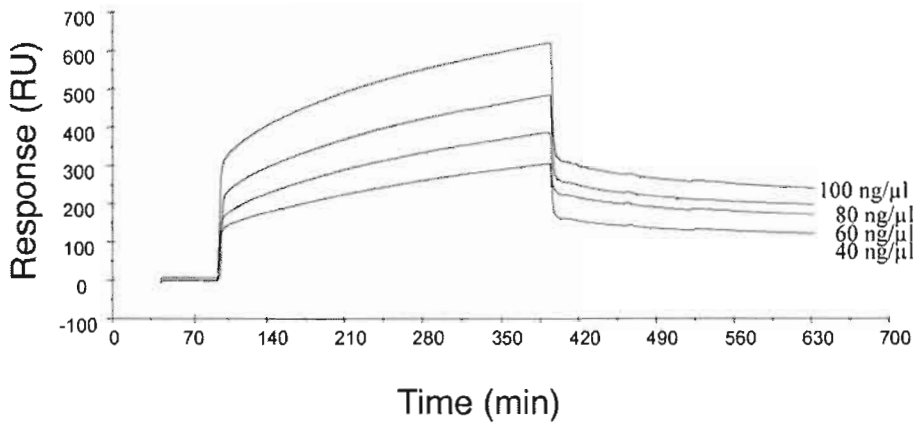

Figure 20 Biospecific interaction analysis in real-time between sMTf and Plg.

sMTf diluted in Buffer A was injected onto immobilized Plg on a sensor chip as in Materials and methods. The SPR response for these proteins was plotted in response units (RU) as a function of time.

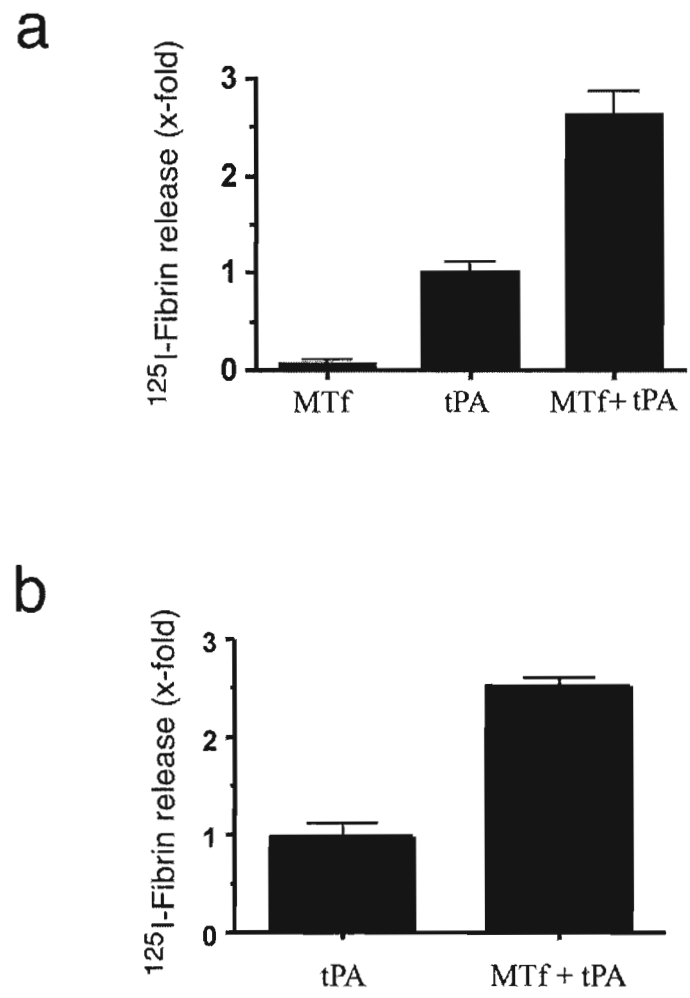

Figure 21 Impact of sMTf on tPA-dependent fibrinolysis.

(a) Release of [^{125}I]-fibrin fragments was measured in the presence of sMTf, tPA (50 pM) or both sMTf and tPA, and was quantified using a gamma scintillation counter. Relative release was established as 1-fold for tPA. Data shown (means and SD) are from 8 experiments. **P < 0.002 vs. baseline. ***P < 0.001 vs. tPA (b) Fibrin release was compared between clots which had been crosslinked with FXIII (1U/ml) using two different conditions, tPA (1 nM) and tPA + sMTf (500 nM). ***P < 0.001 vs. tPA. Data shown (means and SD) are from 8 experiments.

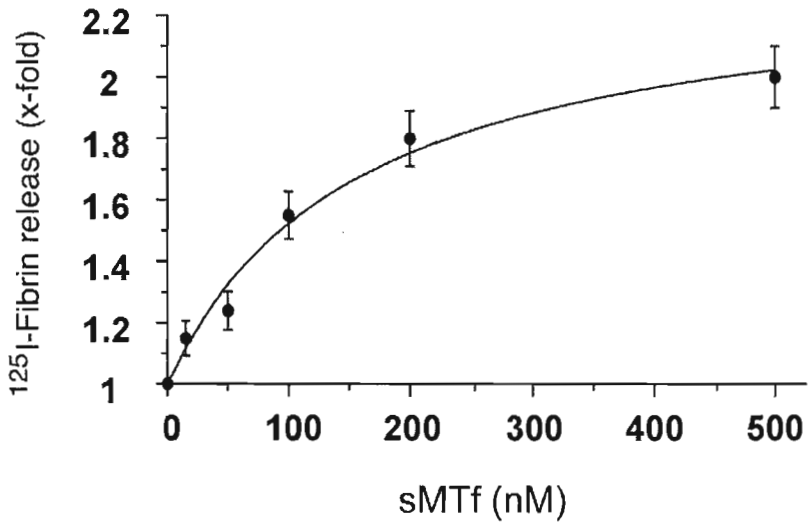

Figure 22 Modulation of tPA-dependent fibrinolysis of PRP clot by sMTf.

Release of [^{125}I]-fibrin fragments was measured in PRP containing tPA with varying concentrations of sMTf. The data shown are from 3 independent experiments. Values are means ± SD. *P < 0.05 vs. tPA.

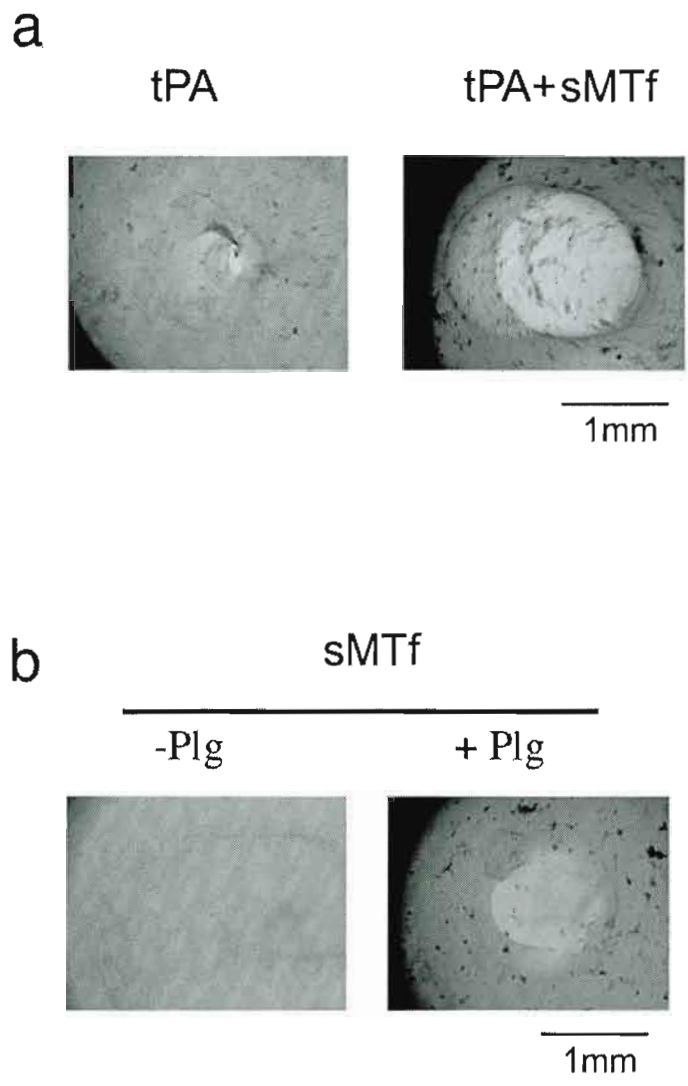

Figure 23 Effect of sMTf on clot fibrinolysis with tPA.

(a) Human fibrinogen was clotted with thrombin in the presence of Plg in a 6 well plate. In each well, a 2 µl volume of either tPA, tPA + sMTf or sMTf alone was laid down onto the fibrin clot. Clots were dyed with Chinese ink and digitally measured. Experiments shown are representative of multiple independent experiments. (b) The fibrin clot was treated with sMTf in the presence or absence of Plg. Photographs were taken at 100× magnification.

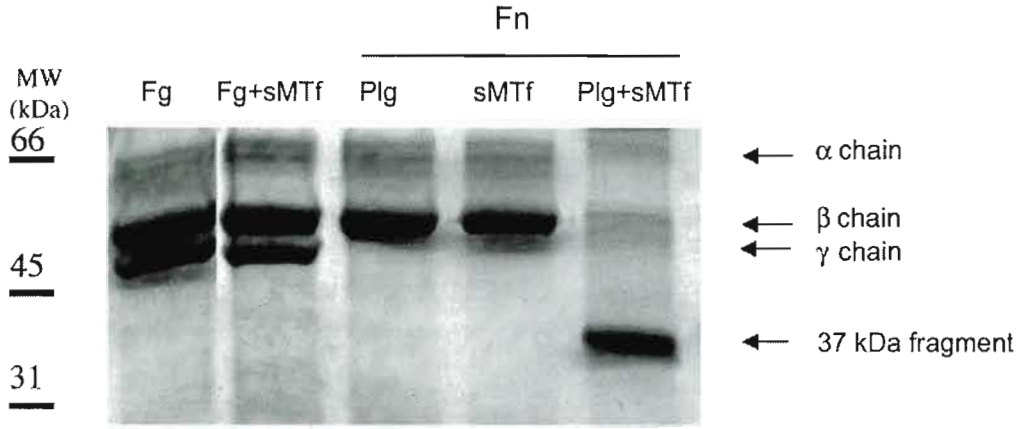

Figure 24 Degradation of human fibrin (Fn).

Human fibrinogen (Fg), Plg and thrombin were incubated at 37 °C for one h. After the clot had polymerized, the clot was treated with sMTf for 8 h at 37 °C. The clot was then dissolved under reducing conditions [20]. Electrophoresis was carried out on a 9% acrylamide gel at 100 V for 2 h. The gel was dyed afterwards with Coomassie blue. Protein sequencing was performed to determine the site of cleavage.

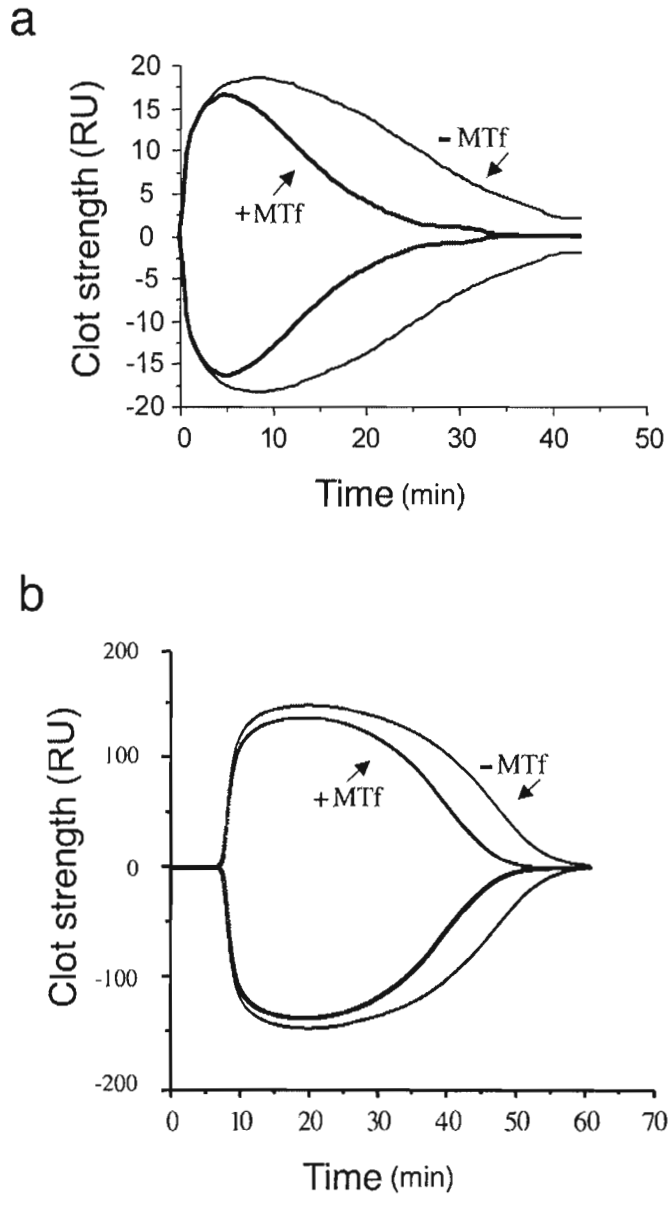

Figure 25 Effect of MTf on clot strength and fibrinolysis.

Representative tracing showing effects of MTf (500 nM) on clot strength in relative units (RU) during the fibrinolysis of clot formation under shear by TEG. (a) Thromboelastogram of the fibrin clot model. (b) Thromboelastogram of PRP clotted after addition of $CaCl_2$ (2 mM). The results shown here are representative of 3 different experiments.

Table 3 Effect of MTf on thromboelastograph parameters

Parameters	**Conditions**	
	tPA	**tPA + MTf**
(a) Artificial fibrin-clot		
(1) MA	498 ± 7	446 ± 17
(2) LY30 %	25.0 ± 8.2	72.5 ± 20.4
(3) CLT min	54.3 ± 10.0	37.8 ± 3.8
(b) Fibrin-clot with PRP		
(1) MA	13465 ± 1586	9560 ± 1626
(2) LY30 %	4.3 ± 0.7	11.8 ± 4.0
(3) CLT min	68.3 ± 1.6	49.1 ± 6.3

The three parameters obtained with the thromboelastograph are presented as follows: 1. MA is the maximum strength of the clot at maximum amplitude of the TEG trace., 2. The percentage of lysis at 30 min (LY 30). 3. The complete clot lysis time in minutes (CLT).

2.3 3e article

2.3.1Résumé :

L'expression de la mélanotransferrine (MTf), une glycoprotéine exprimée dans les mélanomes, est corrélée avec la progression et la vasculatisation des tumeurs suggérant une fonction pro-invasive associée aux tumeurs malignes. Pour tester cette hypothèse, nous avons fait produire un si-RNA dirigé contre l'expression de la MTf pour examiner l'activité plasminolytique et le pouvoir invasif des cellules de mélanomes humains (SK-Mel-28). In vitro, l'activité plasminolytique est inhibée par 58 % dans les cellules silencieuses en MTf. De plus, la diminution d'expression de la MTf réduit la migration cellulaire. *In vivo*, nous avons utilisé un modèle de souris immunodéficientes dans lequel l'injection du facteur tissulaire (TF) induit le dépôt de caillots de [^{125}I]-fibrine. Dans ce modèle, le TF induit le dépôt de [^{125}I]-fibrine et l'invasion des cellules [^{3}H-thymidine]-SK-Mel-28 dans le poumon lorsque ces cellules sont injectées dans la circulation. En utilisant ce modèle métastatique, le potentiel invasif au poumon est réduit de 5 fois lorsque l'expression de MTf est inhibée par un si-RNA. Cette étude suggère que la surexpression de la MTf dans les mélanomes contribue à l'agressivité de la tumeur en stimulant la génération de plasmine qui augmente la migration et l'invasion cellulaires.

2.3.2Titre: Melanotransferrin induces human melanoma SK-Mel-28 cell invasion *in vivo*

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Gestion Valeo s.e.c. to R.B.. R.B. is holder of a Research Chair in Cancer Prevention from Université du Québec à Montréal (UQÀM). J.M.L. is a recipient of a Ph.D. scolarship from the Fonds de la Recherche en Santé du Québec (FRSQ).
The authors hold a patent related to the work that is described in this manuscript (Pat.#WO2004099410).
Word count: 2962

2.3.3Abstracts

The expression of melanotransferrin (MTf), a membrane-bound glycoprotein highly expressed in melanomas, is correlated with tumor vascularization and progression, suggesting a proinvasive function associated with MTf in malignant tumors. To test this hypothesis, we silenced MTf in human melanoma SK-Mel-28 cells using small interfering RNA (siRNA) and examined the plasmin activity and invasiveness of MTf-silenced melanoma. *In vitro*, the siRNA-mediated MTf knockdown inhibited by 58% the cell surface activation of plasminogen into plasmin. In addition, decreased expression of MTf in melanoma cells reduced cell migration. *In vivo*, we used a nude mice invasion model in which tissue factor (TF), a procoagulant cofactor, induces vascular [^{125}I]-fibrin deposition following injection. TF induces stronger [^{125}I]-fibrin deposition into the lungs than into other organs and increases invasion of [^{3}H-thymidine] SK-Mel-28 into the lungs. Using this metastasis model, the invasive potential of MTf-silenced cells into the lungs was reduced 5-fold. Altogether, these findings strongly suggest that MTf overexpression in melanoma cells contributes to tumor progession by stimulating plasmin generation as well as cell migration and invasion.
Abstract word count: 175

Key word: melanoma, melanotransferrin, siRNA. invasion, *in vivo* metastasis assay.

2.3.4Article Outline

Materials and methods
Materials
Cell culture
siRNA-mediated MTf knockdown
Fluorescence-activated cell sorting (FACS) analysis
Plasminolytic activity assay
Cell migration assay
Radial clot lysis assay
In vivo [^{125}I]-fibrin deposition assay
In vivo TF-induced metastasis assay

Data analysis
Results
siRNA-mediated MTf knockdown in SK-Mel-28 cells
siRNA-mediated MTf knockdown reduces cell surface plasminogen activation and cell migration
Recombinant sMTf stimulates radial clot dissolution by plasminogen activators
MTf stimulates fibrinolysis and SK-Mel-28 melanoma organ invasion
siRNA-mediated MTf knockdown reduces TF-induced lung metastases in nude mice.
Discussion
Acknowledgements
References

2.3.5Introduction

Melanotransferrin (MTf, Genbank locus ID 4241) is a 97 kDa glycoprotein that shares substantial amino acid sequence similarity and iron-binding properties with transferrin, lactoferrin and ovotransferrin. MTf exists as soluble (sMTf) and membrane-bound (mMTf) forms, depending on whether the protein possesses a GPI anchor. MTF was first identified on the surface of melanoma cells and is a marker for metastatic melanoma cells[1]. However, the expression of MTf is correlated with tumor vascularization and progression, suggesting a proinvasive function associated with MTf in malignant tumors[2]. Moreover, MTf is know to play a minor role in the transport of iron into cells[3]. Recently, we reported that both forms of MTf could stimulate the plasminogen activator- (PA)-mediated activation of plasminogen (Plg) into plasmin [4-6]. We also demonstated that sMTf is able to increase fibrinolysis [7].

To invade tissues, metastatic cells secretesproteases which are required for the degradation of the extracellular matrix [8]. Several studies have shown that plasmin, a serine protease released from Plg. promotes fibrin clot dissolution as well as cell migration and invasion through extracellular matrices when activated at the cell surface [9, 10]. Tissue-type PA (tPA) is a key player in fibrinolysis, mediating the conversion of inactive Plg into the enzymatically active plasmin [11], which could represent a crucial step during cell invasion [12]. We therefore investigated whether MTf could modulate the invasiveness of tumor cells.

The ability of tumor cells to establish new metastatic colonies from blood vessels involves a multistep process and represents a major obstacle to any cancer cure [10]. Briefly, the tumor cells access the vasculature by local invasion followed by circulation within the blood. Eventually, the tumor cell implants in the capillary bed of an organ and proliferates into metastasis [13]. Clinical evidence supports the notion that metastasis aggressiveness involve the hemostasis system [14]. In addition, efficient metastasis of tumor cells has long been suggested to be

dependent on plasma coagulation cascades. In this hypothesis, the formation of an initiating complex on host cells triggers a local coagulation cascade which represents an important step during tumor cell implantation [15]. It has been shown that tumor-derived tissue factor (TF) as well as other procoagulants are involved in the cancer hypercoagulable states [16]. TF is a single-chain, 263-amino acid, membrane-bound glycoprotein whose primary sequence indicates structural similarity with the cytokine receptor family. TF binds active factor VII and serves as the catalytic cofactor for this serine protease, thereby initiating the plasma coagulation cascades [17]. This process produces a fibrin clot that serves as an anchor matrix for metastatic cells to invade tissues [18]. Here, we used small interfering RNA (siRNA) to block MTf gene expression in human SK-Mel-28 melanoma cells. Our results demonstrate that the downregulation of MTf in SK-Mel-28 cells reduces their ability to generate plasmin and to migrate. We also used an *in vivo* nude mice metastasis model, in which the injection of TF induces fibrin clot deposition and leads to an increased accumulation of [^{3}H]-thymidine labeled-SK-Mel-28 cells in various organs. The siRNA-mediated MTf knockdown also decreased the accumulation of radiolabeled SK-Mel-28 into the lung. Overall, these results provide evidence that mMTf expression facilitates coagulation cascade-dependent melanoma invasion *in vivo*.

2.3.6Materials and Methods

2.3.6.1Materials

The L235 monoclonal antibody (mAb) against MTf was kindly provided by Biomarin Pharmaceutical (Novato, CA). Specific pathogen-free, male CD-1 mice as well as female athymic Crl:CD-1- nuBR nude mice were obtained from Charles River Laboratories (Lasalle, QC). Other biochemical reagents were from Sigma-Aldrich (Oakville, ON)

2.3.6.2Cell culture

Human melanoma SK-Mel-28 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in modified Eagle's medium (MEM) containing 1 mM Na pyruvate and 10% foetal bovine serum (FBS) under 5% CO_2/95% air atmosphere.

2.3.6.3siRNA-mediated MTf knockdown

siRNA SK-Mel-28 cell transfection was performed with siRNA duplex sense r(gggcgaaguguacgaucaa)dTdT and antisense r(uugaucguacacuucgccc)dAdC using HiPerFect transfection reageant from Qiagen (Mississauga, ON). As a control, SK-Mel-28 cells were transfected with the same vector containing a inoperant duplex sense r(GGGCGAAGUGUACGAUACA)dTdT and antisens r(UUGAUCGUACACUUCGCCC)dAdC, that did not effect the MTf production and the transfection reageant alone. The siRNA transfection were performed according to the manufacturer's protocol. Briefly, 7.5 μl of HiPerFect transfection reagent (diluted in 100 μl of serum-free medium) was added dropwise to 1ng of siRNA. After 15 min of incubation, the mix was added to 2.5 x 10^5 cells. The cells were then incubated for 1 to 8 days. 72 hours incubation with siRNA was used for the MTf-knockdown in subsequent assays. SK-Mel-28 cells treated with the transfection reagent only were used as a control.

2.3.6.4Fluorescence-activated cell sorting (FACS) analysis

SK-Mel-28 were dissociated by incubation with phosphate-buffered NaCl solution (PBS)-citrate (138 mM NaCl, 2.8 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2PO_4, 15 mM sodium citrate, pH 7.4) for 10 minutes. SK-Mel-28 cells (1 X 10^4) were resuspended in MEM media and incubated at 37 °C for 20 minutes with either 1μg/ml mAb L235 or with non-specific, control immunoglobulin G (IgG). The cells were then washed twice with MEM medium and incubated in the dark at 37 °C for 20 minutes with 1 μg/mL goat anti-mouse IgG-Alexa488 (Molecular Probes, Eugene, OR). After two washes with Ringer/HEPES solution (150 mM NaCl, 5.2

mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 5 mM HEPES and 2.8 mM glucose, pH 7.4), the cells were analyzed by flow cytometry on a Becton Dickinson FACSCalibur with a 488 nm argon laser. Cell surface expression levels of MTf were corrected for the background fluorescence intensity measured in the presence of non-specific IgG and were expressed as a percentage of the mean fluorescence intensities measured for the control and inoperant duplex .

2.3.6.5Plasminolytic activity assay

The *in vitro* plasminolytic activity of SK-Mel-28 cells (control and MTf-silenced) was measured using a colorimetric plasmin activity assay. Briefly, SK-Mel-28 cells were grown to 85% confluency and were dissociated by incubation in PBS-citrate solution for 10 min. Cells were then washed twice with incubation buffer (50 mM Tris–HCl buffer (pH 7.5), 150 mM NaCl and 50 mM $CaCl_2$). In the plasminolytic assay, 1 X 10^5 cells were incubated in the presence of 25 nM Glu-Plg (Calbiochem, Novato, CA) and 15 µg of the chromogenic plasmin substrate D-Val-Leu-Arg *p*-Nitroanilide (VLK-pNA) in 200 µl of incubation buffer containing 1% bovine calf serum (BCS). In this assay, the cleavage of VLK-pNA results in a *p*-Nitroanilide molecule that absorbs at 405 nm. The plasmin activity was monitored at 37°C for 6 hours at 405 nm using a ThermoMAX microplate reader (Molecular Devices, Sunnyvale, CA).

2.3.6.6Cell migration assay

SK-Mel-28 cell migration was performed using Transwell filters (Costar, Corning, NY; 8 µm pore size) precoated with 0.15% gelatin. Briefly, 1 X 10^5 SK-Mel-28 cells were resuspended in 100 µL of serum-free medium and added into the upper chamber of each Transwell (the lower chamber of the transwell contained 10% serum). The plates were then placed at 37°C in a 5% CO_2/95% air

atmosphere for 18 hours. Cells that migrated to the lower surface of the filters were fixed with 3.7% formaldehyde in PBS, stained with 0.1% crystal violet/20% MeOH. Migrating cells were visualized at 100X magnification using a Nikon Coolpix 5000 digital camera (Nikon Canada, Mississauga, ON, Canada) attached to a Nikon TMS-F microscope (Nikon Canada). The average number of migrated cells per field was assessed by counting at least 4 random fields per filter.

2.3.6.7Radial clot lysis assay

Radial clot lysis was assayed as described by Mosesson [19] , with minor modifications. Briefly, fibrin clots were obtained by the incubation of fibrinogen (8.2 μM), Glu-Plg (2 μM) and 0.4 U/ml thrombin in buffer A (50 mM Tris/HCl pH 7.4, 150 mM NaCl, and 50 mM $CaCl_2$) at 37 °C for 60 min. Clot lysis was initiated by adding pro-uPA (2 nM) or tPA (2 nM) with or without recombinant sMTf (500nM). Clots were then incubated for 30 min at 37 °C and stained with chinese ink. Clots were visualized at 40X magnification using a Nikon Coolpix 5000 digital camera attached to a Nikon TMS-F microscope. The clot diameters were measured in order to quantify the fibrinolysis observed.

2.3.6.8In vivo [^{125}I]-fibrin deposition assay

Male CD-1 mice were anesthetized by intraperitoneal (IP) injection of pentobarbital (50 mg/kg). Human plasma [^{125}I]-fibrinogen ($2x10^6$ CPM) were injected intravenously (IV) in the right jugular vein. Microthrombi were induced 5 minutes after the injection of fibrinogen by TF infusion (0.4 μg/kg). The mice were sacrificed 20 min after the TF infusion. Several organs (kidney, lung, heart and brain) were excised and weighed. Organ-associated radioactivity, representing the level of [^{125}I]-fibrin deposition, was quantified using a gamma counter.

2.3.6.9*In vivo* TF-induced metastasis assay

The TF-induced SK-Mel-28 cell metastasis assay was based on the method of Muto [20] with minor modifications. All mice used were 5-10 weeks of age. All animal studies were conducted in accordance with recommendations from the Canadian Council on Animal Care (CCAC) for care and use of experimental animals. Briefly, SK-Mel-28 cells were incubated with [^{3}H]-thymidine for 72 hours. After the metabolic labeling, 2.5 X 10^5 SK-Mel-28 cells (control and MTf-silenced) were injected into the tail vein. Microthrombi were then induced by injection into the tail vein of 0.1 µg/kg TF as described [20]. The mice were sacrificed 24 hours after TF injection and the lungs were excised and dissolved in solvent (Solvable, Perkins; Wellesley, MA) for 24 hours. The amount of lung associated [^{3}H]-thymidine labeled SK-Mel-28 cells was measured by liquid-scintillation spectrometry.

2.3.6.10Data analysis

Statistical analyses were performed using Student's paired t-test via GraphPad Prism (San Diego, USA). Significant difference was assumed for *P* values less than 0.05.

2.3.7Results

2.3.7.1siRNA-mediated MTf knockdown in SK-Mel-28 cells

Cell surface mMTf expression in SK-Mel-28 cells was determined by FACS using the mAb L235, which recognizes a conformational epitope on MTf, following siRNA-mediated MTf knockdown (Fig. 26). The intensity of the green fluorescence (Alexa488; FL1 detection) associated with detection of cell surface mMTf by the mAb L235 is much lower in MTf-silenced than in control SK-Mel-28 cells (Fig. 26A). The inhibition of mMTf expression by siRNA was optimal after 72 hours, reaching a maximal inhibition of 70% (Fig. 26B) . Eight days after the siRNA knockdown of MTf, the mMTf cell expression inhibition was still about 45% of control levels. These results demonstrated that siRNA vectors significantly reduced mMTf protein expression. As control, SK-Mel-28 cells were transfected with a inoperant duplex had not effect on MTf expression like the SK-Mel-28 cells treated with the transfected agent alone (Fig.26C)

2.3.7.2siRNA-mediated MTf knockdown reduces cell surface plasminogen activation and cell migration

We previously established that mMTf affects the activation of Plg as well as cell migration and invasion *in vitro* [4, 5]. Here, we investigated whether siRNA-mediated MTf knockdown could modulate the SK-Mel-28 cell surface activation of Plg. The plasmin activity at the surface of control and MTf-silenced SK-Mel-28 cells was measured (Fig. 27A). The Plg activation initial velocity (v) was 48.3±2.9 mOD/min for control cells and decreased to 20.7±2.7 mOD/min for MTf-silenced cells, which represents 58% inhibition of plasmin generation at the cell surface. Since mMTf expression affects the activation of Plg *in vitro* and since Plg activation is required for tumor cell invasion and metastasis [21], the impact of mMTf knockdown on *in vitro* SK-Mel-28 cell migration was examined. Cell migration was measured using Transwell filters coated with gelatin. The migration assay showed that MTf-silenced cells exhibited less cell migration than did

control cells (Fig. 27B). The siRNA-mediated MTf knockdown decreased, by about 78%, the migration of SK-Mel-28 melanoma cells (Fig. 27C). These results strongly suggest that the siRNA-mediated MTf knockdown inhibits both cell surface Plg activation and cell migration in SK-Mel-28 melanoma cells. As another control, the inoperant duplex as not effect on the migration like the control. Since not difference was found in mMTf expression and cell migration between the inoperant duplex with transfection reagent and the transfection reagent alone. The inoperant duplex was discarded.

2.3.7.3Recombinant sMTf stimulates radial clot dissolution by plasminogen activators

During tumor cell migration, the invading cell needs to degrade the extracellular matrix [8]. Since melanoma aggressiveness is linked to the coagulation system, [22] we used a radial clot lysis assay to demonstrate the effect of recombinant sMTf on plasminogen activator (PA)-induced clot lysis (Fig. 28A). In this assay, the fibrin clot contains Plg. The addition of MTf to both PAs enhanced their fibrinolysis properties, leading to 3-fold increased dissolution of the fibrin clots. These results indicate that recombinant sMTf stimulates fibrin clot dissolution by PAs (Fig 28B).

2.3.7.4MTf stimulates fibrinolysis and SK-Mel-28 melanoma organ invasion

A previous study has shown that the injection of TF increases the formation of fibrin clots *in vivo* [20]. To evaluate the capacity of TF to induce fibrin deposition in organs, [^{125}I]-labeled fibrinogen is injected into mice. Circulating [^{125}I]-fibrinogen is then cleaved by thrombin into [^{125}I]-fibrin by the extrinsic coagulation pathway As shown in Fig. 29A, the [^{125}I]-fibrin deposition in the *in vivo* assay is highest in the lung with 55 X 10^3 CPM/g of tissue, followed by the kidney with 41 X 10^3 CPM/g of tissue. In heart and brain, [^{125}I]-fibrin deposition was much lower at 4 X 10^3 CPM/g and 0.4 X 10^3 CPM/g of tissue, respectively. These results show that TF can increase [^{125}I]-fibrin deposition within several organs. We used

this TF-induced clot *in vivo* model to investigate the link between clot deposition and the ability of SK-Mel-28 melanoma cells to invade various organs (Fig. 29A).

2.3.7.5siRNA-mediated MTf knockdown reduces TF-induced lung metastases in nude mice.

The Plg system is considered the primary effector of cell invasion. During cell invasion, this system mediates destruction of the extracellular matrix by fibrinolysis. To determine whether MTf is involved during *in vivo*, TF-induced SK-Mel-28 melanoma invasion into lung, metabolically radiolabeled SK-Mel-28 were injected intravenously into mice that were preteated (or not) with TF. The melanoma cell invasion into the organs was assessed by measuring the infiltration of SK-Mel-28 cells into these organs in untreated and TF-treated mice. [^{3}H-thymidine] labeled SK-Mel-28 accumulation was observed to increase in lung by 3-fold whereas the kidney, heart and brain accumulation was similar to that in the control animals. Altogether, these resuts demonstrated a correlation between the levels of [^{125}I]-fibrin deposition and of SK-Mel-28 cell accumulation in the lung (Fig 29B). However, siRNA-mediated MTf knockdown reduces by about 80% the lung invasion by TF-induced SK-Mel-28 cells in nude mice (Fig 29C). This reduction of SK-Mel-28 cell metastasis in the lung observed with MTf-silenced cells indicates that MTf may be involved during melanoma cell lung invasion.

2.3.8Discussion

In this study, we demonstrated the involvement of endogenous MTf in *in vitro* Plg activation and cell migration as well as *in vivo* invasion of melanoma cells by using siRNA-mediated MTf knockdown. When siRNA is introduced into mammalian cells, transitory sequence-specific destruction of endogenous target mRNAs occurs and gene expression is effectively suppressed [23-25].

The melanoma cell line SK-Mel-28, which highly expresses MTf, [4] has been used to investigate the involvement of MTf in Plg activation. The siRNA-mediated

knockdown of MTf decreased Plg activation at the SK-Mel-28 cell surface. This result is in agreement with our previous studies, where the overexpression of MTf in Chinese hamster ovary (CHO) cells stimulated Plg activation by PA [4-6]. However, the use of siRNA to inhibit the expression of MTf provides the first evidence that a downregulation of MTf leads to a reduction in Plg activation at the cell surface. Interestingly, the disruption of MTf also led to decreased cell migration, suggesting that MTf do play a role in plasminogen activation by uPA or tPA. An earlier study showed that the activation of Plg at the cell surface is a crucial step during cell migration [12]. A recent study confirm using others si-RNA that mMTf is involved in migration of SK-Mel-28 *in vitro* [26]. Our study futher caracterize the involvement of mMTf *in vivo*. The TF initiate the coagulation of the blood clots that promote an favorable site of implantation for tumor cells and increase there invasion [8]. Here, ours results showed for the first time that the inhibition of the expression of mMTf antagonise the action of TF.

To invade tissues, metastatic cells secrete proteases which are required for the degradation of the extracellular matrix [8]. Several studies have shown that plasmin could promote fibrin clot dissolution as well as cell migration and invasion through extracellular matrices when activated at the cell surface [9, 10]. The fibrinolytic system is important in tumor spreading and involves the dissolution of the fibrin matrix [14]. Recently, we showed that recombinant sMTf could increase both tPA- and uPA-dependent fibrinolysis [4, 7]. Therefore, the involvement of recombinant sMTf in PA-mediated fibrinolysis as well as the implication of MTf in the migration of SK-Mel-28 cells are sufficient to suggest that MTf could be involved in melanoma invasiveness.

To verify this hypothesis, we determined whether TF-induced fibrin clot deposition leading to the stimulation of melanoma invasion was dependent on MTf expression. The TF is known to activate thrombin which cleaved fibrinogen into fibrin (Fig. 30A). Fibrin polymerized an form a clot with other adhesive

proteins, such as vitronectin, laminin and fibronectin, fibrin forms a provisional matrix [27]. This TF model shows that the fibrin clot accumulates in the lung. This is in agreement with another study showing that fibrin clot has a tendency to deposit in the lung because of this tissue's ability to serve as a filter for fibrin clots [28]. Early and high metastatic formation is typical in human melanomas [29] Also, thrombin has been shown to strongly enhance metastasis *in vivo* [18]. In the present study, human melanoma SK-Mel-28 cells demonstrate increased TF-induced melanoma invasion into the lung (Fig. 29B). This finding corroborates another study where the presence of fibrin clots markedly increased the incidence of spontaneous macroscopic metastasis of melanoma into the lung [30]. Using siRNA-mediated MTf knockdown, we showed that MTf suppression reduced the TF-induced cell invasion, suggesting that MTf is directly implicated in melanoma cell metastasis (Fig. 29C).

In conclusion, we have shown for the first time that MTf gene expression knockdown using siRNA inhibits invasiveness of malignant human melanoma cells in nude mice. We are also reporting that the loss of mMTF expression in SK-MEL-28 cells reduces Plg activation at the cell surface which, in turn, affects cell migration. Collectively, our findings suggest that endogenous mMTf can be considered as a potential therapeutic target for future therapy aimed at blocking MTf-expressing tumor cell tissue invasion.

2.3.9Acknowledgments

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2.3.10Références

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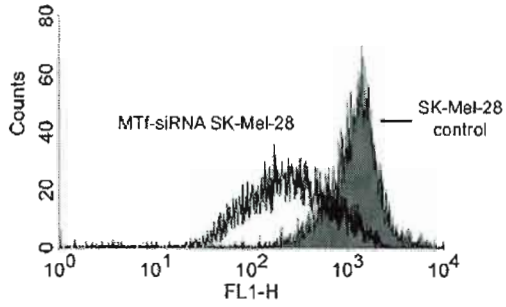
A



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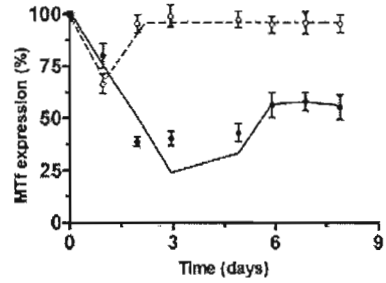

Figure 26 SiRNA-mediated MTf knockdown in SK-Mel-28 cells.

(A) Flow cytometry analysis of cell surface MTf levels was performed as described in the Materials and Methods section. Control SK-Mel-28 (filled line) and MTf-silenced SK-Mel-28 (empty line) cells were probed with anti-MTf mAb L235 and detected with goat anti-mouse IgG-Alexa488. (B) Flow cytometry analysis of cell surface MTf levels was performed at different times as described in the Materials and Methods section. Cell surface expression levels of MTf (●) si-RNA duplex were corrected for the background fluorescence intensity measured in the presence of a non-specific IgG and were expressed as a percentage of the mean fluorescence intensities compared to control cells and inoperant dulex exposed cells. Statistically significant differences, as compared to control conditions, are indicated by ***P < 0.001 (Student's t test) (N = 3).(○) A inoperant duplex with the transfection reagant was compared the the transfected reagent alone, not significatif difference was found (N=3).

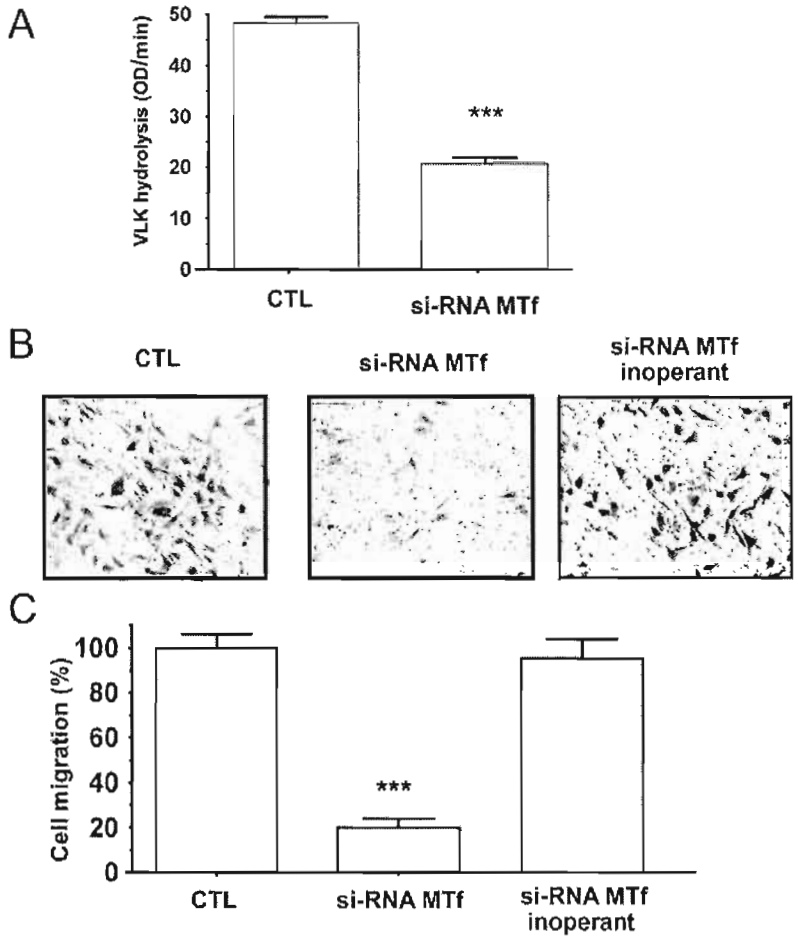

Figure 27 SiRNA-mediated MTf knockdown reduces cell surface plasminogen activation and cell migration.

(A) Effect of siRNA-mediated MTf knockdown on SK-Mel-28 cell surface plasminolytic activity. The plasminolytic activity was measured for control and MTf-silenced SK-Mel-28 cells as described in the Materials and Methods section (N = 8) (B) SK-MEL-28 cell migration was performed using modified Boyden chambers with filters caoted with gelatin. Cells that had migrated to the lower surface of the filters were fixed, stained and counted as described in the Materials and Methods section. Photos (original magnification, ×100) obtained from a representative experiment are shown. (C) Cell migration quantification represent the means ± SEM of two independent experiments performed in triplicate. Statistically significant differences from control values are indicated by ***P<0.001 (Student's t test) (N = 6).

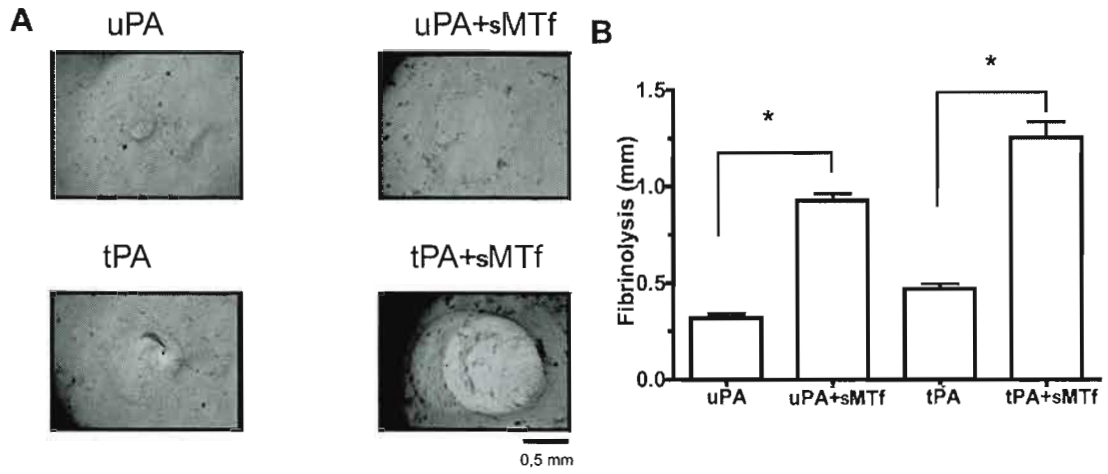

Figure 28 Stimulation of fibrinolysis by MTf.

(A) MTf was added to tPA or pro-uPA in a radial fibrinolysis assay. Fibrinolysis was performed as described in the Materials and Methods section. Photos (original magnification 40X) obtained from a representative experiment are shown (upper panel) (field = 2 mm) . (B) The clot diameters were measured (lower panel). Results represent the means ± SEM of five different experiments.

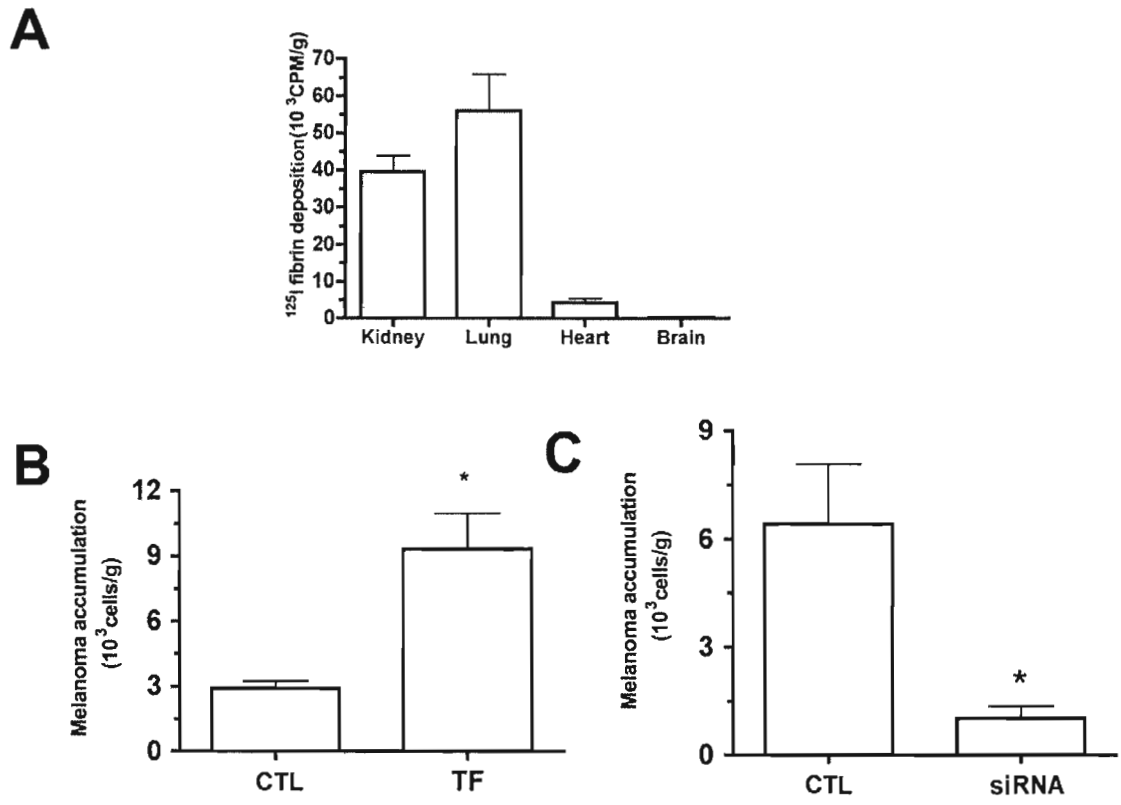

Figure 29 SiRNA-mediated MTf knockdown reduces TF-induced lung metastases in nude mice.

(A) Fibrin clot deposition was performed in mice. Mice were treated with vehicle or with TF by i.v. injection. After 5 min [^{125}I]-fibrinogen was injected and [^{125}I]-fibrin deposition was detected at 20 min using a gamma counter. Several organs (kidney, lung, heart and liver) were excised to monitor fibrin deposition. Data represent the mean ± SEM of five different experiments. (B) Cultured human melanoma SK-MEL-28 cells were radiolabeled with [H^{3}]- thymidine for 72 hours. TF and cells were injected into nude mice via the tail vein. Radiolabeled SK-Mel-28 cells were measured in the lung with or without Tf injection. Lung-associated radioactivity was determined for controls and for TF-induced SK-Mel-28 metastasis. Data represent the mean ± SEM of five different experiments. (C) Effect of siRNA-mediated MTf knockdown on SK-Mel-28 cells lung metastasis. Lung-associated radioactivity was determined in control and MTf-silenced SK-Mel-28 metastasis.Background invasion was soustracted. Data represent the mean ± SEM of five different experiments. Statistically significant differences from control values are indicated by ***P<0.001 (Student's t test).

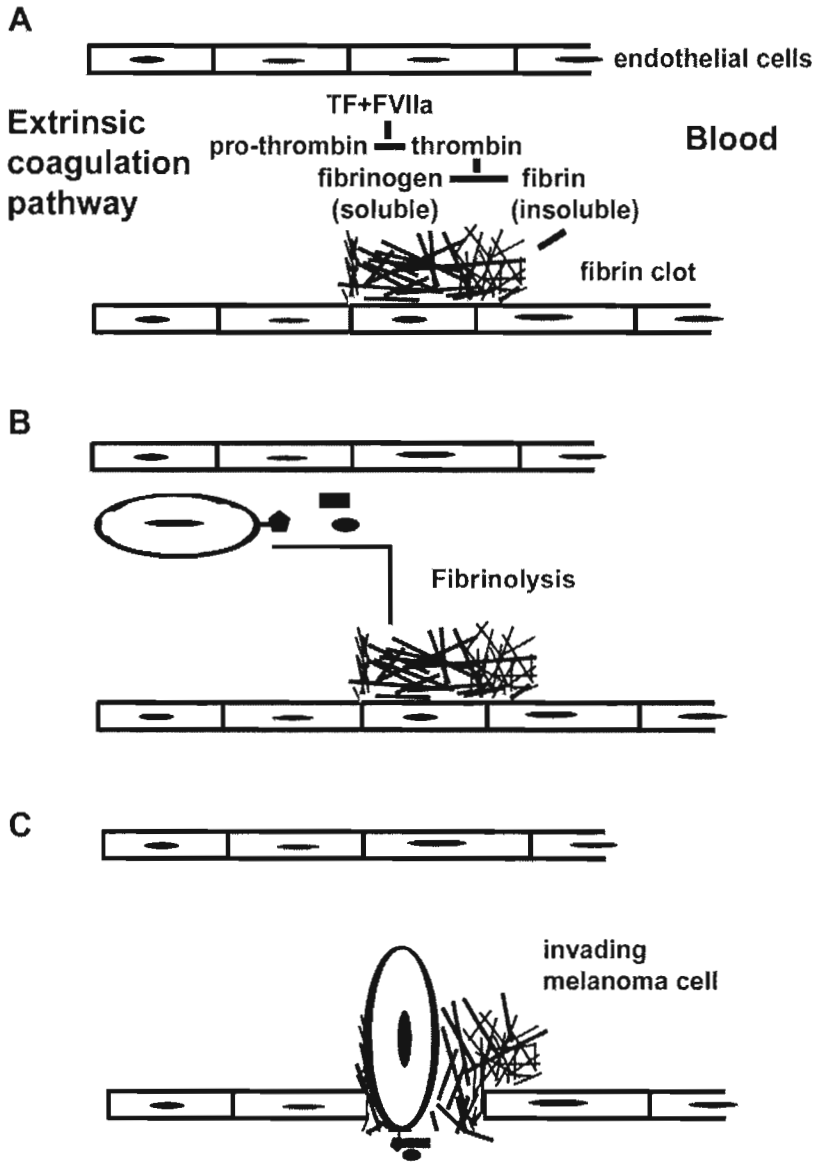

Figure 30 Schematic representation of the TF-induced melanoma cell invasion process.

(A)The extrinsic coagulation pathway. TF initiates the deposition of a fibrin clot into the lung. (B) Melanoma cells released from the primary tumour to the blood circulation digest the fibrin clot with the cell surface MTf-induced plasmin production. MTf (⬟) binds to plasminogen (■) and plasminogen activator (●) and stimulates the fibrinolysis of the fibrin clot. (C) The cells invade the clot and migrate through the extravascular region, invading the lung.

2 Discussion

L'activation du plasminogène est au cœur de plusieurs étapes importantes liées au cancer. Ma thèse démontre que la MTf agit comme modulateur dans l'activation du Plg, avec ses deux activateurs : l'uPA, lié au cancer et le tPA, lié à la fibrinolyse. Son action ambivalente, à la fois protumorale et antitumorale, est caractérisée par son site d'action au niveau cellulaire. La mMTf agit comme un modulateur pour amplifier l'activation du Plg à la membrane, tandis que la sMTf entre en compétition avec la mMTf pour inhiber son effet. Cet effet de compétition peut constituer une stratégie thérapeutique comme cela a déjà été décrit pour plusieurs autres récepteurs (Monteiro et coll., 2006; Perry et coll., 2006).

Cette discussion est divisée en cinq parties. Nous débuterons dans la première partie avec les liens physiques entre la MTf et les composantes du système plasminolytique. Dans la seconde partie, nous caractériserons l'influence de la MTf sur l'activation du plasminogène. Dans la troisième partie, nous discuterons de la participation de la MTf dans les étapes liées au cancer. Dans la quatrième partie, nous caractériserons l'influence de la MTf sur la matrice extracellulaire provisoire de fibrine (PEFM) responsable du microenvironnement favorisant la dispersion des cellules tumorales. Dans la cinquième partie, nous discuterons de l'impact de la MTf sur l'invasion tumorale. Nous conclurons avec les perspectives qui découlent de ma thèse.

2.1 Le lien entre la MTf et le système plasminolytique

Dans la présente thèse, la technologie du BIAcore montre les interactions potentielles entre la MTf et deux composantes du système plasminolytique : la pro-uPA, précurseur de l'uPA et le Plg. Ces interactions sont spécifiques, car

aucune interaction entre la MTf et d'autres protéines liées au système plasminolytique, incluant le tPA, le PAI-1, la plasmine et l'angiostatine, n'a été observée. De plus, ces résultats sont les premiers à décrire des interactions potentielles entre la MTf et ces deux composantes du système plasminolytique.

Le modèle mathématique de conformation à deux étapes, utilisé pour modéliser le lien entre la MTf et le système plasminolytique, donne les meilleures représentations des interactions de la pro-uPA et du Plg avec la MTf immobilisée sur le BIAcore. De telles représentations expérimentales avec un modèle d'interaction à plusieurs étapes indiquent qu'un changement de conformation peut survenir. Nous avons montré que le Plg peut se lier à la MTf et vice versa. Les K_D sont semblables dans les deux cas.

Le motif de dégradation du Plg suggère que la MTf induit un changement de conformation, ce qui augmente l'activité de la pro-uPA sans coupure apparente de la pro-uPA. Fait intéressant, les fragments de Plg produits en ajoutant la MTf sont différents de ceux provenant de la dégradation du Plg avec l'uPA seul, suggérant encore une fois un changement de conformation de la part du Plg. Un autre exemple de changement de conformation du Plg par la MTf est le modèle de dégradation de la fibrine en présence du Plg et de la sMTf qui démontre un clivage de la fibrine β. Une approche plus directe, telle que la protéolyse limitée, la spectrométrie circulaire de dichroïsme ou la technologie nucléaire de résonance magnétique serait utile pour confirmer et fournir des informations additionnelles sur l'interaction entre la MTf et la pro-uPA ou le Plg.

2.1.1 La MTf : un stimulateur de l'activation du Plg

En plus de son interaction avec le pro-uPA et le Plg, la MTf stimule l'activation du Plg en diminuant le K_m du pro-uPA pour le Plg et en augmentant le Vmax de la réaction. La conversion du pro-uPA en uPA actif se produit par le clivage

protéolytique d'un lien peptidique simple (Lys^{158}-Ile^{159} dans l'uPA humain) (Dano et coll., 1985). Cette conversion peut être catalysée par la Pln ou par plusieurs autres protéases, telle que la kallikréine plasmatique, le facteur de coagulation sanguin XIIa, la cathepsine B, la cathepsine L et l'antigène spécifique de la prostate (Andreasen et coll., 1997).

Nous rapportons aussi que la MTf augmente la formation de la plasmine par le tPA en agissant sur le Plg. Lors de l'homéostasie, le tPA est principalement sécrété par les cellules endothéliales et clive le Plg présent dans la circulation sanguine en plasmine, qui est l'enzyme responsable de la dégradation protéolytique de la fibrine (Collen et Lijnen 1995). L'action de la MTf sur le Plg réduit de 5 fois le K_m pour le tPA.

L'inhibition par le mAb L235 de la capacité de la sMTf à induire l'activation du Plg par le tPA ou l'uPA suggère que son interaction avec le Plg est spécifique et pourrait impliquer l'épitope conformationnel identifié par ce mAb (Kennard et coll., 1996). La capacité de l'anticorps anti-MTf de bloquer cet effet d'induction confirme que le facteur biologiquement actif est la sMTf elle-même plutôt qu'un facteur contaminant. Ainsi, la MTf comcidérée comme modulateur positif de l'activation du Plg pour le tPA ou pour l'uPA. Les résultats démontrent que l'effet de la MTf est dépendante de la concentration et que l'activité de la MTf est dans la gamme des nanomolaires. Il a récemment été démontré que si le Plg est lié à la cellule, la génération de plasmine par les activateurs du Plg est nettement stimulée, comparativement à la réaction en solution. Nos données indiquent également que l'activité plasminolytique des cellules exprimant la MTf à la surface est inhibée par le mAb L235.

Nous avons également observé que la capacité d'activation du Plg par les cellules HMECs-1 est diminuée par le traitement avec la sMTf. La lignée cellulaire SK-Mel-28, une lignée de mélanomes, qui exprime fortement la MTf

(Demeule et coll., 2003), a été employée pour étudier la participation de la MTf dans l'activation du Plg. L'utilisation d'un siRNA dirigé contre la MTf a diminué l'activation du Plg sur la surface des cellules SK-Mel-28. Ce siRNA MTf a donc permis de démontrer que la mMTf participe à l'activation du Plg à la surface des cellules. Ce résultat est en accord avec nos études précédentes, où la surexpression de la MTf sur des cellules CHO stimule l'activation du Plg par ses PA (Demeule et coll., 2003; Michaud-Levesque et coll., 2005; Michaud-Levesque et coll., 2005). Cependant, l'utilisation du siRNA pour empêcher l'expression de la MTf fournit une première démonstration directe qu'une diminution de MTf mène à une réduction de l'activation du Plg à la surface des cellules.

2.1.2 Les étapes cancéreuses affectées par la MTf

La première corrélation entre le cancer et l'activation du Plg a été faite il y a plus de 50 ans (Butler et coll., 1979). Beaucoup d'études ont été réalisées sur la valeur pronostique de l'uPA, du tPA et du PAI-1 (Duffy et coll., 1996). Plusieurs études portant sur des modèles expérimentaux de tumeurs ont été publiées. Ces études suggèrent que l'inhibition des enzymes fibrinolytiques empêche la croissance tumorale et la formation de métastases (Bugge et coll., 1998; Praus et coll., 2002).

L'activation du Plg à la surface des cellules est une étape cruciale dans la migration des cellules (Collen et coll., 2000). La plasmine initie l'activation des autres protéases, telles que la MMP-2 et la MMP-9, qui dégradent la matrice extracellulaire (Tan et coll., 2006). La Pln est donc un élément important dans l'invasion cancéreuse menant aux métastases.

Nous démontrons dans cette thèse que la MTf intervient dans le processus menant aux métastases. L'invasion cancéreuse menant aux métastases est un processus complexe qui comprend des étapes séquentielles. Ces étapes exigent

qu'une cellule tumorale module sa capacité à adhérer, à dégrader la matrice extracellulaire environnante, à migrer et à proliférer à un autre emplacement (Buo et coll., 1995). De plus, les tumeurs doivent stimuler l'angiogenèse pour atteindre des dimensions macroscopiques. Pendant ces étapes, les cellules tumorales subissent une série de modifications liées les unes aux les autres. Le tissu de la tumeur tend vers une croissance incontrôlée pendant que les cellules se développent progressivement dans le corps en tant qu'envahisseurs ectopiques.

Le développement d'une tumeur implique les étapes suivantes : déclenchement, promotion et progression (Tan et coll., 2006). Indépendamment de l'événement de transformation initiale, plusieurs autres facteurs favorisent la propagation de la tumeur. L'inflammation est pour la plupart un phénomène principal des tumeurs, de même que la néo-vascularisation, qui permet une augmentation de la taille de la tumeur primaire (Werb et coll., 1997). Les cellules néoplasiques se détachent par la suite de la tumeur primaire et dérivent dans la circulation par l'intermédiaire du sang ou du système lymphatique. Ceci est suivi de leur adhérence à l'endothélium dans des organes éloignés. Habituellement, la tumeur extravase et pénètre dans un nouveau tissu (Chambers et coll., 1995). Quand la tumeur quitte le système sanguin, une tumeur secondaire apparaît dans un autre endroit que la tumeur primaire. Cette nouvelle tumeur est une métastase. Nous avons donc une étape de migration cellulaire et d'angiogenèse, suivie d'une activation de la cascade de coagulation dissoute par la fibrinolyse, puis d'une étape finale d'invasion tumorale dans cette matrice et dans les tissus.

2.1.3 L'effet de la MTf sur la migration cellulaire et l'angiogenèse

Le système plasminolytique est impliqué dans plusieurs pathologies qui impliquent une migration cellulaire, telles que l'invasion des cellules tumorales et

les métastases. Plusieurs études démontrent que le système plasminolytique joue un rôle principal dans la transduction de signaux aussi bien que dans la régulation de la migration et de l'angiogenèse des mélanomes (Stahl et Mueller, 1997; Weaver et coll., 1997). Une étude récente a démontré que la MTf favorise la migration et l'angiogenèse de cellules HMEC-1 quand elle est utilisée comme un chimioattractant (Sala et coll., 2002). Dans nos conditions, quand la MTf est ajoutée sous forme soluble, la migration de cellules HMECs-1 est diminuée de plus de 50 %. La MTf affecte donc la progression de l'angiogenèse. Une étude antérieure a prouvé que l'activation du Plg à la surface des cellules est une étape cruciale pendant la migration des cellules (Collen et coll., 2000). Nos données indiquent également que la migration et l'activité plasminolytique des cellules exprimant la MTf sont inhibées par le mAb L235, suggèrant ainsi que la mMTf est impliquée dans les processus cancéreux et d'angiogenèse. Le fait que la migration des cellules endothéliales HMECs-1 et des cellules de mélanomes SK-Mel-28 soit diminuée quand la sMTf est ajoutée, suggérent que la sMTf affecte la régulation de l'activation du Plg sur la surface des cellules. Ainsi, en affectant l'équilibre entre la sMTf et la mMTf, il est possible d'affecter la migration des cellules de HMEC-1 et de SK-Mel-28. Notre étude comparative sur la migration de cellules endothéliales exprimant la MTf, les HMECs-1 et une autre lignée endothéliale n'exprimant pas de MTf, les HUVECs, démontre qu'en présence du mAb L235, seule la lignée HMEC-1 est affectée. Nous avons également mesuré l'impact du mAb L235 et d'un siRNA dirigé contre la MTf sur la migration du mélanome SK-Mel-28. En présence du mAb L235 ou utilisant des cellules de mélanomes où l'expression de la MTf est inhibée par un siRNA, la migration des cellules SK-Mel-28 est réduite significativement. Pendant le processus dynamique de la migration cellulaire, la cellule tumorale active les protéases pour dégrader localement les composantes de la matrice provisoire de fibrine (PEFM) générée pendant le phénomène de l'angiogenèse en périphérie de la cellule.

La sMTf agirait dans ce phénomène comme un inhibiteur compétitif à la mMTf.

2.1.4 La fibrinolyse de la matrice provisoire (PEFM)

Le système plasminolytique, qui mène à la formation de la Pln et à la fibrinolyse subséquante, joue un rôle important dans la dissolution du PEFM, un événement important dans l'angiogenèse (Reijerkerk et coll., 2000). Nous démontrons que la sMTf module la dissolution induite par le tPA des fragments [^{125} I]-fibrine d'un caillot artificiel et d'un caillot sanguin. Bien que l'introduction du facteur XIII diminue la fibrinolyse induite par le tPA (Lorand, 2001), l'addition de la sMTf augmente toujours la libération de fragments [^{125}I]-fibrine. L'analyse du caillot fournit un résultat supplémentaire que l'activation du Plg par le tPA ou l'uPA est augmentée par la sMTf et contribue à la dissolution du PEFM.

La fibrine n'est pas requise pour l'angiogenèse induite par la tumeur. Cependant, la fibrine dans un modèle *in vivo* augmente fortement l'agressivité de la tumeur (Palumbo et coll., 2002). Plusieurs études montrent que la plasmine peut favoriser la dissolution de caillots de fibrine aussi bien que la migration de cellules et l'invasion de la matrice extracellulaire, une fois activée à la surface de cellules (Gong et coll., 2001; Rakic et coll., 2003). Le système fibrinolytique est donc important dans la dispersion de tumeurs et implique la dissolution de la matrice de fibrine (Sudhoff et Schneider 1992). Par conséquent, l'implication de la sMTf dans la fibrinolyse tPA-dépendante et dans la migration des cellules SK-Mel-28 est suffisante pour suggérer que la MTf est impliquée dans l'invasion des mélanomes.

2.1.5 L'invasion tumorale

La Pln est un joueur clé dans la dispersion tumorale. Les enzymes protéolytiques activées par l'action de la Pln sont essentielles pour la migration cellulaire, mais également pour l'intravasation et l'extravasation de tumeurs dans les vaisseaux sanguins. Dans le modèle « *grip and go* » (attachement et déplacement) proposé par Reijerkerk et coll., la plasmine est un élément important. Ce modèle décrit 2 étapes : une étape où la cellule s'attache à la MEC en aval de la cellule pour lui permettre d'avoir un appui pour s'avancer. Dans autre étape, les protéases s'activent pour dégrader la MEC en amont et libérer la cellule de ses attaches dans la direction où elle se dirige. Ce modèle est reconnu pour former un cycle alternatif d'attachement et de dégradation de la MEC qui fait progresser la cellule vers sa destination (Reijerkerk et coll., 2000; Gong et coll., 2001). Nos données suggèrent que la mMTf puisse être impliquée dans ces processus qui sont associés à l'invasion. Pour envahir le tissu, les cellules tumorales utilisent le système plasminolytique qui dégrade la MEC (Im et coll., 2004). Par conséquent, pour vérifier l'hypothèse sur l'invasion, nous avons établi un modèle animal où le dépôt de fibrine induit par l'injection de TF stimule l'invasion des cellules de mélanomes SK-Mel-28. Le TF est connu pour activer la thrombine qui clive le fibrinogène. Le clivage du fibrinogène soluble en fibrine insoluble forme une matrice provisoire (Reijerkerk et coll., 2000). Ce modèle animal avec l'injection intraveineuse (i.v.) de TF démontre que la fibrine entraîne une accumulation de caillots de fibrine dans le poumon. Ces résultats sont en accord avec une autre étude qui montre que les caillots de fibrine ont tendance à se déposer dans le poumon, qui fait office de filtre (Shoji et coll., 1998). De plus, la formation rapide et élevée de métastases est typique dans les mélanomes humains (Liotta et coll., 1987). D'ailleurs, la thrombine augmente fortement les métastases *in vivo* (Klepfish et coll., 1993). Dans la présente thèse, les cellules humaines de mélanome SK-Mel-28 démontrent une augmentation de l'invasion

induite par le TF dans le poumon. Ces résultats sont en accord avec ceux d'une autre étude où la présence de caillots de fibrine augmente nettement l'incidence des métastases de mélanomes dans les poumons (Palumbo et coll., 2000). La suppression du gène de la MTf par un siRNA démontre que l'invasion des cellules SK-Mel-28 induite par le TF. Ce résultat indique que la MTf est directement impliquée dans la propagation des métastases des mélanomes. L'incidence de la MTf sur l'activation du Plg entraîne des événements consécutifs qui affectent l'invasion tumorale des poumons par les mélanomes.

Cette thèse identifie un lien entre la MTf et le système plasminolytique. Nous avons démontré une interaction entre la MTf et deux composantes du système plasminolytique : le Plg et le précurseur de l'uPA. Par la suite, nous montrons que la MTf augmente l'activation du Plg avec ses deux activateurs : l'uPA et le tPA. Pour l'uPA, la MTf affecte la migration cellulaire alors que pour le tPA, elle influence la dégradation de la fibrine dans la matrice extracellulaire. Ces deux phénomènes modulent l'invasion tumorale induite par l'activation de la coagulation intravasculaire où la MTf joue un rôle clé. D'ailleurs, l'inhibition de l'expression de la MTf sur les cellules de mélanomes compromet l'agressivité invasive induite par la coagulation intravasculaire dans les poumons. Ma thèse ouvre la possibilité d'un traitement thérapeutique pour le cancer.

2.2 Réalisation

Cette thèse a permis de réaliser un brevet international. Ce brevet protège la propriété intellectuelle sur l'utilisation de la MTf soluble pour le traitement du cancer et dans la dissolution des caillots sanguins dans les troubles vasculaires. Les renseignements supplémentaires sont disponible à l'annexe 1.

2.3 Perspectives

Les résultats de cette thèse ouvrent de nouvelles perspectives quant à l'étude de l'invasion des cellules cancéreuses. Quatre avenues pourraient être approfondies :

1— L'identification du ligand membranaire de la MTf.

2— L'effet de la MTf soluble sur la croissance tumorale.

3— La caractérisation de la spécificité de l'interaction selon le site d'implantation des métastases.
4— La MTf et l'invasion cérébrale des cellules de mélanomes.
5— La MTf et les maladies coronariennes.

2.3.1 L'identification du ligand membranaire de la MTf

Danse ses travaux, nous avons observé que la MTf joue un rôle dans l'invasion tumorale. La mMTf joue un rôle de catalyseur alors que la forme soluble sMTf inhibe son effet invasif. Les résultats obtenus apportent des informations nouvelles sur un rôle possible de la MTf par rapport aux résultats obtenus lors d'autres études *in vitro* ou sur des modèles murins. Dans le cas des mélanomes, les résultats sont simplement nouveaux. De plus, ces résultats soulèvent un intérêt pour le récepteur membranaire de la MTf qui reste à identifier. Des études sur le transport transendothélial par l'ablation d'un gène associé au LRP ou des études de co-immunoprécipitation pourraient être un moyen efficace d'identifier le récepteur.

2.3.2 L'effet de la MTf soluble sur la croissance tumorale

La prochaine étape porterait sur l'impact de la sMTf sur la croissance tumorale. Le modèle d'injection sous-cutanée de cellules de mélanomes SK-Mel-28 sera

préconisé chez des souris immunodéficientes. Pour le traitement, deux méthodes semblent préférables, soit une injection sous-cutanée ou par voie orale. L'injection sous-cutanée est effectuée grâce à une pompe osmotique (Alzet) qui libère une solution contenant de la sMTf. Pour la voie orale, l'absorption devra être déterminée selon son rendement. Une mesure quotidienne de la tumeur pourra déterminer son volume dans le temps. Le traitement peut être seul ou en combinaison avec un agent chimiothérapie comme le taxol.

2.3.3 La caractérisation de la spécificité de l'interaction selon le site d'implantation des métastases

Afin de vérifier la spécificité de l'interaction entre les cellules cancéreuses et l'endroit d'invasion, deux approches pourraient être utilisées. Premièrement, le modèle d'adhérence *in vitro* permet de comparer les interactions entre différents types de cellules cancéreuses à un endothélium spécifique (cérébral, rénal, pulmonaire, cardiaque ou hépatique). Comme la spécificité d'interaction semble associée à un site spécifique, l'intérêt serait de trouver la séquence utilisée pour chaque type d'endothélium. Deuxièmement, les mêmes cellules cancéreuses pourraient être injectées dans une souris afin d'étudier le rôle de ces mêmes molécules *in vivo*. Par exemple, suite à une injection dans la veine de queue, les cellules SK-Mel-28 forment des métastases dans les poumons et le cerveau de souris immunodéficientes (nu/nu). Le même modèle de métastases pourrait être utilisé pour vérifier l'implication de la MTf dans l'adhérence des cellules SK-Mel-28 *in vivo*, confirmant ainsi les résultats d'adhérence statique. On peut même imaginer qu'utiliser d'une certaine combinaison des molécules d'adhérence permettrait de mettre en évidence le potentiel métastatique de différentes lignées exprimant la MTf.

2.3.4 La MTf et l'invasion cérébrale des cellules de mélanomes

Ces nouvelles données nous amènent à évaluer si la MTf peut être utilisée comme un agent thérapeutique pour les métastases cérébrales puisque la BBB est un filtre empêchant la plupart des agents de chimiothérapie de la traverser. Avec un modèle d'injection sous-crânienne dans les souris immunodéficientes, le traitement à la sMTf par gavage ou par des pompes osmotiques (Alzet) permettrait une mesure quotidienne des signes vitaux des souris pourra déterminer une courbe de survie. Le traitement peut être seul ou en combinaison avec un agent chimiothérapie comme le taxol.

2.3.5 La MTf et les maladies coronariennes

Le développement du volet fibrinolyse pourra aboutir à un traitement dans les pathologies impliquant une obstruction des vaisseaux sanguins. En utilisant le modèle animal où le TF induit des caillots avec la fibrine marquée radioactivement, des données seront générées sur la possibilité d'utiliser la sMTf avec le tPA.

ANNEXE 1

COMPOUND AND METHOD FOR REGULATING PLASMINOGEN ACTIVATION AND CELL MIGRATION

Inventeur: Richard Béliveau, Michel Demeule, Yanick Bertrand, Jonathan Michaud-Levesque, Yannève Rolland et Julie Jodoin

Contribution : Pour le brevet, moi-même, le Dr Demeule et Dr Béliveau avons découvert l'idée originale : l'implication de la MTf comme modulateur de l'activité plasminolytique. Le champ de couverture préconisait un traitement contre le cancer et la dissolution des caillots dans la circulation sanguine. Par la suite, Yannève Rolland, Julie Jodoin et Jonathan Michaud-Levesque ont contribué à élargir avec le concept impliquant l'angiogenèse et le détachement cellulaire. Dans la déclaration d'invention, les parties portant sur l'angiogenèse et le détachement cellulaire sont résumées.

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Accès internet :

http://www.wipo.int/pctdb/en/fetch.jsp?DISP=25&IDB=0&SORT=1167664-KEY&LANG=ENG&LANGUAGE=ENG&SERVER_TYPE=19&FORM=SEP-0%2FHITNUM%2CB-ENG%2CDP%2CMC%2CPA%2CABSUM-ENG&IA=CA2004000697&TOTAL=1&C=0&SEARCH_IA=CA2004000697&START=1&QUERY=WO%2F2004%2F099410&DBSELECT=PCT&TYPE_FIELD=256&RESULT=1&IDOC=47634&DISPLAY=STATUS

Résumé : L'invention concerne la régulation de l'activation Plg afin de moduler la migration de cellules, l'angiogenèse et la fibrinolyse dans le traitement du cancer et des maladies thromboemboliques telles que l'obstruction artérielle. En outre, la présente invention concerne les compositions pharmaceutiques réglant la migration de cellules, l'activité plasminolytique et l'angiogenèse. Elle intéresse plus particulièrement une méthode qui permet de régler l'activation du Plg entrant en contact pendant un certain temps avec une solution contenant les activateurs du Plg (uPA et tPA) et le Plg à de l'aide la MTf, et ce, dans le but de contrôler le phénomène.

WHAT IS CLAIMED IS : method for increasing plasminogen activation, said method comprising contacting a solution containing pro-uroquinase plasminogen

activator (pro-uPA) with (p97) or an enzymatically active fragment thereof for a time sufficient to cause increased plasminogen activation.

2. The method of claim 1, wherein said p97 increase plasminogen activation and fibrinolysis through tissue plasminogen activator (t- PA).

3. A method for inhibiting plasminogen activation, said method comprising the step of activator (pro-uPA) with membrane bound (p97) for a time sufficient to prevent plasminogen activation.

4. A method for preventing cell migration, said method comprising the step of contacting a cell expressing (p97) on its surface with 97 or an antibody, or an antigen binding fragment thereof, directed to said p97 expressed on the surface of said cell, said soluble p97 competing with the p97 expressed on the cell surface, activating plasminogen in solution instead of membrane-bound plasminogen, thus preventing cell migration, said antibody, or active fragment thereof binding p97 on the surface of the cell thus preventing activation of membrane-bound plasminogen, preventing cell migration.

5. The method of claim 4, wherein the antibody is a monoclonal antibody.

6. The method of claim wherein said monoclonal antibody is selected from the group consisting of L235, HybC, HybE, HybF, 9B6 and 2C7.

7. The method of claim 5, wherein said monoclonal antibody is L235.

8. The method of claim 4, wherein said cell is a tumor cell.

9. The method of wherein said cell is selected from the group consisting of human vascular or endothelial cells and human melanoma cells.

10. A method for treating cancer caused by cells expressing (p97) at their surface, said method comprising the step of administering to a patient in need thereof exogenous soluble p97 or an antibody an antibody, or active fragment thereof, directed to said p97 expressed on the surface of said cell, said soluble p97 competing with the p97 expressed on the cell surface, activating plasminogen in solution instead of membrane-bound plasminogen, thus preventing cell migration, said antibody, or active fragment thereof binding p97 on the surface of the cell thus preventing activation of membrane-bound plasminogen, preventing cell migration, preventing cancer cells from spreading.

11. The method of claim 10, wherein the antibody is a monoclonal antibody.

12. The method of claim wherein said monoclonal antibody is selected from the group. consisting of L235, HybC, HybE, HybF, 9B6 and 2C7.

13. The method of claim 11, wherein said monoclonal antibody is L235.

14. The method of claim 10, wherein said cell is a tumor cell.

15. The method of claim 10, wherein said cell is selected from the group consisting of human vascular or microvascular endothelial cells and human melanoma cells.

16. A method for regulating capillary tube formation, said method comprising the step administering to a patient in need thereof soluble 97, wherein said soluble p97 prevents or reduces capillary tube formation and thus angiogenesis.

17. The method according to claim 16, wherein said administering is carried out orally, intravenously, intramuscularly, intraperitoneally, intraarterially, or via a mucus membrane.

18. A pharmaceutical composition for use in regulating activation of plasminogen, said composition comprising a therapeutically effective amount of (p97) or an active fragment thereof in association with a pharmaceutical acceptable carrier.

19. The pharmaceutical composition of claim 18, wherein said p97 is soluble p97 for increasing activation of plasminogen.

20. A method of regulating the activation of plasminogen, comprising administering to an individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to claim 18.

21. The method according to claim 20, wherein said administering is carried out orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, intraarterially, or via a mucus membrane.

22. A pharmaceutical composition for use in regulating cell migration of a cell showing p97 activity, comprising a therapeutically effective amount of one of p97, an active fragment thereof, or an antibody recognizing specifically p97, or an antigen binding fragment thereof, in association with a acceptable carrier.

The pharmaceutical composition of claim 22, wherein said p97 is exogenous soluble p97 for preventing cell migration.

24. The pharmaceutical composition of claim 22, wherein the antibody is a monoclonal antibody.

25. The pharmaceutical composition of claim 24, wherein said monoclonal antibody is selected from the group consisting of L235, HybC, HybE, HybF, 9B6 and 2C7.

26. The pharmaceutical composition of claim 24, wherein said monoclonal antibody is L235.

27. A method of regulating cell migration of a cell showing p97 activity, comprising administering to an individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to claim 22. 28. The method of claim 27, wherein the cell showing p97 activity is a tumor cell..

29. The method of claim 27, wherein said cell is selected from the group consisting of human vascular or microvascular endothelial cells and human melanoma cells.

30. The method of claim 27, wherein said p97 is exogenous soluble p97 for preventing cell migration.

31. The method of claim 27, wherein said administering is carried out orally, subcutaneously, intravenously, intramuscularly, intraperitoneally, transdermally or via a mucus membrane.

32. A pharmaceutical composition for use in treating cancer comprising a therapeutical effective amount of one of (p97), an active fragment thereof, or an antibody recognizing specifically p97, or an antigen binding fragment thereof, in association with a acceptable carrier.

33. The pharmaceutical composition of claim 32, wherein the antibody is a monoclonal antibody.

34. The pharmaceutical composition of claim 33, wherein said monoclonal antibody is selected from the group consisting of L235, HybC, HybE, HybF, 9B6 and 2C7.

35. The pharmaceutical composition of claim 33, wherein said monoclonal antibody is L235.

36. A method of treating cancer, comprising administering to an individual a effective amount of a pharmaceutical composition according to claim 32.

37. The method according to claim 36, wherein said administering is carried out orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, intraarterially, transdermally or via a mucus membrane.

38. The method according to claim 36, wherein said cancer is selected from the group consisting of melanoma, prostate cancer, leukemia, hormone dependent cancer, breast cancer, colon cancer, lung cancer, skin cancer, ovarian cancer, pancreatic cancer, bone cancer, liver cancer, biliary cancer, urinary organ cancer (for- example, bladder, testis), lymphom, retinoblastoma, sarcoma, epidermal cancer, liver cancer, esophageal cancer, stomach cancer, cancer of the brain, cancer of the kidney, and metastasis thereof.

39. A pharmaceutical composition for use in regulating angiogenesis comprising a therapeutically effective amount of (p97) or an enzymatically active fragment thereof in association with a pharmaceutically acceptable carrier.

40. A method of regulating angiogenesis, comprising administering to an individual a effective amount of a pharmaceutical composition according to claim 39.

41. The method according to claim 40, wherein said administering is carried out orally, intravenously, intramuscularly, intraperitoneally, intraarterially, or via a mucus membrane. Use of p97, or an enzymatically active fragment thereof, for regulating fibrinolysis.

Use of p97, or an enzymatically active fragment thereof, for regulating the activation of plasminogen.

Use of p97, or an active fragment thereof, for regulating cell migration of a cell showing p97 activity.

Use of p97, or an enzymatically active fragment thereof, for treating cancer.

Use of p97, or an enzymatically active fragment thereof, for regulating angiogenesis.

Use, of p97, or an enzymatically active fragment thereof, in the manufacture of a medicament for regulating fibrinolysis.

Use of p97, or an enzymatically active fragment thereof, in the manufacture of a medicament for regulating the activation of plasminogen.

Use of p97, or an enzymatically active fragment thereof, in the manufacture medicament for regulating cell migration of a cell showing p97 activity.

Use of or an enzymatically active fragment thereof, in the manufacture of a medicament for treating cancer.

Use of p97, or an enzymatically active fragment thereof, in the manufacture of a medicament for regulating angiogenesis.

52. A method for treating thrombo-embolic disorders, said method comprising the step of administering to a patient in need thereof exogenous soluble p97, said soluble p97 increasing clot permeability and dissolution, thereby treating said disorders.

53. The method of claim 52, wherein said disorders are selected from the group consisting of venous or arterial thrombosis, thrombophlebitis, pulmonary or cerebral embolism, thrombotic microangiopathy and clotting.

54. The method of claim 53, wherein said disorders cause heart or cerebral strokes.

BACKGROUND OF THE INVENTION (a) Field of the Invention The invention relates to novel regulators of plasminogen activation and their use for regulating cell migration and treating cancer.

Furthermore, the present invention relates to novel pharmaceutical compositions form regulating cell migration and treating cancer.

(b) Description of the Prior Art Melanotransferrin (p97) possesses a high level of homology (37- 39%) with human serum transferrin, human lactoferrin and chicken transferrin. It is a glycosylated protein that reversibly binds iron and was first found at high levels in malignant melanoma cells. Two forms of p97 have been reported, one of which is bound to cell membranes by a glycosylphosphatidylinositol anchor while the other form is both soluble and actively secreted. The exact physiological role of either membrane-bound p97 or secreted p97 is largely unexplored.

In the early 1980s, p97 was found to be expressed in much larger amounts in neoplastic cells and fetal tissues than in normal tissues. More recently, it was

reported that p97 mRNA is widespread in normal human tissues. p97 is also expressed in reactive microglia associated with amyloid plaques in Alzheimer's disease. Normal serum contains very low levels of p97, which were reported to increase by 5-to 6-fold in patients with Alzheimer's disease.

It was previously demonstrated that recombinant human melanotransferrin (p97) is transported at high rate into the brain using both an in vitro model of the blood brain barrier (BBB) and in situ mouse brain perfusion (Demeule et coll., 2002). It was also shown that p97 transcytosis might involve the low-density lipoprotein related protein (LRP). This receptor is also known to mediate the internalization of the urokinase:plasminogen activator inhibitor: urokinase receptor complex (uPA:PAI-1: uPAR). Briefly, single-chain proenzyme-uPA is activated upon binding to its cell surface receptor uPAR, which is a glycosylphosphatidylinositol (GPI)-anchored membrane protein. After its activation, uPA (which catalyzes the conversion of plasminogen to plasmin) is quickly inhibited by the plasminogen activator inhibitor type-1 (PAI-1).

The inactive uPA: PAI-1 complex binds to uPAR and then is rapidly internalized by LRP. The uPA: PAI-1 complex is degraded in lysosomes whereas the uPAR is recycled at the cell surface. Other LRP ligands include pro-uPA, PAI-1, receptor-associated protein (RAP) and a diverse spectrum of structurally unrelated proteins.

Heart disease has topped the list of killer diseases every year but one since 1900. (The exception was 1918, when an influenza epidemic killed more than 450,000 Americans.) Stroke is the third leading cause of death in the United States, following cancer. Much of the progress is due to the development of effective medicines to control blood pressure and cholesterol, according to officials of the National Heart, Lung and Blood Institute. But, experts warn, the war against heart disease and stroke is not yet won. Every 33 seconds, an American dies of either heart disease or stroke. Nearly 62 million Americans have one or more types of cardiovascular disease, and these diseases cost our society more than $350 billion a year.

Two strategies are presently used to restore the flow after thrombosis: 1) clot dissolution with administration of plasminogen activators and 2) clot permeation by surgical intervention. The tissue-type plasminogen activator (tPA) and its conventional substrate plasminogen, are key players involve in fibrinolysis. Currently, tPA is used as a stroke therapy, however, its associated adverse effects might limit its efficiency.

It would be highly desirable to be provided with novel regulators of plasminogen activation and their use for regulating cell migration and treating cancer.

It would also be highly desirable to be provided with novel pharmaceutical compositions form regulating cell migration and treating cancer.

It would be highly desirable to be provided with a new treatment for thromboembolic disorders such as venous or arterial thrombosis, thrombophlebitis, pulmonary and cerebral embolism, thrombotic microangiopathy and intravascular clotting. Some of these disorders will lead for example in heart and cerebral strokes.

It would be also desirable to be provided with a new method for increasing fibrinolysis or for preventing angiogenesis.

SUMMARY OF THE INVENTION One aim of the present invention is to provide novel regulators of plasminogen activation and their use for regulating cell migration and treating cancer.

Another aim of the present invention is to provide novel pharmaceutical compositions form regulating cell migration and treating cancer.

A further aim of the present invention is to provide a new treatment for thromboembolic disorders such as, for example, without limitation, venous or arterial thrombosis, thrombophlebitis, pulmonary or cerebral embolism, thrombotic microangiopathy or intravascular clotting, some of which will lead for example in heart or cerebral strokes.

An additional aim of the present invention is to provide a new method for increasing fibrinolysis or for preventing angiogenesis.

In accordance with one embodiment of the present invention there is provided a method for increasing fibrinolysis, said method comprising contacting a solution containing pro-uroquinase plasminogen activator (pro-uPA) with melanotransferrin (p97) or an enzymatically active fragment thereof for a time sufficient to cause increased fibrinolysis.

In a preferred embodiment, p97 increase plasminogen activation through tissue plasminogen activator (t-PA).

In accordance with another embodiment of the present invention there is provided a method for inhibiting plasminogen activation, said method comprising the step of contacting pro-urokinase plasminogen activator (pro-uPA) with membrane bound melanotransferrin (p97) for a time sufficient to prevent plasminogen activation.

In accordance with a further embodiment of the invention, there is provided a method for preventing cell migration, said method comprising the step of contacting a cell expressing melanotransferrin (p97) on its surface with exogenous soluble 97 or an antibody, or an antigen binding fragment thereof, directed to said p97 expressed on the surface of said cell, said soluble p97 competing with the p97 expressed on the cell surface, activating plasminogen in solution instead of membrane-bound plasminogen, thus preventing cell migration, said antibody, or active fragment thereof binding p97 on the surface of the cell thus preventing activation of membrane-bound plasminogen, preventing cell migration.

In a preferred embodiment of the invention, the antibody is a monoclonal antibody, and more preferably one of L235, HybC, HybE, HybF, 9B6 or 2C7.

The cell can be for example, without limitation, an endothelial cell or a tumor cell, such as one selected from the group consisting of human microvascular endothelial cells (HMEC-1) and human melanoma SK- MEL28 cells.

Still in accordance with the present invention, there is provided a method for treating cancer caused by cells expressing melanotransferrin (p97) at their surface, said method comprising the step of-administering to a patient in need thereof exogenous soluble p97 or an antibody an antibody, or active fragment thereof, directed to said p97 expressed on the surface of said cell, said soluble p97 competing with the p97 expressed on the cell surface, activating plasminogen in solution instead of membrane- bound plasminogen, thus preventing cell migration, said antibody, or active fragment thereof binding p97 on the surface of the cell thus preventing activation of membrane-bound plasminogen, preventing cell migration, preventing cancer cells from spreading.

Further in accordance with the present invention, there is provided a, method for regulating capillary tube formation, said method comprising the step administering to a patient in need thereof soluble 97, wherein said soluble p97 prevents or reduces capillary tube formation.

Also in accordance with the present invention, there is provided a pharmaceutical composition for use in regulating activation of plasminogen, said composition comprising a therapeutically effective amount of melanotransferrin (p97) or an enzymatically active fragment thereof in association with a pharmaceutically acceptable carrier.

Preferably, p97 is soluble p97 for increasing activation of plasminogen.

In accordance with the present invention there is also provided a method of regulating the activation of plasminogen, comprising administering to an individual in need thereof a therapeutically effective amount of the aforementioned pharmaceutical composition.

In accordance with the present invention there is also provided a pharmaceutical composition for use in regulating cell migration of a cell showing p97 activity, comprising a therapeutically effective amount of one of p97, an enzymatically active fragment thereof, or an antibody recognizing specifically p97, or an antigen binding fragment thereof, in association with a pharmaceutically acceptable carrier.

Further in accordance with the present invention there is also provided a method of regulating cell migration of a cell showing p97 activity, comprising administering to an individual in need thereof a therapeutical effective amount of the aforementioned pharmaceutical composition.

In accordance with the present invention there is further provided a pharmaceutical composition for treating cancer comprising a therapeutically effective amount of one of melanotransferrin (p97), an enzymatically active fragment thereof, or an antibody recognizing specifically p97, or an antigen binding fragment thereof, in association with a pharmaceutically acceptable carrier.

Also in accordance with the present invention there is further provided a method of treating cancer, comprising administering to an individual a therapeutically effective amount of the aforementioned pharmaceutical composition.

The cancer can be, for example, without limitation, selected from the group consisting of melanoma, prostate cancer, leukemia, hormone dependent cancer, breast cancer, colon cancer, lung cancer, skin cancer, ovarian cancer, pancreatic cancer, bone cancer, liver cancer, biliary cancer, urinary organ cancer (for example, bladder, testis), lymphom, retinoblastoma, sarcoma, epidermal cancer, liver cancer, esophageal cancer, stomach cancer, cancer of the brain and cancer of the kidney.

In accordance with the present invention there is also provided a pharmaceutical composition for use in regulating angiogenesis comprising a therapeutically effective amount of melanotransferrin (p97) or an enzymatically active fragment thereof in association with a pharmaceutically acceptable carrier.

Still in accordance with the present invention there is also provided a method of regulating angiogenesis, comprising administering to an individual a pharmaceutically effective amount of the aforementioned pharmaceutical composition.

In accordance with the present invention, there is provided the use of p97, or an enzymatically active fragment thereof, or of any of the aforementioned composition for the various uses described herein or for the manufacture of medication for the various use described herein..

For the purpose of the present invention the following terms are defined below.

The term"p97"is also referred to in the present invention as Melanotransferrin, MTf, or P97. All of these terms are being used interchangeably. The term soluble p97 thus make reference to soluble p97 or soluble'melanotransferrin.

The term"cancer"is intended to mean any cellular malignancy whose unique trait is the loss of normal controls which results in unregulated growth, lack of differentiation and ability to invade local tissues and metastasize. Cancer can develop in any tissue of any organ. More specifically, cancer is intended to include, without limitation, melanoma, prostate cancer, leukemia, hormone dependent cancers, breast cancer, colon cancer, lung cancer, skin cancer, ovarian cancer, pancreatic cancer, bone cancer, liver cancer, biliary cancer, urinary organ cancers (for example, bladder, testis), lymphomas, retinoblastomas, sarcomas, epidermal cancer, liver cancer, esophageal cancer, stomach cancer, cancer of the brain and cancer of the kidney. cancer is also intended to include, without limitation, metastasis, whether cerebral, pulmonary or bone metastasis, from various types of cancers, such as melanomas, or from any types of cancer mentioned above.

The terms"treatment", "treating"and the like are intended to mean obtaining a desired pharmacologic and/or physiologic effect, e. g. , inhibition of cancer cell growth. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment"as used herein covers any treatment of a disease in a mammal, particularly a human, and includes : (a) inhibiting the disease, (e. g. , arresting its development) ; or (B) relieving the disease (e. g. , reducing symptoms associated with the disease).

The term"administering"and"administration"is intended to mean a mode of delivery including, without limitation, oral, rectal, parenteral, subcutaneous,

intravenous, intramuscular, intraperitoneal, intraarterial, transdermally or via a mucus membrane. The preferred one being orally.

One skilled in the art recognizes that suitable forms of oral formulation include, but are not limited to, a tablet, a pill, a capsule, a lozenge, a powder, a sustained release tablet, a liquid, a liquid suspension, a gel, a syrup, a slurry, a suspension, and the like. For example, a daily dosage can be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

The term"therapeutically effective"is intended to mean an amount of a compound sufficient to substantially improve some symptom associated with a disease or a medical condition. For example, in the treatment of cancer, a compound which decreases, prevents, delays, suppresses, or arrests any symptom of the disease would be therapeutical effective. A therapeutically effective amount of a compound is not required to cure a disease but will provide a treatment for a disease such that the onset of the disease is delayed, hindered, or prevented, or the disease symptoms are ameliorated, or the term of the disease is changed or, for example, is less severe or recovery is accelerated in an individual.

The compounds of the present invention may be used in combination with either conventional methods of treatment and/or therapy or may be used separately from conventional methods of treatment and/or therapy.

When the compounds of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to an individual. Alternatively, pharmaceutical compositions according to the present invention may be comprised of a combination of a compound of the present invention, as described herein, and another therapeutic or prophylactic agent known in the art.

It will be understood that a specific"effective amount"for any particular individual will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, and/or diet of the individual, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing prevention or therapy.

Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like. Salts

derived from organic acids include citric acid, lactic acid, tartaric acid, fatty acids, and the like.

As used herein,"pharmaceutically acceptable carrier"includes any and all solvents (such as phosphate buffered saline buffers, water, saline), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art.

Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in therapeutic compositions is contemplated.

Supplementary active ingredients can also be incorporated into the compositions.

DETAILED DESCRIPTION OF THE INVENTION Materials and Methods Soluble human recombinant p97 which is produced by introducing a stop codon following the glycine residue at position #711 (Fig. 1) and monoclonal antibodies (mAbs) directed against p97 were kindly provided by Biomarin Pharmaceutical Inc. (Novato, CA). TPA, PAI-1 and plasmin are from Calbiochem (La Jolla, CA). Pro-uPA and plasminogen are from American Diagnostica (Greenwich, CT). Angiostatin is purchased from Angiogenesis Laboratories (Tucson, AZ) whereas uPA is from Roche Biochemicals (Laval, QC). CM5 sensor chips are from BIAcore (Piscataway, NJ). The plasmin substrate (D-val-leu-lys-p-nitraniline or VLK-pNA) and other biochemical reagents are from Sigma (Oakville, ON).

Blood-brain barrier model and transcytosis experiments The in vitro model of the blood-brain barrier (BBB) is established by using a c-culture of bovine brain capillary endothelial cells (BBCEC) and newborn rat astrocytes as previously mentioned (Demeule* et al. 2002). p97 is radioiodinated with standard procedures using an iodo-beads kit and D-Salt Dextran desalting columns from Pierce, as previously described (Demeule* et al.. 2002). Transcytosis experiments are performed as follows : one insert covered with BBCECs is set into a six-well microplate with 2 ml of Ringer-Hepes and is pre-incubated for 2 h at 37 °C. [^{125}I]-p97 (0.5-1. 5 uCi/assay), at a final concentration of 25 nM, is then added to the upper side of the insert. At various times, the insert is sequentially transferred into a fresh well to avoid possible reendocytosis of p97 by the abluminal side of the BBCECs. At the end of the experiment, [^{125}I]-p97 is assayed in 500 ul of the lower chamber of each well following TCA precipitation.

Cell culture Cells are cultured under 5% C02/95% air atmosphere. Human microvascular endothelial cells (HMEC-1) are from the Center for Disease Control and Prevention (Atlanta, GA) and are cultured in MCDB 131 media

(Sigma) supplemented with 10 mM L-glutamine, 10 ng/ml epidermal growth factor (EGF), 1 pg/ml hydrocortisone and 10% inactivated foetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) and SK- MEL28 are obtained from ATCC (Manasas, VA). HUVECs are cultured in EGM-2 medium (bullet kit, Clonetics #CC-3162) and supplemented with 20% FBS. Melanoma SK-MEL28 cells are grown in MEM supplemented with 1 mM Na-pyruvate, 100 U/ml penicillin-streptomycin, 1.5 g/L Na- bicarbonate and 10 % FBS.

BIAcore analysis p97, PAI-1 and plasminogen are covalently coupled to a CM5 sensor chip via primary amine groups using the N-hydroxysuccinimide (NHS)/N-ethyl-N'- (dimethylaminopropyl) carbodiimide (EDC) coupling agents. Briefly, the carboxymethylated dextran is first activated with 50 pi of NHS/EDC (50 mM/200 mM) at a flow rate of 5 pl/min. p97, PAI-1 or plasminogen (5 ug) in 20 mM acetate buffer, pH 4.0 are then injected and the unreacted NHS- esters are deactivated with 35 ul of 1 M ethanolamine hydrochloride, pH 8.5. Approximately 8000 to 10000 relative units of p97, PAI-1 or plasminogen are immobilized on the sensor chip surface. Ringer solution or a 50 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl and 50 mM CaCl2 is used as the eluent buffer. Proteins are diluted in the corresponding eluent buffer and injected onto the sensor chip surface.

Protein interactions are analyzed using both the Langmuir binding model, which is the simplest model for 1: 1 interaction between analyte and immobilized ligand, and a two-state conformational change model which describes a 1: 1 binding of analyte to immobilized ligand followed by a conformational change.

Enzymatic assay and cell treatment with soluble p97 The enzymatic activity of pro-uPA is measured using a colorimetric assay. The reaction is performed in a final volume of 200 pi in an incubation medium consisting of 50'mM Tris/HCl buffer (pH 7.5), 150 mM NaCl, and 50 mM Cal2. This incubation medium also contains 15 pg/mi.

VLK-pNA with or without plasminogen. Enzymatic activity is assessed in the absence or presence of p97. The reaction is started by the addition of pro-uPA. In this assay, the cleavage of VLK-pNA results in a p-nitraniline molecule that absorbs at 405 nm. The reaction product is monitored at 405 nm using a Microplate Thermomax Autoreader (Molecular Devices, CA).

HMEC-1 are grown to 85% confluency in 6-well plates and are incubated 18 hrs under 5% C02/95% air atmosphere in cell culture medium with or without p97 (100 nM). Endothelial cells are washed twice with Ringer solution and mechanically scraped from the wells. Cells are counted and frozen at-80 °C until used. A volume corresponding to 100,000 cells is incubated in the plasmin assay

as above and plasmin activity is monitored at 405 nm for 60 min. HMEC-1 are also individualized by PBS citrate solution (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH2PO4, 8.1 mM Na2HP04-7H20, 15 mM Na citrate pH 6.8) for 15 min. Cells are washed twice in Ringer-Hepes solution (150 nM NaCl, 5.2 mM KCl, 2.2 mM Cal2, 0.2 mM MgCl2-6H2O, 6 mM NaHCO3, 5 mM Hepes, 2.8 mM Glucose, pH 7.4) and counted. A volume corresponding to 100,000 cells is incubated in the plasmin assay with mAb L235 (325 nM) or IgG control.

Plasmin activity is monitored at 405 nm for 480 min.

Cell migration assay HMEC-1, HUVEC and SK-MEL28 cell migration is performed using Transwell filters (Costar; 8 um pore size) precoated with 0.15% gelatin for 2 hrs at 37°C. The transwells are assembled in 24-well plates (Falcon 3097) and the lower chambers filled with 500 pl of cell culture medium. To study the effect of p97, mAb L235 or mouse IgG on cell migration, HMEC- 1, HUVEC and SK-MEL28 cells are harvested by trypsinization and centrifuged. Approximatively 10,000 cells are resuspended in 100 pi fresh DMEM medium with or without p97 (native or boiled for 30 minutes at 100°C), mAb L235 or mouse IgG and added into the upper chamber of each transwell (lower chamber of the transwell also contains p97, mAb L235 or non-specific mouse IgG). The plates are then placed at 37°C in 5% C02/95% air for 18 hrs. Cells that had migrated to the lower surface of the filters are fixed with 3.7% formaldehyde in PBS (Ca2+/Mg2+ free), stained with 0. 1% crystal violet/20% MeOH, and counted (4 random fields per filter). Photomicrographs at 100x magnification are taken using a Polaroid Microcam or Nikon Coolpix 500 digital camera attached to a Nikon TMS-F microscope.

Capillary tube formation on Matrigel Matrigel (BD Bioscience, Mississauga, ON) was thawed on ice and 50 pL were added to a 96-well plate and incubated for 10 min at 37 C.

HMEC-1 or HUVEC cells were harvested by trypsinization. 2.5 x 104 cells were resuspended in 100 uL fresh medium and added to Matrigel-coated wells for 30 min at 37 C. After cell adhesion, the medium was removed and 100 uL of fresh cell culture medium with or without soluble p97 was added.

Cells were then incubated for 18 hrs at 37°C. After incubation, tubular structures were visualized at a 40X magnification using a digital Nikon Cooipix 5000 camera attached to a Nikon TMS-F microscope. The length of the total capillary network was quantified using a map scale calculator by measuring and summing the lenght of all tubular structures observed in a chosen field.

Western Blot Analysis HMEC-1 (3 x 106 cells) were plated into a 75 cm2 culture flask and exposed to complete medium containing 0,10 or 100 nM soluble p97. After 18 hours treatment, the cells were washed twice with PBS (Ca+2/Mg+2 free) and solubilized in lysis buffer (1% Triton-X-100, o. 5% NP-40,150 mM NaCI, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 2% N-octylglucoside, 1 mM orthovanadate, pH 7,5) for 30 minutes on ice.

Human plasma Human blood samples were collected into a citrated Vacutainer (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 300 x g for 5 minutes at 4°C. Plasma were aliquote in eppendorfs and used fresh or frozen at-80°C until used.

Thromboelastography analysis Thromboelastography analysis was performed with citrated plasma or artificial clot model using a computerized dual-channel thromboelastograph (TEG) analyzer (model 5000; Haemoscope Corp., Niles, IL). For the artificial clot model fibrinogen (8.2 pM), glu-plasminogen (3.3 pM) and tPA (4.5 nM) diluted in buffer A were transferred into the analyzer cups. Artificial clots were polymerized with thrombin (0.4 U/ml).

For the plasma clot model, 350 ul of citrated plasma were transferred into the analyzer cups with tPA (4.5 nM). $CaCl_2$ (0.2 M) was added to initiate the polymerisation of plasma clot. The thromboelastograph analysis for both artificial and plasma clots were performed in the presence or absence of1uMp97.

Radial clot lysis assay Radial clot lysis assay was performed. Briefly, fibrin-clots were obtained by incubating fibrinogen (8.2 pM), glu-plasminogen (2 uM) and 0.4 U/ml of thrombin in buffer A at 37°C for 60 min in a 6-wells plate. Clot lysis was initiated by dropping 2 pi of tPA (2 nM) with or without p97. Clots were incubated for 30 min at 37°C and dyed with chinese ink. Photomicrographs at 40x magnification were taken using a digital camera Nikon Coolpix 5000 camera (Nikon Canada, Mississauga, ON) attached to a Nikon TMS-F microscope (Nikon Canada).

Data analysis Statistical analyses are made with the Student's paired t-test using GraphPad Prism (San Diego, USA). Significant difference is accepted for p values less than 0.05.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I Transcytosis of p97 through BBCEC monolayers Transcytosis experiments are performed at 37°C for 2 hrs. [^{125} I]-p97 (25 nM) is added to the

upper side of the cell-covered filter in the absence or presence of RAP (650 nM) or BSA (5 uM). At the end of the experiment, radiolabelled proteins are measured in the lower chamber of each well by TCA precipitation. Results represent means SE (n=6) (Fig. 10A). In the second part of the experiment (Fig. 10B), p97 is immobilized on a sensor chip surface (CM5) as described in the Materials and Methods section above and p97, RAP and BSA (5 pg/100 pl) are injected over the immobilized p97.

The first evaluation was the transcytosis of p97 across an in vitro model of the BBB at 37°C (Fig. 10A). A significant (>50%) reduction in the transport of [^{125}I]-p97 (25 nM) from the apical (blood side) to the basolateral side (brain side) of BBCEC monolayers was observed in the presence of 640 nM RAP. Transcytosis of [^{125}I]-p97 was unaffected by a 200-fold molar excess of BSA. The permeability coefficient for sucrose is similar in the absence or presence of RAP indicating that the integrity of the BBCEC monolayers was unaffected by this protein. The results with RAP also indicate that LRP is involved in p97 transcytosis since it has been reported to be an LRP ligand, whereas BSA was shown to bind to megalin, another member of the LDL receptor family, probably via cubilin (Kozyraki R et al., 2001). To determine whether protein interaction could occur between p97 and RAP, leading to a reduction in p97 transcytosis, protein interactions were investigated by using biological interaction analysis in real-time (Fig. 10 B). For this analytical approach, p97 was first immobilized on the surface of a sensor chip. Using standard NHS/EDC coupling procedures about 8 to 10 ng/mm2 of p97 were immobilized. RAP or BSA (0. 05 ug/pl) were then injected over immobilized p97. No interactions could be observed between these proteins and p97, indicating that the inhibition of [^{125}I]-p97 transcytosis is not related to protein interactions between p97 and RAP.

EXAMPLE II Pro-uPA and p97 interaction Biospecific interaction analysis between p97 and anti-p97 mAbs Biospecific interaction analysis in real-time between p97 and various anti-p97 mAbs is performed as follows. p97 is immobilized on a sensor chip (CM5) using standard coupling procedures incorporating NHS, EDC and ethanolamine. Different mAbs directed against p97 (HybC, HybE, HybF, L235,2C7, 9B6), diluted to 0. 05 pg/pi in Ringer/Hepes, are injected into the BIAcore at a flow rate of 5 pl/min. The surface plasmon resonance response obtained for these mAbs is plotted (in relative units (RU)) as a function of time. After each injection immobilized p97 is regenerated with 0.2M glycine at pH 2 for 2 min (n =4).

To evaluate the impact of immobilization procedures on the structural integrity of p97 different mAbs directed against various conformational epitopes of p97 were injected over p97 (Fig. 11). The surface plasmon resonance (SPR) signal generated by the interaction between p97 and various mAbs varied from 250

relative units (RU) to 2500 RU. These data show that the mAbs could still recognize p97, indicating that the protein is intact following its immobilization on the sensor chip surface. Table 1 shows the kinetic parameters estimated by the BIAevaluation software for antibody interactions with p97. From these values, the affinity constant (KA=ka/Kd) of these mAbs for immobilized p97 ranged from 0. 08 to 1.6 nM-1 and for the relative affinities are HybE;L235;9B6;2C7, HybC<HybF.

TABLE 1 Kinetics of interaction between immobilized p97 and mAbs.

 <P>Antibodies ARU Ka Kd KA=Ka/Kd KD=Kd/Ka

 (M-1s-1) (s-1) (M-1) (M) L235 1055~82 4. 4x104 5. 3x10-5 0. 9x109 0.1x10-10 HybC 1509184 7. 2x104 4. 5x10-5 1. 6x109 6. 4x10-10 HybE 23252 0. 9x104 9. 8x10-5 0.08x109 0.01x10-10 HybF 2199+150 8. 0x104 3. Ox10-5 2. 7x109 3. 8x10-10 9B6 244013 1. 2x104 9. 1x10-5 1. 3x109 7.9x10-10 2C7 229087 5. 9x104 3. 8x10-5 1. 6x109 6.5x10-10 The difference between the relative units measured after and before injection of mAbs directed against p97 are presented (ARU) as well as the apparent association (Ka) and dissociation (Kd) constants. The affinity (KA) and dissociation (KD) constants were calculated from the Ka and Kd.

Molecular interactions of p97 and various components of the PA: plasmin system Determining the molecular interactions between p97 and various components of the PA: plasmin system was as follows. Pro-uPA and tPA (0. 05 ug/ui), diluted in Ringer/Hepes, are injected onto immobilized p97 on a sensor chip at a flow rate of 5 pl/min. The SPR response for these proteins is plotted in RU as a function of time. p97 (0.05 pg/ui) is also injected over immobilized PAI-1 (p97/PAI-1). Plasminogen, plasmin or angiostatin (0. 05 ug/ul) are also injected onto immobilized p97. The SPR response for these proteins is plotted in RU as a function of time. The results indicate that pro-uPA and plasminogen interact with p97. After each injection the sensor chip surface with immobilized p97 is regenerated by injecting 10 mM glycine, pH 2.2 for 2 min.

When pro-uPA and tPA (0.05 µg/ul) were injected over immobilized p97, protein interaction occurred between pro-uPA and p97 but not between tPA and p97 (Fig. 12A). About 8-10 ng/mm2 of PAI-1 was also immobilized onto another well of a sensor chip surface using NHS/EDC coupling conditions. No interaction between p97 and immobilized PAI-1 could be detected (Fig. 12A). However a strong interaction could be observed when tPA was injected over PAI-1, indicating that PAI-1 can still interact with tPA following immobilization. In addition, plasminogen, plasmin and angiostatin (0. 05 pg/pl) were injected over immobilized p97 (Fig. 12B). According to the SPR, plasminogen also interacts with immobilized p97 whereas plasmin and angiostatin, two plasminogen fragments, do not. The kinetic data obtained from binding of pro-uPA or

plasminogen to immobilized p97 biosensor surface were evaluated using both the 1: 1 Langmuir binding model and the two state conformational change model. Interestingly, the two state conformational change model was a better fit than the 1: 1 Langmuir binding model when comparing a single concentration of either pro-uPA and plasminogen over p97 biosensor surface. Kinetic data obtained with the two state conformational model are presented in Table 2. Kinetic data for the interaction between pro-uPA and p97 shows an association constant (kas) of 6.6 x 103 M-1s-1 and a dissociation rate constant (kd1) of 1.7 x 10-3 s-1. Furthermore, the forward rate constant (ka2= 3.2 x 10-3 S 1) and backward rate constant (kd2= 7.1 x 10- 4 1) for the conformational change provide. an apparent equilibrium dissociation constant ((KD= kd1/ka1)/(kd2/ka2)) of 65 nM. The kinetic analysis of plasminogen interaction with p97 shows an association constant (ka1) of 2.1 X104 M-1s-1. The dissociation rate constant (kd1=4. 3 x10-2 s-1), as well as the forward rate constant (ka2) of 6.0 x10-2 s-1 and backward rate constant (kd2) of 1.1 x10-3 s-1, are different from those seen for the pro- uPA interaction with p97. However, the apparent equilibrium dissociation constant (KD) between p97 and plasminogen is 350 nM, which is different from that observed for the interaction of pro-uPA with immobilized p97.

TABLE 2 Kinetics of interaction between immobilized p97 and pro-uPA or plasminogen using the two state conformational model Immobilized Ligands ka1 ka2 kd1 kd2 KD

 proteins (x 104 M-1s-1) (x10-3 s-1) (x10-3 s-1) (x10-4 s-1) (x10-9 M)

 p97, pro-uPA 66.2 3.2 6.0 7.1 65 plasminogen 2.1 6.0 3.1 11.2 350 Kinetic parameters of Table 3 were based on a two state conformational change binding model using the biosensorgram shown in Fig. 12. This model describes a 1: 1 binding of analyte to immobilized ligand followed by a conformational change in the complex. It is assumed that the conformationally changed complex can only dissociate through the reverse of the conformational change: A + B = AB = ABx. The dissociation constants (KD) were derived using both association (ka) and dissociation (kd) rates (Ko = (kd1/ka1) x (kd2/ka2). The parameters are: ka1, association rate constant for A+B1= AB1 (M-1s-1) ; kd1, dissociation rate constant for AB1=A+B1 (s') ; ka2, forward rate constant for AB=ABx (s-1) ; kd2, backward rate constant for AB=ABx (s-1). The mean Chi2 values for the sensorgram fits were less than 0.4.

Effect of p97 on pro-uPA, tPA and plasminogen To evaluate the effect of p97 interaction on pro-uPA, tPA and plasminogen, the serine activity (VLK-pNA hydrolysis) of 90 nM pro-uPA and 75 nM tPA were measured in the absence (o) or presence (•) of 70 nM p97 without plasminogen using a colorimetric assay, both with and without p97 (Figs. 13 and 19). The reaction was performed in a final volume of 200 pl as described in the Materials and Methods section above,

in both Figs. 13A and 13B, controls were also performed with p97 (g) but without pro- uPA or tPA (n=9, for pro-uPA; n=6, for tPA). In the absence of p97, only a slight activity was measured for both pro-uPA and tPA. However, the VLK- pNA hydrolysis by pro-uPA goes from less than 50 AU/min in the absence of p97 to more than 450 AU/min when p97 is added into the incubation (Fig. 13A). Addition of p97 to tPA elicits no observable effect and p97 alone had no proteolytic activity (Fig. 19B). The results from both SPR and enzymatic activity indicate that the change in pro-uPA conformation induced by p97 increased its ability to degrade the plasmin substrate.

To determine whether interaction with p97 leads to a cleavage of pro-uPA, the proteins were co-incubated for 5 min. at 37°C in the presence or absence of plasminogen. They were then separated by SDS-PAGE under reducing conditions using a 12.5% acrylamide gel and stained with standard Coomassie Blue. The results are shown in Fig. 13C. The lanes of the gel are as follows (Fig. 13C) : 2 pg of p97 (lane 1), 1 pg pro-uPA (lane 2) and 2 pg plasminogen (lane 3) were incubated for 5 min. at 37°C alone as controls. Pro-uPA (2 pg) was incubated at 37°C for 5 min. with 2 ug of p97 (lane 4). Plasminogen and Pro-uPA were added without incubation (lane 5) and with 5 min. incubation at 37°C (lane 6). Pro-uPA with 2 ug of both p97 and plasminogen were added without incubation (lane 7) or with 5 min. incubation at 37°C (lane 8). Tc-uPA (2 μg) was also loaded as a control (lane 9). Under these conditions p97 and uPA migrated as 97 kDa and 33 kDa bands, respectively, whereas pro-uPA migrated as a single band at 55 kDa. No major degradation of either protein could be detected, indicating that the incubation of pro-uPA with p97 under the conditions used to perform the VLK-pNA hydrolysis did not cleave either protein. Even after 6 hours incubation at 37°C, both proteins were stable. In the presence of plasminogen, pro-uPA was cleaved after an incubation of 5 min. at 37°C and two major fragments of 33 kDa and 29 kDa could be observed. When p97 was added to the incubation medium, the generation of these fragments did not change.

The impact of p97 on plasminogen fragmentation by pro-uPA was further estimated using 6 hours incubation at 37°C and the results are shown in Fig. 13D. The lanes of the gel are as follows : 3 μg of p97 (lane 1), glu-plasminogen (lane 2) and lys-plasminogen (lane 3) were incubated alone for 6 hours at 37°C as controls. In lane 4, 3pg of both glu- plasminogen and p97 were also incubated for 6 hours at 37°C. Pro-uPA (20 ng) was added to plasminogen for the same period of incubation at 37°C (lane 5). p97 was added to pro-uPA and plasminogen for 6 hours at 37°C (lane 6) or 4°C (lane 7). In lane 8, 3 μg of angiostatin (lane 8) was also added as a control. Proteins were separated on a 7.5% acrylamide gel under non-reducing conditions and stained with Coomassie blue.

When p97 is added to glu-plasminogen no apparent fragment was generated. In contrast, the addition of a low amount (10 ng) of pro-uPA, which could not be detected using standard Coomassie blue staining, induced degradation of Glu-plasminogen with the appearance of fragments which migrated at the same molecular weight as Lys-plasminogen.

Moreover, when p97 is added to glu-plasminogen and pro-uPA, the degradation profile of glu-plasminogen is changed. In the presence of p97 with glu-plasminogen and pro-uPA, higher levels of bands migrating at the same molecular weight as lys-plasminogen were observed and two other fragments appeared at 50 and 30 kDa. These fragments do not seem to be related to angiostatin since they migrated at a different molecular weight than did the control angiostatin at 42 kDa. These results indicate that p97 alters the cleavage of glu-plasminogen by pro-uPA.

EXAMPLE III Plasminogen activation by p97 The interaction of p97 with pro-uPA was further characterized by measuring the activation of plasminogen by pro-uPA in the presence of p97 (Fig. 14). The plasminolytic activity of 1 nM uPA was measured without (o) or with (•) 70 nM p97 in the presence of 30 nM plasminogen. The reaction was performed in a final volume of 200 μl as described in the Materials and Methods section above. As a control, the enzymatic activity in the presence of p97 alone was also measured (■). When p97 is added to pro- uPA and plasminogen, the VLK-pNA hydrolysis is 4-fold higher after 180 min (Fig. 14A). Control experiments performed with p97 indicated that this protein alone does not generate plasmin when it is added to plasminogen.

The plasmin activity in the presence of various concentrations of p97 was also measured (Fig. 14C). Plasmin activity induced by pro-uPA was measured in the presence of various p97 concentrations. Since the generation of plasmin proceeds at a constant rate under the assay conditions used, plotting the experimental data as a function of time (t) 2 allowed for the determination of the initial rate of plasmin formation. From these linear curves, the initial plasmin activity measured in the absence of p97 was subtracted from the activities obtained in the presence of various p97 concentrations. Thus, the data represent the initial rates of plasmin activity (corresponding to the slopes) in the presence of various p97 concentrations. p97 stimulates the plasminogen cleavage by pro-uPA in a dose-dependent manner with half-maximal stimulation occurring at 25 6 nM.

The effect of p97 on plasmin activity in the presence of various concentrations of plasminogen was also measured (Fig. 14B). Plasmin activity induced by pro-uPA

was measured without (o) or with (•) 250 nM p97 and various concentrations of plasminogen. Initial rates of plasmin activity calculated at several plasminogen concentrations were plotted as a function of plasminogen concentrations. The resulting experimental data were fitted using nonlinear regression analysis. p97 decreased the apparent Km of pro-uPA for plasminogen from 188 22 to 102 17 nM and increased the Vmax from 6.9 0.4 to 8.9 0.6 AU/min. These results indicate that p97 positively affects the activation of plasminogen by pro- uPA by increasing the catalytic efficiency by a factor of 2. 4-fold.

To determine whether the induction of plasmin formation by p97 was specific, the formation of plasmin by pro-uPA in the presence of either the mAb L235 (directed against p97) or a non-specific IgG was measured (Fig. 14D). The plasminolytic activity of pro-uPA was measured in the presence of 70 nM p97 and 65 nM of either mAb L235 (o) or non-specific mouse IgG (•). One representative experiment is shown and data represent the means SD of values obtained from triplicates (n=3). MAb L235 (50 nM) inhibited the pro-uPA activation induced by p97 by 50%. These results indicate that the effect of p97 upon pro-uPA's activation of plasminogen is specific and involves the epitope recognized by the mAb L235.

EXAMPLE IV Inhibition of cell migration by mAb L235 Since p97 affects the activation of plasminogen in vitro and since the uPA/uPAR system is important in cell migration, it was further investigated whether endogenous p97 might be associated with this process. Cell migration of HMEC-1, SK-MEL28 cells or HUVEC was measured using modified Boyden chambers as described in the Materials and Methods section above. Because p97 was first identified in melanoma cells (Brown JP et al., 1981 Proc Natl Acad Sci USA 78: 539-543), the impact of the mAb L235 on the migration of human melanoma (SK-MEL28) cells was also measured (Fig. 15A). Cells that had migrated to the lower surface of the filters were fixed and stained with crystal violet. Images obtained from a representative experiment are shown. Cells that had migrated in the presence of 50 nM mAb L235 or a non-specific mouse IgG were also counted. The results were expressed as the percentage of the control measured in the presence of a non-specific mouse IgG and represent the means SD (n=5 for HMEC-1; n=4 for SK-MEL28; n=3 for HUVEC).

Statistically significant differences are indicated by ***$p < 0.001$ (Student's t-test). In the presence of mAb L235 (50 nM), the migration of HMEC-1 and SK-MEL28 cells was inhibited by 54% and 48%, respectively.

However, cell migration of HUVEC was unaffected by this concentration of mAb L235.

Endogenous p97 was immunodetected in lysates or serum-deprived culture media (18 hours) from HMEC-1, SK-MEL28 and HUVEC cells. Fig. 15B shows the detection of endogenous p97 by Western blot analysis.

Proteins were separated by SDS-PAGE and were electrophoretically transferred to PVDF membranes. p97 was detected by Western blotting using mAb L235 and a secondary anti-mouse IgG linked to peroxidase. p97 migrated under unreduced conditions at 73 and 60 kDa, as previously observed. It was highly expressed in lysates from HMEC-1 and SK-MEL28 cells and at lower levels in their respective conditioned culture media. In HUVEC cells, p97 was however almost undetectable. In fact, the exposure time was at least 30 times greater to detect a much lower level of p97 in HUVEC compared to HMEC-1 and SK-MEL28 cells. These results indicate that mAb L235, by interacting with endogenous p97, inhibits the migration of HMEC-1 and SK-MEL28 cells. This also indicates that the endogenous p97 in these cells is involved in cell migration.

EXAMPLE V Effect of exogenous p97 on cell migration It was also estimated whether exogenous p97 could affect the migration of HMEC-1 and SK-MEL28 cells. HMEC-1 and SK-MEL28 cell migration was performed using modified Boyden chambers as described in the Materials and Methods section above. Cells that had migrated in the presence or absence of p97 (100 nM) to the lower surface of the filters were fixed and stained with crystal violet. The results are shown in Figs.

16A and 16B. Cells that had migrated were also counted and expressed as a percentage of the control cells, measured in the absence of p97 (n=4, for HMEC-1; n=3, for SK-MEL28). Exogenous p97, at 10 nM and 100 nM, inhibited the migration of HMEC-1 cells by 34% and 50% (Fig. 16C). The migration of SK-MEL28 cells was inhibited by 44% and 70% in the presence of 10 and 100 nM p97. Migration of HUVEC cells was unaffected by these concentrations of p97. Moreover, this inhibition of cell migration is not related to a reduction of endothelial or melanoma cell adhesion since the same concentrations of p97 did not affect adhesion on gelatin of either HMEC-1 or SK-MEL28 cells.

EXAMPLE VI Inhibition of plasminolytic activity at the cell surface by soluble p97 and mAb L235 The effect of p97 on plasminolytic activity was determined as follows. HMEC-1 cells were treated for 18 hours with 100 nM p97 (+p97) or Ringer solution (Control). Following this treatment the plasminolytic activity was measured using standard conditions, as described in the Materials and Methods section above. When cells were treated with p97 (100 nM), plasminogen activation was inhibited by 95% (Fig. 17A). This marked reduction in the plasminolytic capacity of these cells by soluble p97 could explain the inhibition of HMEC-1 migration. The effect of mAb L235 on plasminolytic activity of HMEC-1

was also determined. HMEC-1 cells (1 x 105 cells) were pre-incubated 1 hr. at 37°C with Ringer solution (Ctl) or with 250 nM of either mAb L235 or non-specific mouse IgG. Following this pre-incubation, the plasminolytic activity was measured for 6 hrs by adding pro-uPA (1 nM) and plasminogen (50 nM) using standard conditions, as described in the Materials and Methods section. The plasminolytic activity of HUVEC was also measured using 1 x 105 cells under the same conditions. Data represent the means SD of three independent experiments performed in triplicate. Statistically significant differences are indicated by *** where $p < 0.001$ (Student's t-test). When HMEC-1 cells were treated with the mAb L235, the plasminolytic activity was inhibited by more than 50% compared to non-specific mouse IgG (Fig. 17B). This inhibition by the mAb L235 indicates that endogenous, membrane-bound p97 participates in plasminogen activation in HMEC-1.

EXAMPLE VII Anti-Angiogenic Properties of p97 Angiogenesis, a complex multistep process that leads to the outgrowth of new capillaries from pre-existing vessels, is an essential mechanism in wound healing, embryonic development, tissue remodeling, and in tumor growth and metastasis. This process involves EC proliferation, migration and morphogenic differentiation into capillary-like structures. One of the key elements in cell migration is the urokinase-type plasminogen activator receptor (u-PAR). The plasminogen activator (PA) family is composed of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA); their inhibitors are the plasminogen activator inhibitor type 1 and 2 (PAI-1 ; PAI-2). u-PAR mediates the internalization and degradation of u-PA/inhibitor complexes via the low-density lipoprotein receptor-related protein (LRP), whereas LRP mediates the internalization and degradation of t-PA/inhibitor complexes. Thus, the u-PAR/LRP system controls cell migration by regulating plasminogen activation by PAs at the cell surface. PAs are therefore involved in angiogenesis by enhancing cell migration, invasion and fibrinolysis. Moreover, plasminogen needs to be first converted to the two-chain serine protease plasmin. When Glu- plasminogen, the native circulating form of the zymogen, is bound to the cell surface, plasmin generation by PAs is markely stimulated compared with the reaction in solution. Optimal stimulation of plasminogen activation at the EC surface requires the conversion of Glu-plasminogen to Lys- plasminogen (Michaud-Levesque et coll., 2005).

In summary, p97 stimulates the plasminolytic activity of single chain urokinase plasminogen activator (sc-uPA or pro-uPA), uPA and tissue plasminogen activator (tPA) in vitro. In addition, low density lipoprotein related protein (LRP) and the urokinase activator receptor (uPAR) are down regulated in p97 treated MHEC-1 cells. Furthermore, HMEC-1 and HUVEC capillary tube formation is inhibited by low concentration of soluble p97 (Michaud-Levesque et coll., 2005).

Overall, these results indicate that soluble p97 stimulates plasmin-and MMP-dependent endothelial cell detachment.

Consequently, these are the first data indicating that exogenous human recombinant soluble p97 have anti-angiogenic properties, by affecting the morphogenic differentiation of EC into capillary-like structures, by interfering with key proteins involved in angiogenesis and by inducing EC detachment (Rolland et coll., 2006).

EXAMPLE VIII Melanotransferrin Increases Human Blood Clot tPA-fibrinolysis Regulation of plasminogen is a key element in blood clot fibrinolysis.

In the present invention, potential interactions between human recombinant p97 with components of the plasminogen activator system in relation with fibrinolysis were investigated. By using biospecific interaction analysis, it is demonstrated herein that p97 interacts with immobilized plasminogen.

Kinetics analysis of the biosensorgrams using two state conformation change model shows an apparent equilibrium dissociation constant KD of 2.6 x10-7 M for this interaction (Fig. 20). Moreover, soluble p97 increased the tPA-dependent plasminogen activation. This induction by p97 is inhibited by the monoclonal antibody L235 directed against p97 indicating that the increase in the plasminolytic activity is specific to p97 (Figs. 19A, 19B and 19C). p97 also enhanced the tPA fibrinolysis of plasma and fibrin clots (Fig. 21,22,23). The thromboelastography of fibrinolysis and clot strength were evaluated with or without p97 (Fig. 25). Complete lysis time (CLT) was reduced in the IVM (in vitro model) and plasma by 50% and 20% respectively when p97 was added to tPA. There was also a difference in the fibrinolysis by tPA at 30 min (LY30) in both models when p97 was added. The LY30 was enhanced by 5-and 2-fold in both artifical and blood clots, respectively. These results indicates that p97, by interacting with plasminogen, enhanced plasminogen activity by tPA reduced time of thrombolysis. In conclusion, these results demonstrate the potential of the present invention in new treatments of arterial disease and thrombosis and to reduce the damages to occluded hearth tissues.

Interaction between p97 and plasminogen using biospecific interaction analysis in real-time Plasminogen was immobilized on BIAcore with standard coupling procedures. Various concentrations of p97 were injected over immobilized plasminogen. The estimated constant of dissociation (KD) estimated from these curves for the interaction between p97 and immobilized plasminogen is 275 nM. The results of this experiment are shown in Fig. 20.

Melanotransferrin (p97) increases the plasminogen activation by tissue plasminogen activator (tPA) Hydrolysis of the peptide VKL was measured in the presence of p97 alone, tPA and tPA+p97. As shown in Fig. 23A, in the presence of p97 the plasminogen activation by tPA was increased by 4-fold. As shown in Fig. 19C, the plasminogen activation by tPA was increased in a dose- dependent manner by p97 with half-maximal stimulation occurring at 12 3 nM.

Inhibition of the p97 effect by the monoclonal antibody L235 The plasmin activity was measured in the presence of tPA and p97 with the monoclonal antibody directed against p97 (mAb L235) or a non- specific mouse IgG (mouse) IgG). As shown in Fig. 19B, the induction caused p97 of the plasminogen activation by tPA is inhibited by the monoclonal antibody directed against p97 indicating that this induction is specific to p97. p97 increases clot fibrinolysis induced by tPA The effect of p97 on fibrinolysis was measured using a thromboelastograph. In the thromboelastography analysis (TEG), 320 gui of citrated plasma or artificial clot model (8. 2, uM fibrinogen, 2, uM glu- plasmingen and 0.4 U/ml thrombin) was transferred into analyser cups with tPA (4.5 nM) and in the presence or absence of p97 (1 uM). The cups were placed in computerized dual-channel TEG analyzer (model 5000; Haemoscope Corp., Niles, IL). In one of the cups (channel 1), tPA was added,, in another cup (channel 2) p97 and tPA were added. All cups containing 20 pl 0.2M $CaCl_2$ were prewarmed to 37°C and analyzed simultaneously. The TEG variables collected from each sample included : CLT (clot lysis time), G (clot strength or Shear elastic modulus in dyn/s2, defined as G= (5000A)/ (100-A)), LY30 and LY 60 (percent of clot lysis at 30 and 60 min after maximun clot strenght is achieved). As shown in Fig. 25A, when p97 was added to the artificial clot, the clot lysis at 30 min was increased by 5-fold. As shown in Fig. 25B, in the presence of p97, the lysis at 30 min of human blood clot by tPA was increased by 2-fold.

Because soluble p97 interacts with glu-plasminogen, the inventors have investigated whether human recombinant p97 might affect fibrinolysis and clot permeation. To show that soluble p97 could modulate fibrinolysis, the impact of human recombinant soluble soluble p97 on plasminogen activation by tPA (Fig. 19A) was first determined. After 180 minutes, the addition of soluble p97 increased by 6-fold the plasminogen activation by tPA measured by the hydrolysis of the VLK-peptide. Soluble p97 alone has no proteolytic or plasmin-like activity. The induction of tPA-dependent plasminogen activation by soluble p97 was also measured in the presence of the mAb L235 directed against soluble p97 or a non-specific IgG (Fig. 19B). The mAb L235, at 50 nM, inhibited by 80% the effect of soluble p97 on plasminogen activation by tPA. These results suggest that the effect of soluble p97 on plasminogen activation is rather specific and involves the conformational epitope recognizes by the mAb L235. In addition,

plasmin activities measured as a function of time allowed us to extract initial rates.

These rates were plotted as a function of soluble p97 concentrations (Fig. 19C). Soluble p97 stimulated the tPA-dependent conversion of plasminogen to plasmin in a dose-dependent manner with half-maximal stimulation occurring at 52 nM. The effect of soluble p97 on plasmin formation by tPA in the presence of various concentrations of plasminogen (Fig. 19D) was further evaluated. Initial rates of plasmin activity plotted as a function of plasminogen concentrations indicate that soluble p97 decreases the apparent Km of tPA for plasminogen by 5-fold from 280 to 52 nM. In Fig. 23A, the plasminolytic activity of tPA (60 ng) was measured without (o) or with 10 pg/ml p97 (•) in-the presence of Plg (0. 5 μg).The reaction was performed in a final volume of 200 u as described in the Materials and Methods section. The plasminolytic activity in the presence of p97 alone was also measured. in Fig. 19B, the plasminolytic activity of tPA was measured in the presence of p97 (5 ug/ml) and either the mAb L235 (o) or a non-specific mouse IgG (o). The reaction was performed in a final volume of 200 ul as described in the Materials and Methods section. In Fig. 19C. plasmin activity induced by tPA was determined by measuring VLK- hydrolysis in the presence of various p97 concentrations. In Fig. 19D. initial rates of VLK-hydrolysis during Plg activation by tPA were measured without (o) or with 50 nM p97 (•) in the presence of various concentrations of Plg.

Data are shown as means of 3 experiments.

To further characterize the soluble p97 effects on the action of tPA in fibrinolysis, the effect of soluble p97 on a radial tPA-fibrinolysis assay (Fig. 24) was evaluated.

The addition of soluble p97 to tPA enhances its action and leads to an increase perforation of the fibrin-clot (Fig. 21A). Surprisingly, in this experiment performed without tPA, soluble p97 in the presence of plasminogen creates a perforation of the fibrin-clot. Moreover, the size of the perforation increases as a function of soluble p97 concentration (Fig. 21B). In absence of plasminogen and tPA, the fibrin-clot is unaffected by soluble p97 alone. To determine whether soluble p97 has an intrinsic fibrinolytic activity, the release of fibrin fragments from clots labeled with [^{125}I]-fibrin (Fig. 22) was measured. In spite of its ability to perforate the clot, soluble p97 alone does not generate [^{125}I]-fibrin fragments. However, soluble p97 in the presence of plasminogen increases the release of [^{125}I]- fibrin fragments by 2.5 fold following plasminogen activation by tPA.

The impact of soluble p97 on clot fibrinolysis by tPA was also measured ex vivo (Fig. 22). The addition of soluble p97 increases by 2.5- fold the action of tPA. In Fig. 22, the fibrinolytic activity of tPA (1 nM) on plasma clot fibrinolyis was measured ex vivo in the presence of increasing concentrations of p97.

In the blood coagulation system, the tissue-type plasminogen activator (tPA) is associated with fibrinolysis. tPA, mainly express by endothelial cells, cleaves the circulating plasminogen to the active proteinase plasmin which is the major enzyme responsible for the proteolytic degradation of the fibrin fiber. Currently, tPA is a stroke therapy which efficacy may be limited by neurotoxic side effects. Since soluble p97 potentialize plasminogen activation by tPA, the impact of soluble p97 on clot formation and lysis by thromboelastography analysis (TEG) has been evaluated using first an artificial fibrin-clot model (Fig. 25A). This model allowed to monitor the effect of soluble p97 on tPA-fibrinolysis in the absence of plasmin inhibitor. The fibrin clot is formed by the action of thrombin on fibrinogen and this clot also contains glu-plasminogen (2 pM).

In Fig. 25, representative tracing showing effects of p97 (1 uM) on the fibrinolysis of clot formation under shear by TEG. In Fig. 25A illustrates a thrombelastogram of the fibrin clot model and Fig. 26B illustrates a Thromboelastogram of plasma recalcified after addition of 2 mM $CaCl_2$.

The results shown here are representative of 3 experiments. The monitoring of the TEG paramaters indicates that the addition of soluble p97 increases the thrombolytic activity of tPA (Table 3). In particular, when soluble p97 (1 pM) is added to tPA, the lysis of the clot after 30 min (LY30) after its complete formation is 5 times higher whereas the complete lysis time (CLT) is 50% shorter. The impact of soluble p97 on fibrin-clot dissolution using human citrated plasma (Fig. 25B) was further evaluated.

For these analysis, $CaCl_2$ is added to initiate the polymerisation of plasma clot. The TEG parameters obtained for these experiments (Table 4) indicate that the addition of soluble p97 to tPA causes a 30% decrease in the clot strength (G), increases twice the fibrinolysis rate and reduces the CLT by 20%.

Table 3 Effects of p97 on thromboelastograph parameters Parameters Conditions tPA tPA + a. Artificial fibrin-clot 1. G d/sc 4987 44617 2. Lys (30) % 6.5 31.9 3. CLT min 54.7 30.3 b. Fibrin-clot with citrate-treated serum 1. G d/sc 134651586 95601626 2. Lys (30) % 4. 30. 7 11. 84. 0 3. CLT min 68. 31. 6 49. 16. 3 G (d/sc) is the maximum strength of the clot at maximum amplitude of the TEG trace.

The present findings are significant for several reasons. First, it was discovered that soluble p97, by interacting with plasminogen, enhances its activation by tPA. Furthermore, it is established that protein-protein interaction could positively regulate the activity of an enzyme by inducing a conformational change which lead to the exposure of active cryptic site. In addition, the data presented here in the radial clot lysis assay and the TEG analysis provide further evidence that soluble p97 positively regulates the tPA-dependent fibrinolysis by mainly decreasing the clot strength and time of lysis. Overall, the data indicate that soluble p97 increases the efficacy of the anti-thrombolysis agent tPA.

Second, perforation of the clot by soluble p97 without any release of fibrin fragments indicates that soluble p97 interaction with plasminogen induces a change in the fibrin-clot structure. Soluble p97 greatly facilitates the tPA action, leading to a localized and accelerated fibrinolysis.

In conclusion, the data presented herein indicates that human recombinant soluble p97 is as a switch activator of plasminogen since its interaction with plasminogen leads to an increase in the clot permeation and fibrinolysis by tPA. Thrombolysis with blood clot dissolving agent like tPA can reduced mortality in acute myocardial infraction.

EXAMPLE IX Inhibition of angiogenesis by Melanotransferrin During angiogenesis, cells must proliferate and migrate to finally invade the surrounding extracellular matrix (ECM). Moreover, metastasis is associated with tissue remodeling and invasion. In fact, when processing from migration to invasion, an additional complexity is added, as invasion comprises not only cell locomotion, but also the active penetration of cells into ECM (Michaud-Levesque et coll., 2006)

DISCUSSION The data clearly show that both pro-uPA and plasminogen interact with p97 and that these interactions are specific since no interaction between p97 and other proteins including tPA, PAI-1, plasmin, angiostatin, BSA, or ovalbumin could be measured. These results are the first to describe potential interactions between p97 and proteins of the uPA system.

In addition to its interaction with pro-uPA and plasminogen, p97 stimulates plasminogen activation by decreasing the Km of pro-uPA for plasminogen and by increasing the Vmax of the reaction. The conversion of pro-uPA to two-chain uPA occurs by proteolytic cleavage of a single peptide bond (Lys158-Ile159 in human uPA). This conversion can be catalyzed by plasmin or several other proteases such as plasma kallikrein, blood coagulation factor XIIa, cathepsin B, cathepsin L and prostate- specific antigen. In the present invention the SPR assay, the enzymatic assay and electrophoresis experiments all indicate that p97 induces a

conformational change that increases pro-uPA activity without any apparent cleavage of pro-uPA. The two-state conformational model gave the best fits for the interactions of both pro-uPA and plasminogen with immobilized p97 on the BIAcore. Such good fits of experimental data to a multi-state model of interaction are an indication that a conformational change is taking place. Interestingly, the fragments of plasminogen generated by adding p97 were different from the plasminogen degradation by pro-uPA alone. These biochemical analyses further suggest that p97 could also be seen as a cofactor in uPA-dependent plasminogen activation.

The uPA/uPAR system has been involved in several pathological and physiological processes which require cell migration, such as tumor cell invasion and metastasis. Several reports showed that the uPA/uPAR system plays a key role in signal transduction as well as in regulation of melanoma cell migration and angiogenesis. As shown in the present invention, when p97 is added to both compartments of the Boyden chamber migration of HMEC-1 is inhibited by more than 50%. Thus, given the important role of plasmin, a protein like p97 which targets the formation of plasmin and acts on the migration of endothelial cells as well as of SK- MEL28 cells will thus affect angiogenesis and cancer progression. It was also observed in the present invention that the basal capacity for plasminogen activation by HMEC-1 decreased following p97 treatment. A recent study demonstrated that the expression of LDL receptor-related protein 1 B (LRP1 B), a new member of the LDL receptor family, lead to an accumulation of uPAR on the cell surface which event inhibits the migration of CHO cells. From these results, it was proposed that LRP1B negatively regulates uPAR regeneration and function whereas the net results of uPAR regeneration, seems to depend on the relative expression of the two receptors.

Recently, it was shown that when glu-plasminogen is bound to cell surfaces, plasmin generation by plasminogen activators is markedly stimulated compared to the reaction in solution. This is a key element for cell migration where the process of"grip and go"would play an important role. The process of plasminogen activation system is regulated by two different mechanisms: 1) cell surface-binding sites which facilitate the productive catalytic interactions with plasminogen and thereby increases plasmin generation, and 2) protein inhibitors such as serpin inhibitors which restrict the activities of the proteases. In light of this, soluble p97 participates in the activation of plasminogen without being in the pericellular environment (Fig. 18A). The present invention also indicates that the migration and the plasminolytic activity of cells expressing p97 are inhibited by mAb L235, indicating that endogenous, membrane-bound p97 are involved in these processes which are associated with cancer and angiogenesis (Fig. 18B). Moreover, both the migration of HMEC-1 and the plasminolytic activity are diminished when exogenous p97 is added, indicating that soluble p97 affects the

regulation of plasminogen activation at the cell surface (Fig. 18C). Thus, by breaking the equilibrium between soluble p97 and membrane bound p97, it is possible to affect cell migration of HMEC-1 and SK-MEL28 cells.

In conclusion, these are the first results indicating that p97 interacts with pro-uPA as well as with plasminogen and regulates the activation of plasminogen by pro-uPA. As shown in the present invention migration of HMEC-1 and SK-MEL28 cells is inhibited by mAb L235 and soluble p97, indicating that active and functional p97 participates in this process.

Collectively, the results thus indicate that the balance between membrane- bound and soluble p97 could affect cell migration.

As mentioned above, these are the first data indicating that exogenous human recombinant soluble p97 have anti-angiogenic properties, by affecting the morphogenic differentiation of EC into capillary- like structures, by interfering with key proteins involved in angiogenesis and by inducing EC detachment.

Also as mentioned previously, the data presented herein indicates that human recombinant soluble p97 can be seen as a switch activator of plasminogen since its interaction with plasminogen leads to an increase in the clot permeation and fibrinolysis by tPA. Thrombolysis with blood clot dissolving agent like tPA can reduced mortality in acute myocardial infraction. However, damage can occur since the blow flow is restored by only 60% after 90 min. The results presented herein suggest that soluble p97 could increase the efficiency of the thrombolytic agent (tPA) when co- administrated, Furthermore, since the reoccluded clots are usually more resistant to tPA, soluble p97 administration could counter this adverse effect by increasing the therapeutic window of tPA. According to the American Heart Association, two million Americans suffer from atrial fibrillation, in which the two small upper chambers of the heart quiver instead of beating effectively. Blood in these quivering chambers can clot, travel and obstruct blood circulation. This phenomenon can also happen in the vein, where, the clot would obstruct as well. Soluble p97 would enhance tPA effectiveness and broaden its therapeutic window. P97 has also the power to modify clot structure. Moreover, p97-containing gel could also be used to control new blood vessel growth and to reduce the need for coronary bypass surgery and provide effective treatment for a debilitating cardiovascular disease.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary

practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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Ma participation comme co-premier auteur est complète.La participation du Dr Demeule comme permier co-auteur avec moi-même, a permis de rehausser la qualité de l'article étant mon premier dans une revue qui possède un très bon facteur d'impact. Il a été rédigé conjointement. J'ai produit les figures 2, 3, 4, 5, 6b, 8 et 9. Le Dr Demeule a produit la figure 1 et les tableaux 1 et 2. Jonathan Michaud-Levesque a corrigé une partie du texte. Yannève Rolland a contribué à la figure 6 et 7.

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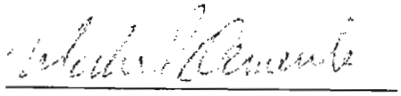
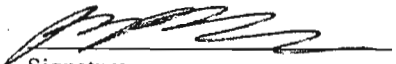
1) Michel Demeule Nom, prénom	 Signature	5 dec 06 Date
2) Richard Béliveau Nom, prénom	 Signature	6 dec 06 Date
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Ma participation est entière pour cet article, le texte et les figures. Dr Demeule a participé au contenant par les corrections du texte et la reformulation. Le Dr Rivard nous a permis d'utiliser son tromboelastogramme pour produire une figure et un tableau.

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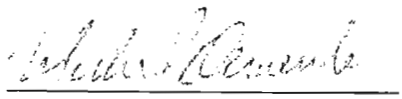
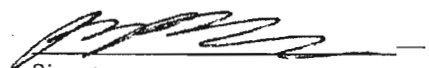
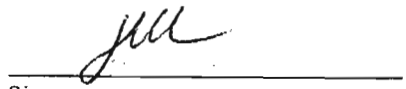
1) Michel Demeule Nom, prénom	 Signature	5 dec 06 Date
2) Richard Béliveau Nom, prénom	 Signature	6 dec 06 Date
3) Jonathan Michaud- Levesque Nom, prénom	 Signature	5 dec 06 Date

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