UNIVERSITÉ DU QUÉBEC À MONTRÉAL

IMPLICATION DES VOIES DE SIGNALISATION INTRACELLULAIRES RÉGULANT LES FONCTIONS DE LA MMP-9:

ACTION D'UN NOUVEL AGENT ANTI-MÉTASTASIQUE

MÉMOIRE PAR ARTICLES
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PAR
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Ibn Sina (Avicenne).

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LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

(p/v)	(poids/volume)
APS	persulfate d'ammonium
AND	acide déoxyribonucléique
ARN	acide ribonucléique
ARNm.	acide ribonucléique messager
AP-1	protéine activatrice 1
BSA	albumine sérique bovine
EDTA	acide éthylènediamine tétraacétique
ELAV	système visuel anormal mortel embryonnaire
ERK1/2	kinase 1 et 2 régulatrices des signaux extracellulaires
FGF	facteur de croissance des fibroblastes
GTPase	guanidine tris phosphatase
HA	acide hyaluronique
JNK	c-Jun- N-terminal kinase
MMPs	métalloprotéinases matricielles
MT-MMPs	métalloprotéinases matricielles membranaires
MEC	matrice extracellulaire
MAPKinase	protéines kinases activées par des mitogènes
NaCl	chlorure de sodium
NFκB	facteur nucléaire κB
NO	oxyde nitrique
P38	protéine 38
PAI-1	inhibiteur des activateurs du plasminogène
PSP94	protéine sécrétoire de la prostate 94

PKA	protéine kinase A
PKC	protéine kinase C
PDGF	facteur de croissance dérivé des plaquettes
PMA	13-acétate de phorbol 12-myristate
PVDF	polyvinylidène difluorure
RhoA	gène homologue de la famille de Ras, Membre A
RT-PCR	transcription inverse- réaction en chaine de la polymérase
SDS	dodécylsulfate de sodium
SDS-PAGE	sodium dodécylsulfate- électrophorèse sur gel polyacrylamide.
TEMED	N, N, N', N'-tétraméthylènediamine
TGF	facteur de croissance transformant
TNFα	facteur de nécrose tumorale alpha
TIMP	inhibiteur tissulaire des métalloprotéinases
Tris	tris (hydroxyméthyl) aminoéthane
TX-100	triton X-100
tPA	activateur tissulaire du plasminogène
uPA	activateur urokinase du plasminogène
VEGF	facteur de croissance de l'endothélium vasculaire
WT	type sauvage

LISTE DES SYMBOLES

%	pourcentage
α	alpha
ß	beta
γ	gamma
□	kappa
ºC	degré Celcius
kDa	kilo Daltons
min	minute
ml	millilitre
µl	microlitre
µM	micromolaire
mM	millimolaire
nm	nanomètre
mg	milligramme
µg	microgramme
rpm	rotations par minute

RÉSUMÉ

Les métalloprotéinases matricielles (MMPs) jouent un rôle crucial dans le développement malin des tumeurs. Elles dégradent la matrice extracellulaire (MEC) permettant ainsi la migration des cellules cancéreuses vers d'autres foyers. Ce processus d'invasion tumorale est appelé "processus métastasique". La MMP-9, appelée aussi gélatinase B, est fortement exprimée lors de ce processus. Son expression peut être régulée à différents niveaux *via* des voies de signalisation intracellulaires, notamment au niveau de l'expression du gène, mais aussi lors de la transcription, la stabilité de son ARNm, la traduction ou la sécrétion de la protéine.

Des agents anti-tumoraux sont en cours de développement ciblant les différentes étapes du développement cancéreux. Le PCK3145, un peptide synthétique, dérivé de la PSP94, dirigé contre le cancer de la prostate avancé et métastasé est au stade d'essais cliniques en phase II. Ce peptide réduit chez la souris immunodéficiente la croissance des xénogreffes de tumeurs prostatiques humaines en diminuant les concentrations plasmatiques de la MMP-9. Nos travaux visent à élucider *in vitro* les mécanismes moléculaires impliqués dans la régulation des fonctions de la MMP-9 *via* l'effet anti-métastasique du PCK3145. Dans un premier temps, nous avons étudié l'interaction de la MMP-9 avec la surface de la cellule cancéreuse. Nous démontrons que le PCK3145 inhibe la sécrétion de la MMP-9 et déclenche le processus de clivage de CD44 de la surface cellulaire *via* une cascade de signalisation intracellulaire impliquant la GTPase RhoA. Dans un second lieu, nous avons étudié le mécanisme d'action du PCK3145 menant à l'inhibition de la sécrétion de la MMP-9. Nous démontrons que cette inhibition requiert le récepteur de la laminine à 67 kDa et est dépendante de l'activation de la voie des MAPKinases. Le PCK3145 entraine la diminution de la sécrétion de la MMP-9 *via* HuR, une protéine intracytoplasmique qui stabilise l'ARNm de la MMP-9 en se liant à sa séquence riche en AU.

Les propriétés anti-métastasiques du PCK3145 peuvent être dirigées contre d'autres types de tumeurs, autres que le cancer de la prostate, liés à l'augmentation de la MMP-9 et à la dégradation de la MEC lors d'un processus métastasique. Par ailleurs, l'identification du mode d'action du PCK3145 *via* le récepteur de la laminine à 67 kDa permet de cibler des cancers dont le taux de ce récepteur est particulièrement élevé telle que la leucémie.

Mots clés : cancer de la prostate, métastase, migration, adhésion, MMP-9, HuR, ERK1/2, RhoA, CD44.

PREMIÈRE PARTIE

INTRODUCTION

CHAPITRE I

REVUE DE LA LITTÉRATURE

1.1 Le cancer

1.1.1 Fréquence du cancer et facteurs de risque

Longtemps considéré comme le mal des pays en développement, le risque de cancer est devenu aujourd'hui un grave problème de santé publique pour ces pays. Avec la difficulté supplémentaire d'un diagnostic beaucoup plus tardif: 80 % des malades sont en effet diagnostiqués à un stade incurable. Partout dans le monde, ils augmentent à un rythme alarmant. La fréquence des cancers pourrait augmenter de 50 % dans le monde avec 15 millions de nouveaux cas en 2020 (contre 10 millions en 2000) selon le *World Cancer Report* (WCR) 2003. En 2004, *l'American Cancer Society* estime qu'il y a eu 1 368 030 nouveaux cas diagnostiqués de cancers et que quelques 563 700 américains en sont décédés. Cela représente environ 1 500 décès par jour.

Depuis 1990, plus de 18 millions de nouveaux cas de cancers ont été diagnostiqués. Le cancer s'avère la seconde cause de mortalité en importance aux Etats-Unis et au Canada. Cette augmentation du nombre de cas de cancers serait principalement due au vieillissement de la population ainsi qu'à l'adoption croissante de modes de vie préjudiciables à la santé, l'importance du tabagisme et l'alimentation déséquilibrée, pour ne nommer que ces derniers (figure 1.1). Ces facteurs de risque sont à l'origine de 43% de tous les décès imputables au cancer en 2000 (WCR) soit

2,7 millions de morts. Les infections sont également la cause de nombreux cancers comme celui de l'estomac, du foie ou du col de l'utérus. Dans les pays en développement, elles sont responsables de 23 % des tumeurs malignes contre 8 % dans les pays développés. Les cancers du foie dus au virus de l'hépatite B peuvent être prévenus par la vaccination, une intervention encore trop rare dans les pays en développement. Le cancer de l'estomac, causé par la bactérie *Helicobacter pylori,* peut être évité en instaurant un traitement antibiotique dans les premières années de la contamination (Doron et Gorbach. 2006).

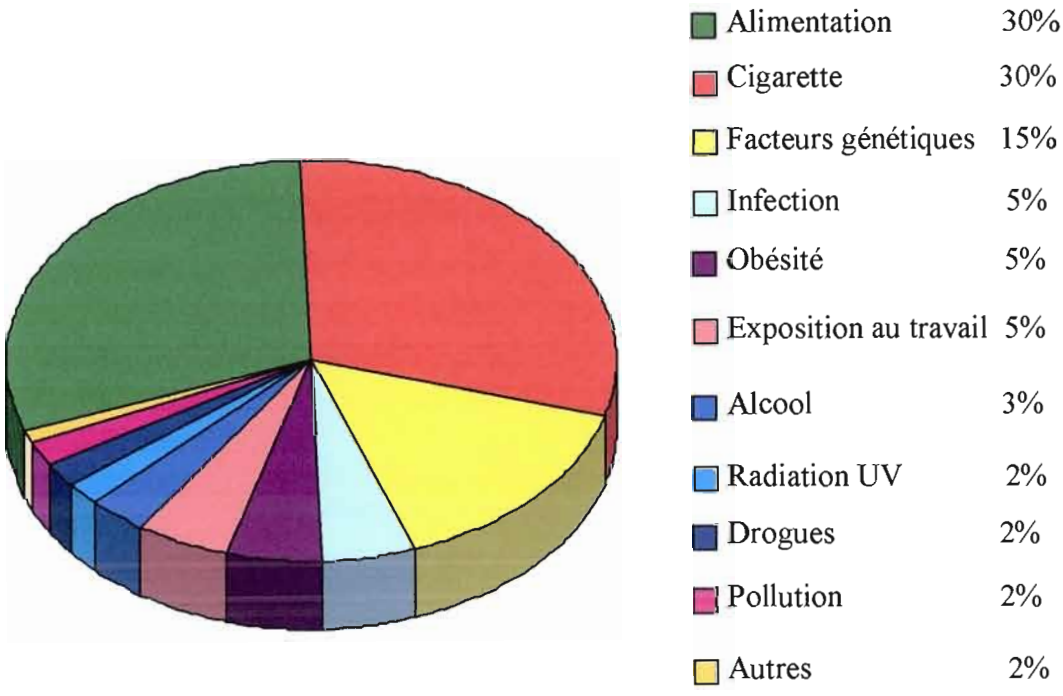

Figure 1.1 Facteurs de risque du cancer (World Cancer Report, 2003)

1.1.2 Caractérisation de la cellule cancéreuse

La cellule, unité microscopique du corps humain, est la plus petite quantité de matière vivante possédant une vie autonome et pouvant se reproduire. La capacité de se diviser, de se spécialiser, et de mourir est inscrite dans le génome ou ADN (acide déoxyribonucléique) de chaque cellule saine. Sous l'influence de multiples facteurs de l'environnement, l'ADN humain subit des altérations et des mutations génétiques, la plupart du temps réparables par des systèmes de réparation de l'ADN. Parmis ces systèmes de réparation, la P53; une protéine suppresseur des tumeurs, responsable de l'activation de la transcription d'une séries de protéines impliquées dans le contrôle du cycle cellulaire, l'apoptose et la sénescence (vieillissement physiologique) (Lavin et Gueven, 2006). Lorsque ces systèmes ne suffisent pas à la réparation, soit parce qu'ils sont dépassés par le nombre de mutations, soit parce qu'ils sont défectueux et ne réparent pas totalement l'ADN muté, les cellules échappent à toute régulation. Elles se multiplient et prolifèrent d'une façon désordonnée et anarchique et ne répondent plus aux signaux environnants. Cette altération génétique constitue la base de tous les types de cancers. Elles s'accumulent généralement en plusieurs années, le génome des cellules tumorales acquiert alors des allèles mutants de proto-oncogènes, de gènes suppresseurs de tumeurs et de gènes contrôlant directement ou indirectement la prolifération et la mort cellulaire. La conséquence de ces mutations génétiques est l'acquisition de nouvelles propriétés dont la capacité de générer leurs propres signaux mitogènes, de résister aux signaux externes d'inhibition de la croissance, de proliférer sans limite (*immortalisation*), d'infiltrer les tissus adjacents et de constituer une néo-vascularisation (*angiogénèse*) (Mosnier et coll., 2005). (Voir aussi section 1.2.2). La prolifération aboutit à la formation de la tumeur, qui, en se développant arrive à détruire les cellules normales avoisinantes et même à envahir d'autres tissus.

Les cancers sont subdivisés en deux très grandes catégories: ceux qui concernent les cellules hématopoïétiques (les cellules du sang) à l'origine des leucémies et des lymphomes, et ceux communément nommés tumeurs solides, associés aux autres tissus, tels que les mélanomes (les cancers de la peau), les gliomes (cancer du cerveau), les carcinomes (les cancers des épithéliums) ou les adénocarcinomes (cancers glandulaires).

1.1.3 Phénotypes tumoraux

La croissance anormale des cellules constitue la propriété typique des tumeurs ou néoplasmes. Elles sont autonomes et la multiplication cellulaire échappent à la régulation de la croissance et ne répondent plus aux mécanismes qui limitent leur prolifération.

Un néoplasme, ressemble plus ou moins au tissu sain qui lui a donné naissance. Cette ressemblance se trouve dans la structure (forme des cellules) avec des anomalies dans les noyaux: la taille (augmentation, inégalité: anisocaryose); le contenu (chromatine irrégulièrement répartie, hyperchromatisme), la forme (contours irréguliers, incisures) et les mitoses (augmentation de nombre, anomalies de forme); ainsi que des anomalies dans le cytoplasme: diminution de taille et augmentation du rapport nucléocytoplasmique et basophilie. Le néoplasme est très différencié si la ressemblance est forte, peu différencié si la ressemblance est floue et complètement indifférencié si elle a fini par prendre un aspect qui n'évoque plus vraiment le tissu normal (Mosnier *et coll.,* 2005).

Les tumeurs sont divisées en deux catégories, les tumeurs bénignes et les tumeurs malignes (ou cancéreuses) (Mosnier et coll., 2005). Les tumeurs bénignes sont bien différenciées, locales, grossissent peu, évoluent lentement, envahissent peu ou pas les tissus voisins, et ne produisent pas de métastases. Les tumeurs malignes, en général, sont peu différenciées, grossissent beaucoup, évoluent plus rapidement. La

propriété la plus importante d'une tumeur maligne est sa capacité, d'une part, à envahir les tissus proches, d'autre part, à se disséminer dans les organes éloignés (Mosnier et coll., 2005). Cette dissémination à distance, par la circulation sanguine ou lymphatique, est appelée métastasique. Cette nouvelle tumeur qui en résulte se met à évoluer pour son propre compte (Hanahan et Weinberg, 2000).

1.2 Cancérogenèse

1.2.1 De la tumeur aux métastases

La dissémination de la tumeur dans l'organisme est l'une des principales caractéristiques d'un cancer (figure 1.2). 1) Les cellules cancéreuses primaires se détachent de leur lieu d'origine, 2) passent dans la circulation sanguine ou lymphatique et vont se loger dans un autre foyer (os, poumons, reins...). 3) Ces groupements de cellules qui se multiplient dans des tissus ou des organes éloignés sont appelés des métastases (une explication plus profonde des mécanismes de dissémination de la tumeur dans l'organisme est élaborée dans la section 1.3). Le développement et la progression tumorale et métastasique sont assurés par l'apport nutritionnel *via* des vaisseaux sanguins. Cette évolution dépend de l'agressivité d'un cancer (invasif, infiltrant, métastasique), et peut avoir lieu rapidement ou se produire très lentement, et même ralentir ou s'arrêter momentanément. Le rythme d'évolution d'un cancer sera donc un facteur déterminant dans le choix du traitement approprié (voir séction 1.5.1).

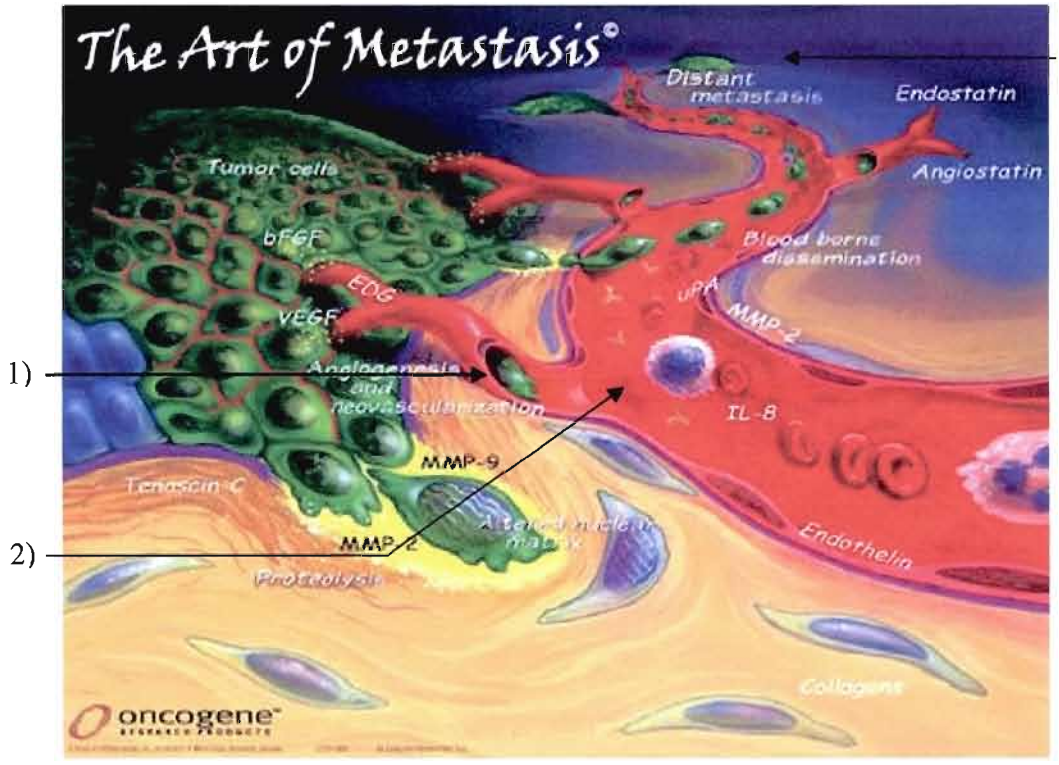

Figure 1.2 Dissémination de la tumeur dans l'organisme (Adaptée d'après Oncogène, 2005) 1) Détachement cellulaire; 2) Passage des cellules dans la circulation sanguine; 3) Formation de métastases dans des foyers distants.

1.2.2 L'angiogenèse tumorale

Le développement des tumeurs et l'invasion métastasique dans l'organisme nécessitent la formation de nouveaux vaisseaux à partir de vaisseaux préexistants. Cette néo-vascularisation est indispensable au développement des tumeurs et cette hypothèse a été découverte il y a plus de trois décennies (Folkman, 1971). Elle alimente les cellules tumorales en nutriments et en oxygène. De nombreuses molécules sont capables d'interagir pour favoriser cette néo-vascularisation.

Lors du processus tumoral, les cellules tumorales augmentent dans la taille seulement par la multiplication de leurs propres cellules, et se retrouvent étroitement collées les unes aux autres dans le même volume d'espace, les cellules se trouvent alors en manque d'oxygène ou hypoxie, celle-ci favorise la migration des cellules de

la moëlle osseuse, les MSC (bone-marrow-derived stromal cells) (Annabi *et coll.,* 2003), et serait impliquée dans la transcription de gènes spécifiques tel le HIF-1 (Hypoxia Inducible Factor-1) qui, à son tour, stimule des voies intracellulaires dépendantes de la RhoA GTPase impliquée dans le développement tumoral (Turcotte, *et coll.,* 2003; Turcotte, *et coll.,* 2004) et active des gènes codant pour des promoteurs angiogéniques tels que le facteur de croissance de l'endothélium vasculaire et son récepteur (VEGF et le VEGFR-1) (Buchler, 2003).

Le VEGF a une activité mitogène spécifique pour les cellules endothéliales *in vitro* et un fort pouvoir angiogénique *in vivo.* L'expression de ce facteur et de ses récepteurs est corrélée avec le développement des tumeurs. Il a été rapporté dans notre laboratoire que l'hypoxie favorise la migration des cellules stromales dérivées de la moëlle osseuse de souris et la formation de tubes capillaires *via* l'activation d'une protéase membranaire, la MT1-MMP (Annabi *et coll.,* 2003). D'autres facteurs, tel le b-FGF (basic fibroblast growth factor), exercent un puissant effet mitogène et chimiotactique pour les cellules endothéliales, fibroblastiques et musculaires lisses des vaisseaux. L'ensemble de cette néo-vascularisation, et toutes les réactions cellulaires et moléculaires associées est appelé angiogenèse. Parmis ces réactions, certaines sont cellulaires: la prolifération des cellules endothéliales, leur migration cellulaire, le détachement et l'adhésion cellulaire et la formation de tubes capilaires; et d'autres moléculaires : la régulation des voies de signalisation dépendantes du VEGF, l'induction des métalloprotéinases, protéines impliquées dans la dégradation de la matrice extracellulaire, l'inhibition de l'apoptose dans les cellules endothéliales par des molécules pro-angiogéniques (tableau 1.1). Ce phénomène confère alors un avantage à la tumeur lui permettant l'infiltration et l'invasion de tissus sains avoisinants et distants par dissémination métastasique.

L'angiogenèse peut être impliquée dans des phénomènes physiologiques lors de l'embryogenèse, la cicatrisation, le cycle menstruel. Cependant, les nouveaux vaisseaux formés se stabilisent par un retour à l'équilibre entre les promoteurs et les

inhibiteurs de l'angiogenèse (tableau 1.1). Un état pathologique survient lorsque cet équilibre est déplacé. Dans le cas d'une surexpression de facteurs d'inhibition d'angiogenèse, survient la maladie d'Alzheimer, l'athérosclérose, l'ostéoporose et l'hypertension artérielle (Carmeliet, 2003). Dans le cas contraire où la surexpression de promoteurs angiogéniques survient, elle provoquerait l'arthrite rhumatoïde, l'asthme, le psoriasis, la rétinopathie diabétique, les maladies cardiovasculaires et le cancer (Carmeliet, 2003). Il a été rapporté que les vaisseaux tumoraux se distinguent des vaisseaux normaux de par leur structure (forme irrégulière, dilatée et possédant une organisation chaotique) et leur physiologie (perméabilité accrue, flux sanguin irrégulier, hémorragique en raison de leur surexpression de VEGF) (Bergers et Benjamin, 2003).

Tableau 1.1 Inhibiteurs et activateurs endogènes régulant l'angiogenèse tumorale

Inhibiteurs	Activateurs
Angiostatine	Facteurs de croissance transformant angiogénine
Récepteur du facteur de croissance basique des fibroblastes	Angiopoiétine
Endostatine	Angiotropine
Interféron-alpha	Facteur de croissance des fibroblastes
Interleukin 1,6, 12	Facteur stimulateur des granulocytes
Protéine reliée à la prolifférine placentaire	Facteur de croissance hépatique
Facteur plaquettaire 4	Interleukine 8
Prolactine	Métalloprotéinases matricielles
Thrombospondine	Facteur de croissance placentaire
Inhibiteurs tissulaires des métalloprotéinases (TIMPs)	Facteur de croissance dérivé des plaquettes
Facteur de croissance transformant-béta	Prolifférine
	Facteur de nécrose tumoral (TNF-α)
	VEGF

Adapté d'après Shepherd et Sridhal, 2003

1.3 Mécanismes cellulaires et moléculaires extracellulaires impliqués dans la cancérogenèse

1.3.1 La matrice extracellulaire

L'espace qui entoure les cellules, appelé matrice extracellulaire (MEC) contient un ensemble de macromolécules : des polysaccharides ou glycosaminoglycanes comme l'acide hyaluronique, le dermatan sulfate, l'héparine; des protéines fibreuses comme le collagène et l'élastine et des protéines d'adhésion comme la fibronectine et la laminine (Egeblad et Werb, 2002). La MEC joue un rôle essentiel dans la constitution, le remodelage de l'architecture tissulaire et le maintien des vaisseaux sanguins. Les constituants de la MEC sont synthétisés et sécrétés par les cellules comme les fibroblastes et les chondroblastes et dégradés par des protéases telles les métalloprotéinases matricielles (MMPs) (Egeblad et Werb, 2002).

1.3.2 Le détachement, l'adhésion et la migration des cellules cancéreuses

Les cellules saines sont liées entre elles et à la MEC par des systèmes de jonction et par des molécules d'adhésion. La modulation d'expression des molécules d'adhésion et la diminution des jonctions intercellulaires entre les cellules tumorales facilitent et conduisent à l'invasion tumorale. Ces molécules d'adhésion sont principalement des récepteurs protéiques extracellulaires. Elles jouent un double rôle lors du développement tumoral, d'abord au niveau de la liaison à la MEC *via* une partie extracellulaire, assurant l'adhésion cellulaire dans le réseau matriciel afin de permettre le mouvement de la cellule lors de la migration cellulaire, puis au niveau de l'induction des signaux intracellulaires *via* leur partie intracytoplasmique. Deux principaux types de récepteurs ont jusqu'à maintenant été étudiés: les membres de la famille des récepteurs intégrines, et les membres de la famille de récepteurs non-intégrines

1.3.2.1 Récepteurs intégrines

Les intégrines sont des glycoprotéines ubiquitaires transmembranaires avec une portion extracellulaire et une portion intracellulaire, représentées par une 'tête' et deux 'jambes' formant deux sous-unités: α (représentée en bleu) et β (représentée en rouge) (figure 1.3).

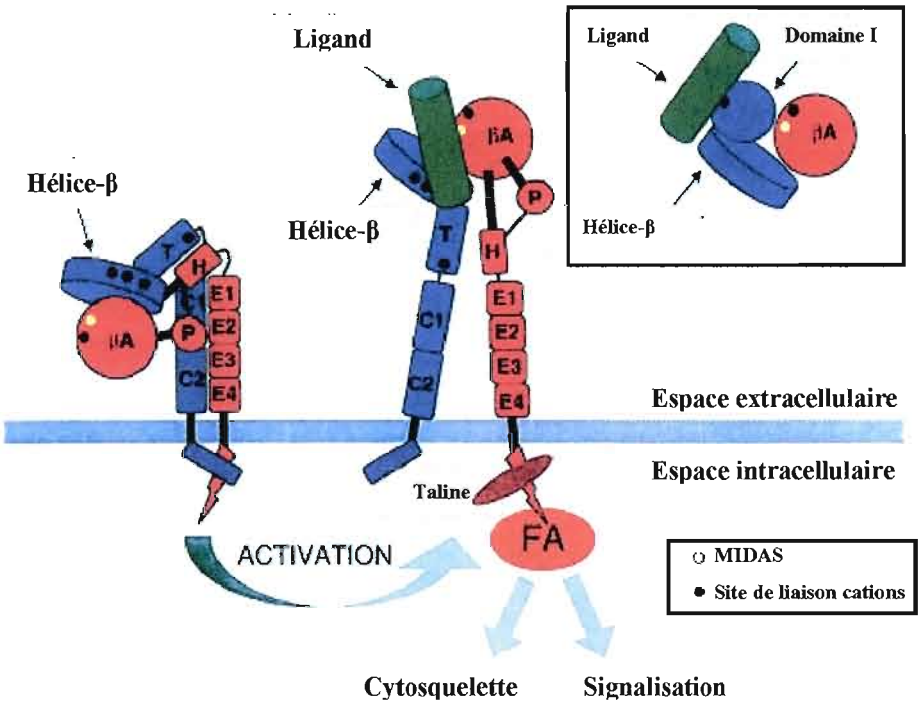

Figure 1.3 Structure et activation des intégrines. (Adaptée d'après Ruegg, C. et Mariotti, A. 2003). FA, foyers d'adhésion ; MIDAS, site d'adhésion dépendant d'ion métallique ; T, domaine "thigh"; C1-2, domaine "calf" 1 et -2; βA, domaine de liaison de l'hélice; H, domaine hybride; P, domaine "plexin-semaphorine-intégrine"; E1-4, facteur épidermique de croissance (EGF)-like repeats 1-4.

Dans la portion extracellulaire, la tête contient un domaine de l'hélice-β au niveau de la sous-unité α, et un domaine de liaison de l'hélice βA pour la sous-unité β. L'association de ces deux domaines forme un hétérodimère stable et la combinaison des deux sous-unités détermine la spécificité du substrat. Dans certaines sous-unités α, un domaine I est associé et assure la liaison du récepteur à son ligand, telles la fibronectine, la vitronectine, la laminine, le collagène. Les jambes contiennent des domaines étroitement liés qui forment une tige rigide avec une région charnière flexible. Cinq sites de liaison aux cations sont situés dans la tête, alors que le sixième est présent sur la sous-unité α près de la région charnière (Ruegg, C. et Mariotti, A. 2003).

La portion intracellulaire est assez courte, et est reliée aux protéines du cytosquelette, en particulier la taline, la vinculine et l'actine. L'association des domaines cytoplasmiques des sous-unités α et β maintient l'intégrine dans un état de repos (figure 1.3). La rupture de l'interaction entre ces domaines déclenche un changement conformationnel menant à l'activation du récepteur. Elle induit alors, une signalisation intracellulaire menant à plusieurs fonctions cellulaires, dont la survie, l'adhésion et la motilité cellulaire (Hynes, 1992; Clezardin, 1998). Le récepteur actif est étiré, possède un site d'adhésion du ligand dans un état conformationnel de haute affinité et un site de coordination altéré au niveau du MIDAS. Des protéines cytoplasmiques additionnelles sont recrutées pour former des adhérences focales (FA). Cependant, les intégrines ne possèdent pas d'activité enzymatique intrinsèque. La transduction du signal requiert le recrutement de molécules de signalisation, telles les protéines kinases, les lipides kinases, les petites GTPases et les phosphatases. De plus, plusieurs voies de signalisation médiées par les intégrines sont également activées par les récepteurs des facteurs de croissance.

A l'heure actuelle, 24 intégrines ont été découvertes, résultant de la combinaison de 18 sous-unités α et 8 sous-unités β. Certaines d'entre elles jouent un rôle très important dans la formation de métastases notamment les sous-unités α_3, α_6, β_1, β_4 et

en particulier les hétérodimères $\alpha_v\beta_3$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ et $\alpha_6\beta_4$ (Engbring et Kleinman, 2003)

1.3.2.2 Récepteurs non-intégrines

√ Récepteur de la laminine à 67 kDa

La laminine, principale glycoprotéine retrouvée dans la membrane basale (Aumailley et Smyth, 1998), est un hétérotrimère composé de trois sous-unités α, ß, et δ liées par des ponts disulfures (Engbring et Kleinman, 2003). Indirectement, la laminine et ses dérivés peptidiques contribuent au développement tumoral (Engringe et Kleinman, 2003). En effet, il a été démontré que le peptide SIKVAV, dérivé de la chaîne α, augmente l'angiogénèse, la croissance tumorale et la formation des métastases (Engringe et Kleinman, 2003). La laminine induit *via* des récepteurs de nature intégrine et non-intégrine (Tableau 1.2) des voies de signalisation intracellulaires impliquant des régulateurs du cytosquelette tels les protéines G hétérodimères, le calcium intracellulaire, la phospholipase D, des phosphatases et des petites GTPases de la famille des Rho (Givant-Horwitz *et coll.,* 2005).

Parmi les récepteurs non intégrines de la laminine, le récepteur à 67 kDa (Ménard, 1997), est impliqué dans plusieurs processus physiologiques telles l'implantation (Zhang *et coll.* , 2000) et l'angiogenèse (McKenna *et coll.,* 2001). L'augmentation d'expression de ce récepteur est corrélée avec la prolifération cellulaire (McKenna *et coll.,* 2001), la migration (Chen *et coll.,* 2002) et l'invasion (Van Den Brule *et coll.,* 1994). La signalisation de la laminine *via* ce récepteur joue un rôle prépondérant dans la progression tumorale (Givant-Horwitz *et coll.,* 2005). Il a été démontré que l'implication du récepteur à 67 kDa dans l'agressivité des tumeurs repose dans sa capacité à modifier la laminine-1. Il active des enzymes protéolytiques telle MT1-MMP qui favorise l'invasion des cellules tumorales par dégradation de la

MEC (Berno *et coll.*, 2005).

Tableau 1.2 Récepteurs à la laminine: intégrines et non intégrines

Récepteur		Ligands
Intégrines	α1ß1	Collagène (I, II, IV), laminine (1,2)
	α2ß1	Collagène (I, II, IV), laminine (1,2), chondroadhérine
	α3ß1	Fibronectine, collagène (I), laminine (2, 5, 8, 10, 11), nidogène, épilegrine, perlecane
	α6ß1	Laminine (1, 2, 5, 8, 10, 11)
	α6ß4	Laminine (1, 2, 5, 10)
	α7ß1	Laminine (1, 2, 8, 10)
Récepteur de la laminine 67 kDa		Laminine
Dystroglycane		Laminine (1, 2), agrine, perlecane
Sulphate d'héparane		Laminine (1, 2), collagène XVIII

Adapté d'après Givant-Horwitz *et coll.*, 2005

√ Le CD44

CD44 est une glycoprotéine transmembranaire ubiquitaire. (Naor *et coll.*, 1997). L'organisation génomique de la partie extracellulaire est constituée de 10 exons variables (v1–v10). Leur épissage conduit à la forme standard de CD44 (CD44s), alors que l'insertion de ces exons conduit aux différentes isoformes de CD44 (CD44v). Dans toutes ces formes, l'exon 18 est épissé de sorte que le domaine transmembranaire codé par l'exon 17 soit suivi du domaine cytoplasmique codé par l'exon 19 (Naor *et coll.*, 1997) (Figure 1.4). La structure protéique est constituée d'un domaine extracellulaire glycosylé liant principalement l'acide hyaluronique (HA) assurant ainsi les interactions intercellulaires et cellules avec la matrice. CD44 lie

aussi le collagène de type 1, les laminines, la fibrine, la fibronectine et la chondroitine sulfate (Naor *et coll.*, 1997). Une étude réalisée dans notre laboratoire a montré que la liaison des cellules de glioblastomes à HA est induite par le collagène de type I et est régulée par les cavéoles (Annabi *et coll.*, 2004). CD44 serait également impliqué dans la transmission de signaux de prolifération cellulaire par liaison aux facteurs de croissance et à HA (Naor *et coll.*, 1997). CD44 est relié aux fibres d'actine du cytosquelette par l'interaction de son domaine cytoplasmique avec les protéines de la famille Ezrine-Radixine-Myosine.

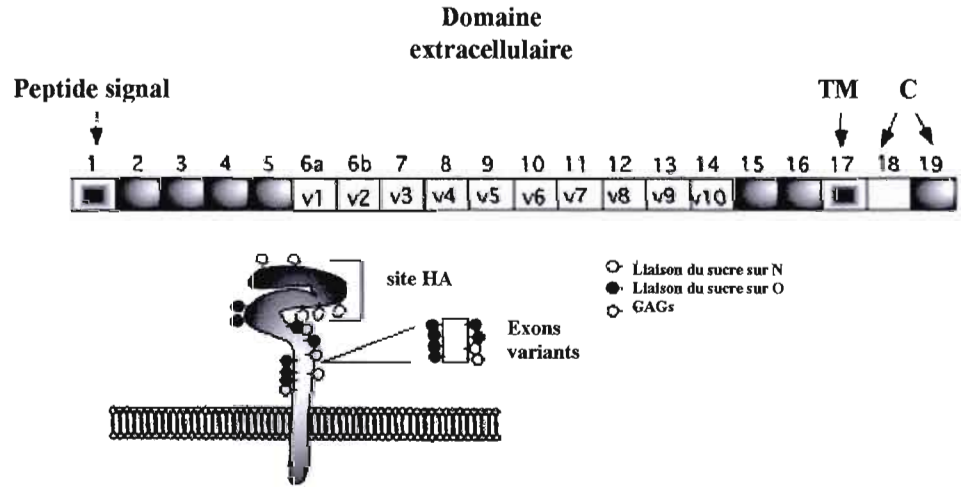

Figure 1.4 Structure de CD44 : Organisation génomique et protéique (Adaptée d'après Isacke et Yarwood, 2002). TM, domaine transmembranaire; C, domaine cytoplasmique; HA, acide hyaluronique.

CD44 lierait une principale métalloprotéinase matricielle, la MMP-9 et intéragit avec certains domaines de la MT1-MMP (Isacke et Yarwood, 2002) (figure 1.5), des protéases fortement exprimées lors du processus métastasique. Il permet l'ancrage de la MMP-9 à la membrane cellulaire, augmentant ainsi la digestion de la

MEC (Yu et Stamenkovic, 1999). La MT1-MMP, quant à elle, serait co-localisée dans les lamellipodes avec CD44 *via* son domaine hémopexine et serait responsable de son clivage (Mori *et coll.*, 2002), entrainant une augmentation de la migration cellulaire. La diminution de son expression à la surface de la cellule par clivage favoriserait les capacités métastasiques des neuroblastomes (Sy MS, 1997). De plus, l'activation de CD44 par le HA induirait la sécrétion de la pro-MMP-2 (figure 1.5) (Isacke et Yarwood, 2002), une métalloprotéinase impliquée dans l'invasion tumorale, activée par la MT1-MMP.

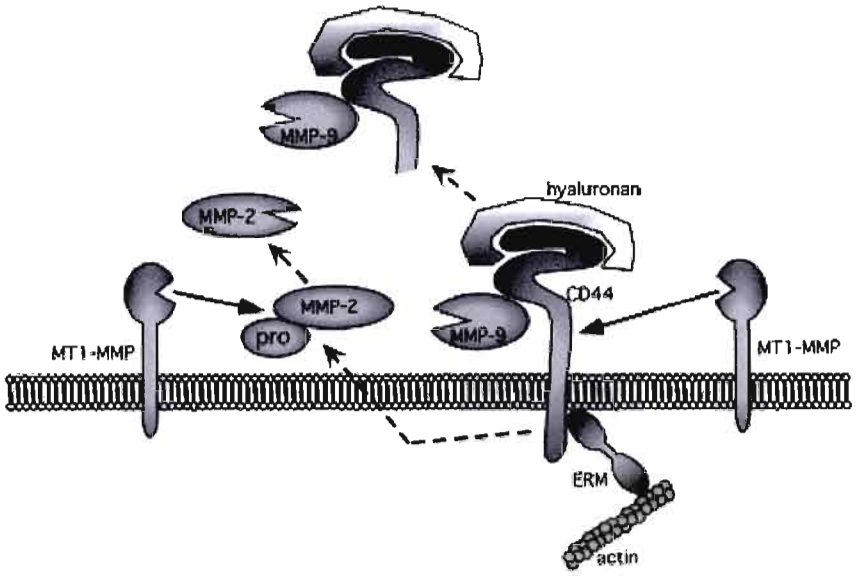

Figure 1.5 : Interaction de CD44 avec les MMPs (Isacke et Yarwood, 2002)

1.3.3 Dégradation de la MEC par les métalloprotéinases matricielles

Les cellules sont capables de dégrader les constituants de la membrane basale et de la MEC. Cette protéolyse menée par des protéases est un processus physiologique fondamental. Les cellules pathologiques font intervenir une rupture d'équilibre entre ces enzymes et leurs inhibiteurs physiologiques. Ces enzymes sont principalement des métalloprotéases matricielles (MMPs) et des sérines protéases, constituants du système de la plasmine. Les MMPs sont caractérisées par une activité endopeptidasique dépendante du cation zinc (Zn^{2+}).

Actuellement, 27 MMPs ont été dénombrées chez les vértèbrés (Sternlicht et Werb, 2001), structurellement homologues (figure 1.6) comprenant quatre domaines. 1) Un peptide signal à l'extrémité N-terminale permettant l'entrée dans le réticulum endoplasmique. Ce peptide est clivé lors de la sécrétion dans le milieu extracellulaire. 2) Un domaine pro-peptidique de 80 acides aminés contenant une séquence conservée PRCGVPD, éliminée lors de l'activation. 3) Un domaine catalytique. 4) Un domaine hémopexine et une région charnière en C-terminal à l'exception de la matrylisine qui n'en contient pas. La région charnière permet la liaison entre le domaine catalytique et le domaine hémopexine afin de piéger le substrat (Egeblad et Werb, 2002).

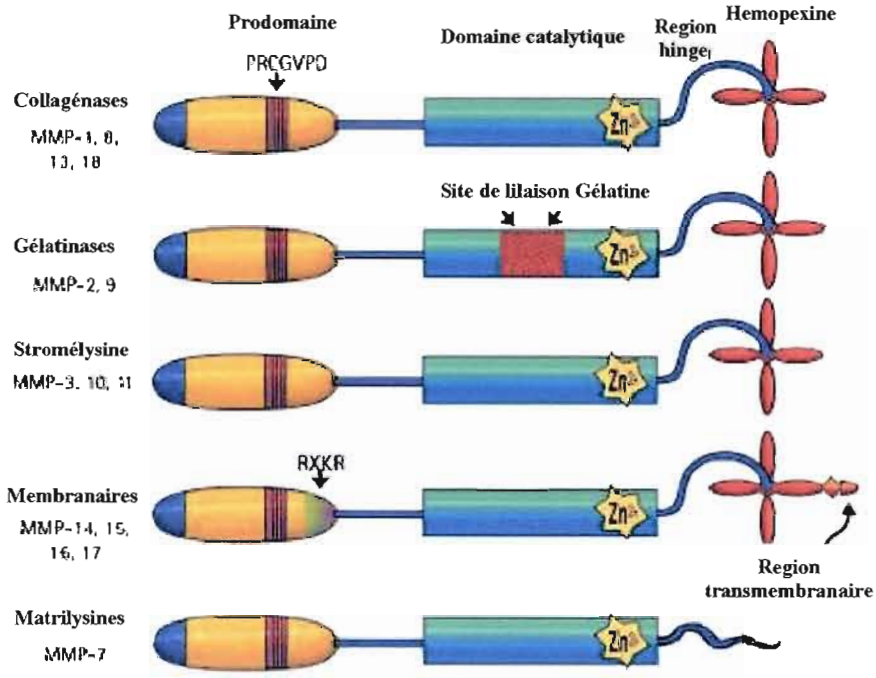

Figure 1.6 Structure homologue des différentes MMPs (Adaptée d'après Egeblad et Werb, 2002)

Certaines MMPs contiennent dans leur structure, des séquences propres à elles qui leur confèrent un caractère particulier. Par exemple, les gélatinases contiennent un domaine fibronectine indispensable pour la reconnaissance du substrat. La MMP-9 et la MT1-MMP possèdent un domaine de liaison au collagène en C-terminal, (Forget *et coll.*, 1999).

Les MT-MMPs contiennent une séquence de 4 acides aminés (RRKR) permettant leur activation et une séquence conservée (YGYL) assurant un rôle de chaperon intracellulaire (Pavlaki *et coll.*, 2002)

Les MMPs sont impliquées dans plusieurs processus physiologiques tels que le développement embryonnaire, l'ovulation, la cicatrisation, l'inflammation, etc,

mais aussi lors de processus pathologiques tels les maladies cardiovasculaires, le cancer, les ulcères, l'Alzheimer, etc. (tableau.1.3).

Tableau. 1.3 Implication des MMPs dans les différents processus physiologiques et pathologiques

Processus normal	Processus pathologique
Développement embryonnaire	Arthrite
Implantation du blastocyste	Cancer
Morphogenèse	Maladie périodontale
Croissance des nerfs	Ulcération de la cornée
Ovulation	Maladie cardiovasculaire
Dilatation cervicale	Scléroses multiples
Involution postpartum utérine	Néphrite
Cycle endométrial	Maladie neurologique
Cycle du follicule pileux	Rupture de la barrière hémato-encéphalique
Remodelage osseux	Ulcération de la peau
Réparation tissulaire	Ulcère gastrique
Angiogenèse	Fibrose hépatique
Inflammation	Emphysème
Apoptose	Cirrhose hépatique
Réponse immune	Fibrose pulmonaire
	Maladies vasculaires
	Dystrophie du fundus de Sorsby
	Maladie d'Alzheimer
	Maladie de Guillian-Barre

Adapté d'après Herold, 2001

Elles ne sont pas exprimées dans tous les types cellulaires et sont classées selon la structure et la spécificité du substrat en 5 familles: les collagénases, les gélatinases, les stromélysines, les matrilysines et les MMP membranaires (MT-MMP) (tableau. 1.4)

Tableau 1.4 : Différentes MMPs, leurs substrats et le type cellulaire d'expression

MMP	Nom Commun	Substrats	Expression
MMP-1	Collagénase-1	CN types I, II, III, V, VII et X, aggrécane, gélatine, et serpines	Fibroblastes, kératinocytes, chondrocytes, cellules épithéliales et endothéliales, ostéoblastes, macrophages et hépatocytes
MMP-2	Gélatinase A	CN types I, IV, V, VII et X, gélatine, élastine, FN, LN, nidogène, MMP-9 et MMP-13 actives	Lymphocytes T, fibroblastes, macrophages et plaquettes
MMP-3	Stromélysine-1	CN types II, IV, IX, X et XI, nidogène FN, protéoglycane, aggrécane gélatine, élastine, proMMP-1, proMMP-8 et proMMP-9	Fibroblastes, cellules épithéliales, endothéliales et du muscle lisse vasculaire, macrophages kératinocytes, chondrocytes, et ostéoblastes
MMP-7	Matrilysine	CN types IV, gélatine, élastine, protéoglycane et glycoprotéine	Macrophages, cellules épithéliales et mésangiales
MMP-8	Collagénase-2	CN types I, II, III, et V	PMN, chondrocytes, fibroblastes, et cellules endothéliales
MMP-9	Gélatinase B	CN type IV, gélatine, aggrécane, LN, et nidogène	LymphocytesT, macrophages kératinocytes, ostéoclastes, mégacaryocytes, trophoblastes, neutrophiles, éosinophiles et cellules endothéliales
MMP-10	Stromélysine-2	CN type IV, LN, nidogène, FN, protéoglycane et gélatine	Fibroblastes, cellules épithéliales, kératinocytes et lymphocytes T
MMP-11	Stromélysine-3	LN, α1-inhibiteur des protéinases et α1-antitrypsine	Cellules mésenchymateuses, cellules épithéliales et fibroblastes
MMP-12	Elastase des macrophages	Elastine	Macrophages
MMP-13	Collagénase-3	CN types I, II, III, IV, V, IX, X et XI, gélatine, LN, ténascine, aggrécane et FN	Fibroblastes et tissu osseux en développement
MMP-14	MT1-MMP	CN types I, II et III, gélatine, FN, LN, VN, aggrécane nidogène, ténascine, perlecan, protéoglycane, proMMP-2, et proMMP-13	Fibroblastes, cellules épithéliales, macrophages et ostéoclastes
MMP-15	MT2-MMP	CN types I, II et III, gélatine, FN, LN, VN, proMMP-13, proMMP-2, nidogène, tenascine, perlecan et aggrécane,	Macrophages et fibroblastes
MMP-16	MT3-MMP	CN types I et III, FN, LN, VN aggrécane, perlecan, gélatine, caséine, proMMP-2 et proMMP-9	Cerveau, placenta et cellules du muscle lisse vasculaire
MMP-17	MT4-MMP	FN, fibrine et gélatine	Cerveau, tissus reproducteurs et leucocytes
MMP-20	Enamélysine	Amélogénine, aggrécane et la protéine matricielle oligomérique du cartilage (COMP)	Cellules odontoblastiques
MMP23			Tissus reproducteurs
MMP24	MT5-MMP	ProMMP-2 et proMMP-13	Cerveau
MMP-25	MT6-MMP	ProMMP-2	Neutrophiles

Adapté d'après Herold, 2001

1.3.4 Mécanisme de régulation des MMPs

La plupart des MMPs ne sont pas exprimées de manière constitutive par les cellules normales. La régulation de leur synthèse et de leur activité enzymatique est contrôlée à trois niveaux: 1) au niveau de la transcription et de la sécrétion après induction par des cytokines et des facteurs de croissance; 2) au niveau de l'activation du pro-enzyme par les facteurs de la plasmine et les MT-MMPs et 3) au niveau de l'inhibition de l'activité protéolytique par des inhibiteurs endogènes: les TIMPs (Tissue inhibitors of metalloproteinases) (Dollery, 1995) (figure 1.7).

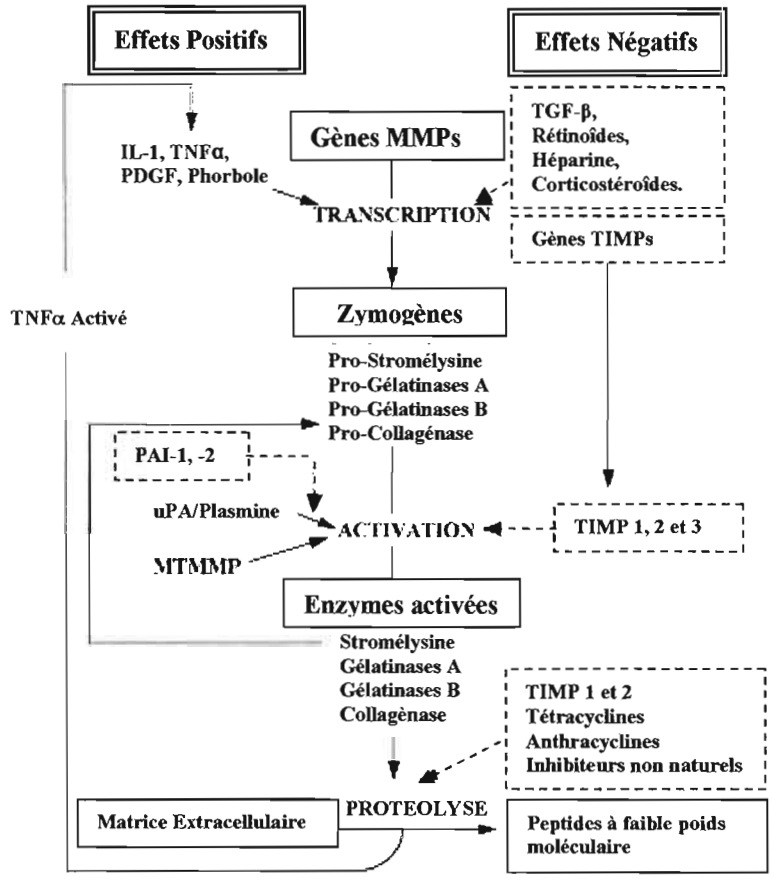

Figure 1.7 Transcription, activation des pro-MMPs, et inhibition de l'activité protéolytique des MMPs. (Adaptée d'après Dollery, 1995)

1.3.4.1 Régulation de la transcription et de la sécrétion

La plupart des MMPs ne sont pas constitutivement exprimées par les cellules normales *in vivo* et leur expression basale dans les cellules en culture est très faible (Stamenkovic, 2000). L'expression des MMPs est régulée principalement au niveau de la transcription. Plusieurs facteurs sont impliqués dans l'induction ou la stimulation des gènes codant pour les MMPs incluant des agents chimiques (Phorbol 12-myristate 13-acetate (PMA)), des cytokines (IL-1, TNF-α), des facteurs de croissances (PDGF) et des hormones (œstrogène, progestérone) (Dollery, 1995). L'étude sur les promoteurs des gènes de ces protéases, principalement des collagénases et des stromélysines a abouti à l'identification d'un nombre de séquences consensus conservées, incluant l'élément *cis* AP-1 (activator protein-1) et *cis* PEA3 (polyomavirus enhancer A binding-protein-3) (Ye, 2000). La séquence de nucléotides AP-1 interagit avec les facteurs de transcription des familles Fos et Jun, tandis que l'élément PEA3 est lié par la famille Ets, ce qui permet l'activation du promoteur des MMPs, et donc leur expression génique (Ye, 2000). L'AP-1 joue aussi un rôle important dans la répression génique des MMPs par des facteurs de croissance, TGF-ß, des rétinoides et des glucocorticoides (Nagase et Woessner, 1999)

1.3.4.2 Activation du pro-enzyme

Le second niveau de contrôle des MMPs est l'activation du pro-enzyme latent. Les MMPs sont synthétisées sous forme de zymogène (pro-MMP) inactif. Leur activation par clivage protéolytique implique le bris du lien entre la cystéine et le zinc de la partie N-terminale (*cysteine switch*). Cette activation est réalisée soit avant d'atteindre la surface cellulaire, c'est le cas des MMPs membranaires (Visse et Nagase, 2003), soit dans la MEC pour les MMPs solubles. *In vitro,* cette activation est assurée par d'autres protéases et divers agents tels les organo-mercuriques, l'aminophenylmercuri acetate (APMA), le dodecyl-sulfate de sodium (SDS) et les ions chaotropiques, qui déstabilisent le lien entre le pro-peptide et l'ion Zn^{2+}

(Cuvelier *et coll.,* 1997). *In vivo,* les MMPs sont activés *via* une cascade enzymatique impliquant des protéases à sérines telles la plasmine, la cathepsine G et la kallikréine. Des activateurs de plasminogène en plasmine de type urokinase et tissulaire (uPA et tPA) initient l'activation des matrilysines la proMMP-1 et proMMP-3 (Forget, Desrosiers et Béliveau, 1999). La MMP-3 active peut activer les proMMP-1, -7, -8, -9, et -13. La MMP-7 peut activer les proMMP-1 et -2, tandis que la MMP-10 clive la proMMP-8. Les MMP-2, -3 et -13 peuvent activer la proMMP-9 (DeClerck et Laug, 1996; Kleiner et Stetler-Stevenson, 1999). Finalement la MT1-MMP peut activer la proMMP-2 (Butler *et coll.,* 1998).

1.3.4.3 Inhibition des MMPs

En dehors des MMPs membranaires, toutes les autres MMPs sont sécrétées dans l'espace extracellulaire, où leur activité catalytique est contrôlée par plusieurs inhibiteurs endogènes spécifiques, tels les inhibiteurs tissulaires des MMPs (TIMPs). Jusqu'à ce jour, quatre TIMPs ont été identifiés (TIMP-1 à TIMP-4). Le premier TIMP identifié est le TIMP-1. Il se lie à la plupart des MMPs actives (collagénase, gélatinase B, matrilysine et stromélysine), mais aussi à la pro-MMP-9. Le complexe TIMP-1/pro-MMP-9 semble recruter la MMP-3, ce complexe ternaire plus stable résulte en l'inactivation de la MMP-3 (Kolkenbrock *et coll.,* 1995). TIMP-2, exerce une faible activité inhibitrice sur les MMPs, mais il serait impliqué dans l'activation de la pro-MMP-2 par la MT1-MMP. Quant aux TIMP-3 et -4, ils inhibent respectivement les MMP-1, -2, -3, -13 (Apte, Olsen et Murphy, 1995) et les MMP-1, -2, -3, -7, -9 (Liu *et coll.,* 1997).

A un moindre degré d'inhibition, l'activité enzymatique des MMPs peut être régulée par des inhibiteurs non spécifiques, tels les inhibiteurs de l'activateur du plasminogène (PAIs) et l'α2-macroglobuline (Forget, Desrosiers et Béliveau, 1999). L'α2-macroglobuline est une glycoprotéine de 772 kDa synthétisée par les

hépatocytes et les macrophages. Elle peut inhiber presque toutes les endopeptidases, quelque soit leur spécificité du substrat. Les complexes α2-macroglobuline–enzyme sont rapidement reconnus par les récepteurs membranaires des macrophages et sont dégradés dans leurs lysosomes. Les inhibiteurs des activateurs du plasminogène (PAI-1 et PAI-2) sont impliqués dans la régulation de l'activité des protéases à sérine l'uPA et le tPA (Irigoyen *et coll.,* 1999), deux activateurs du plasminogène en plasmine. La plasmine peut dégrader la majorité des composés de la MEC par l'activation des MMPs. Ainsi l'inhibition du tPA et de l'uPA résulte en une répression de l'activation des MMPs (Kucharewicz *et coll.,* 2003).

Nos travaux ont été dirigés contre deux principales MMPs dont le rôle est crucial lors de la dégradation de la MEC et la migration cellulaire: la MMP-9 (gélatinase B) et la MMP-14 (MT1-MMP).

1.3.5 La MMP-9

1.3.5.1 Caractéristiques

MMP-9, appelée aussi gélatinase B, est une protéase soluble dégradant le collagène (IV, V, VII, X) la gélatine, l'élastine, la fibronectine et les protéoglycanes. Elle fait partie du sous-groupe des gélatinases, un sous-groupe distinct de par sa structure, son interaction avec TIMP-1 et sa spécificité de substrat. MMP-9 est activée par d'autres protéases telles: MMP-2 (Fridman *et coll.,* 1995) MMP-3, MMP-7 (Von Bredow *et coll.,* 1998) et MMP-13 (Knauper *et coll.,* 1997). Son inhibiteur physiologique est TIMP-1. MMP-9 a souvent été étudiée dans le contexte du développement tumoral et dans l'angiogenèse, mais aussi dans la dégradation de la MEC lors des maladies cardiovasculaires (Ducharme *et coll.,* 2000), tel l'anévrisme abdominal aortique. (Annabi *et coll.,* 2002).

1.3.5.2 Association à la surface de la cellule

MMP-9 est associée à la cellule en se liant à des protéines membranaires. Celles-ci ont été identifiées dans de nombreux types cellulaires humains et murins normaux ou tumoraux: les neutrophiles (Gaudin *et coll.*, 1997), les cellules endothéliales (Olson, 1998), les cellules cancéreuses du pancréas (Zucker, 1990), de l'ovaire (Ellerbroek, 2001), de la prostate (Ellerbroek, 2000), les fibrosarcomes (Mazzieri, 1997) et les carcinomes mammaires de souris (Yu et Stamenkovic, 1999). Quatre protéines de surface on été identifiées liant la MMP-9 (figure 1.8), la chaine α2(IV) du collagène (Olson *et coll.*, 1998), CD44 (Bourguignon *et coll.*, 1998; Yu et Stamenkovic, 1999), RECK (reversion-inducing cysteine-rich protein with Kazal motifs) (Takahashi *et coll.*, 1998) et LRP (low-density lipoprotein receptor-related protein) (Hahn-Dantona *et coll.*, 2001).

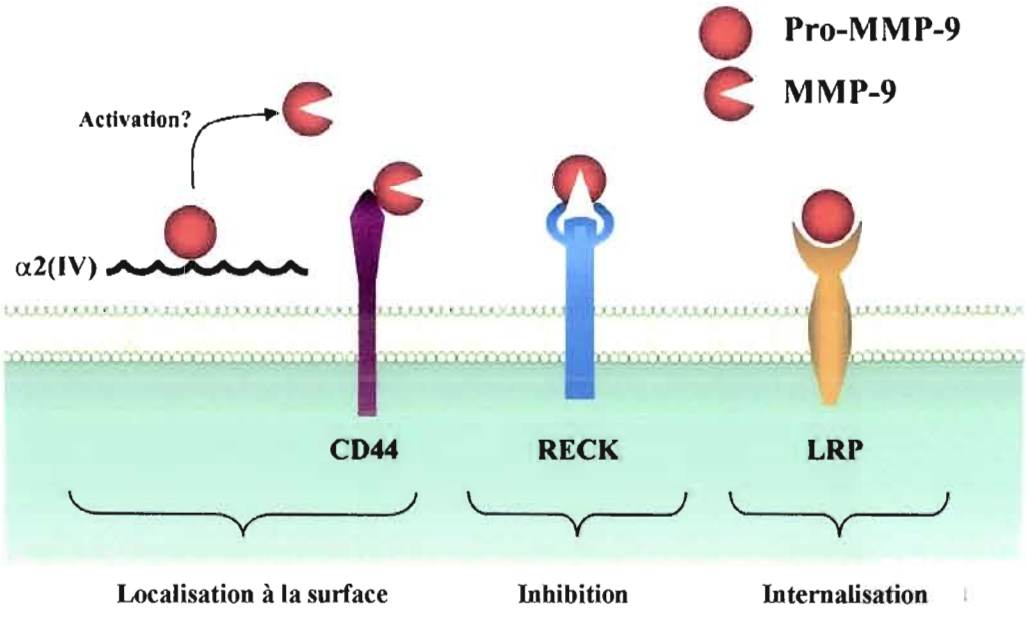

Figure 1.8 Présentation schématique des protéines associées à la pro-MMP-9 et MMP-9 (Adaptée d'après Yu, Stamenkovic, 1999).

√ Association à la chaine α2(IV) et à CD44 : localisation membranaire

La pro-MMP-9 contient un domaine liant la gélatine (collagène dénaturé). Ce qui permet à l'enzyme *in vivo* d'interagir avec les molécules de collagène de la MEC. (Olson *et coll.*, 1998). L'une des protéines liant la pro-MMP-9 à la surface des cellules est la chaîne α2 (IV) formant un complexe avec TIMP-1. Cette liaison ne jouerait pas un lien direct dans la régulation (activation ou inhibition) de l'activité enzymatique de la pro-MMP-9 (Olson *et coll.*, 1998).

La forme active de MMP-9 lie le récepteur de l'acide hyaluronique, CD44 dans les cellules de carcinomes mammaires de souris et les mélanomes humains (Bourguignon *et coll.* ,1998; Yu et Stamenkovic, 1999). L'association de la MMP-9 avec CD44 augmenterait la liaison des cellules à la MEC, la cellule acquiert alors une activité protéolytique péricellulaire de dégradation des composants de la MEC médiée par une expression plus importante de MMP-9 (Bourguignon *et coll.*, 1998) et favoriserait l'invasion des cellules tumorales *in vitro* (Bourguignon *et coll.*, 1998). En effet, nous avons observé l'effet inverse lors de l'inhibition de l'activité de la MMP-9 avec un anticorps synthètique ou de l'expression génique de l'ARNm avec un oligonucléotide anti-sens. Cet inhibition réduit le phénotype invasif des cellules exprimant CD44 à la surface liant la MMP-9, ce qui suggère que la MMP-9 liée à CD44 joue un rôle important dans la dégradation et l'invasion des cellules tumorales. (Bourguignon *et coll.*, 1998). De plus, la MMP-9 liée à CD44 serait impliquée dans l'activation de TGF-ß (Yu et Stamenkovic, 2000) qui favoriserait le développement des tumeurs par dégradation protéolytique (Overall, 1989) et par induction de l'angiogenèse (Yu et Stamenkovic, 2000).

√ Association à RECK et LRP: régulation négative

La liaison de la MMP-9 à la surface de la cellule peut aussi induire une régulation négative de sa fonction. A l'heure actuelle, deux protéines membranaires

liant la MMP-9 peuvent réguler négativement son activité: RECK (Takahashi *et coll.*, 1998) et LRP (Hahn-Dantona *et coll.*, 2001).

Le gène de RECK code pour une protéine avec un sucre glycosylphosphatidylinositol (GPI). Il code donc une glycoprotéine contenant un domaine de liaison à une protéase à sérine et une région homologue au facteur EGF (Epidermal growth factor) (Takahashi *et coll.*, 1998). L'expression de RECK est inhibée dans plusieurs lignées cellulaires suggérant un rôle de suppression tumorale. Il a été démonté que l'expression de RECK recombinant soluble et membranaire est associée à une réduction du niveau de sécrétion de la MMP-9 (Takahashi *et coll.*, 1998), la MMP-2 et la MT1-MMP (Oh, 2001). Le mécanisme par lequel RECK inhibe l'activité enzymatique n'est pas encore élucidé. (Oh, 2001). Par contre, seule la forme soluble de RECK reconnaît la pro-MMP-9 (Takahashi *et coll.*, 1998).

LRP, un récepteur membranaire de la famille des LDL, est reconnu pour réguler la fonction de certaines protéines extracellulaires en induisant leur internalisation, telles les lipoprotéines, les complexes protéases-inhibiteurs, les facteurs de croissance, les composants de la MEC, les virus et les toxines bactériennes. Il a été démontré dans les fibroblastes d'embryon de souris que LRP a une forte affinité pour la pro-MMP9 seule ou complexée à TIMP-1 (Hahn-Dantona *et coll.*, 2001). Ce complexe est dégradé par un mécanisme dépendant de la chloroquine (Hahn-Dantona *et coll.*, 2001). Il a été rapporté aussi que le taux de MMP-9 dans le milieu de culture conditionné de cellules est sensiblement augmenté dans les fibroblastes embryonnaires de souris cultivés en présence de RAP (Hahn-Dantona *et coll.*, 2001). RAP (receptor associated protein), une protéine intracellulaine et un antagoniste de ligand liant la protéine LRP, joue un rôle important de chaperonne pour LRP, son inhibition diminurait la liaison de la pro-MMP-9 à LRP (Herz, 2001).

1.3.6 La MT1-MMP

1.3.6.1 Caractéristiques

Chaque MMP a dans sa structure une séquence additionnelle qui lui confère une fonction unique. Le domaine hydrophobe en C-terminal présent uniquement dans les métalloprotéinases membranaires permet l'ancrage de l'enzyme à la membrane et limite son activité à la surface des cellules. La MT1-MMP une fois active digère les composants de la MEC telles la fibronectine, la vitronectine, la laminine-1, -5, la fibrine, le dermatan sulfate, les protéoglycanes, la gélatine, la caséine et l'élastine (Pei et Weiss, 1996; Ohuchi, 1997) mais aussi les collagènes de types I, II et III (Ohuchi, 1997). Une étude réalisée dans notre laboratoire a révélé la localisation de la MT1-MMP dans les cavéoles (Annabi *et coll.,* 2002). La MT1-MMP est impliquée aussi dans la migration des cellules, la formation de tubules dans les cellules endothéliales sur une matrice de fibrine ou de collagène de type 1. (Hiraoka, 1998; Haas, 1998). *In vitro*, Galvez et ses collaborateurs ont démontré que l'inhibition de la MT1-MMP réduit la migration des cellules endothéliales et la formation de tubes capillaires (Galvez, 2001). Pour caractériser le rôle de la MT1-MMP durant ce processus, ils ont utilisé un model de migration de cellules lors de la réparation tissulaire au cours d'une blessure et un anti-corps monoclonal anti-MT1-MMP regénéré. La regénération de cet anti-corps a été réalisée dans des souris BALB/c immunisées avec les peptides REVPYAYIREGHEK (LEM-1) et CAEPWTVRNEDLNGNDIC (LEM-2) localisés dans le domaine catalytique de la MT1-MMP. La spécificité de ces anti-corps monoclonaux a été confirmée par analyse des cellules COS-1 transfectées avec de l'ADN de MT1-MMP par éléctroporation, et ont été purifiées par chromatogragraphie d'affinité sur colonne de Sepharose-A (Amersham Pharmacia Biotech) (Galvez, 2001).

L'activité enzymatique de la MT1-MMP est spécifiquement inhibée par TIMP-2, TIMP-3 et TIMP-4 mais pas par TIMP-1 (Will, 1996). D'autres protéines

membranaires autres que les TIMPs peuvent inhiber son activité tel RECK (Takahashi *et coll.,* 1998), le testicane 3 et son dérivé le N-Tes 9 (Nakada, 2001). De plus, la MT1-MMP a été identifiée comme activateur spécifique de la pro-MMP-2 (pro-gélatinase A) à la surface de la cellule (Sato, 1994). Cette activation implique TIMP-2 formant ainsi un complexe trimoléculaire (Will, 1996). Cette activation est favorisée par l'association de la Claudin (Miyamori, 2001). La pro-MMP-13 est aussi activée par la MT1-MMP dans les cellules mammaires (Knauper, 1996). La MT1-MMP est reconnue pour son clivage des protéines d'adhésion comme le CD44 (Mori *et coll.,* 2002), de la sous-unité αv des intégrines (Deryugina *et coll.,* 2000), et de la transglutaminase (Belkin *et coll.,* 2001)

1.3.6.2 Protéines associées à la MT1-MMP

La MT1-MMP fait partie des protéines les plus impliquées lors de la migration des cellules. Cette fonction permet à la cellule d'acquérir un caractère invasif. La MT1-MMP est munie d'une queue cytoplasmique, assurant l'association avec les protéines du cytosquelette comme l'actine (Mori *et coll.,* 2002). CD44, impliqué aussi dans la migration cellulaire et lié également à l'actine, a été identifiée pour favoriser l'association de la MT1-MMP à l'actine (Mori *et coll.,* 2002). La MT1-MMP et CD44 forment un complexe *via* le domaine hémopexine de la MT1-MMP. Ainsi, CD44 joue un rôle de pivot dans la régulation de la distribution polarisée de la MT1-MMP durant la migration cellulaire et l'invasion tumorale (Mori *et coll.,* 2002). L'intégrine $\alpha_v\beta_3$, liant la vitronectine, est exprimée dans les cellules endothéliales durant l'angiogenèse et le développement tumoral. L'activation de ce récepteur en une forme active est assurée par la MT1-MMP (Ratnikov *et coll.,* 2002).

1.4 Voies de signalisation intracellulaires impliquées dans la cancérogenèse

1.4.1 Généralités

Tous les effets cellulaires et moléculaires décrits auparavant font intervenir des voies de signalisation intracellulaires: la cellule par l'intermédiaire de facteurs moléculaires externes liant des récepteurs à sa surface, communique avec son environnement en répondant de différentes manières comme la dégradation de la MEC, la migration, la prolifférération, la mort cellulaire, etc. Ces facteurs moléculaires perçus par les cellules, cheminent à travers le cytoplasme où ils déclenchent des cascades de réactions biochimiques; certaines aboutissent dans le noyau où elles régulent l'expression des gènes. Les mécanismes par lesquels ces signaux exercent leurs actions (voies de transduction intracellulaires), mettent en jeu un nombre important de protéines accomplissant des fonctions biologiques diverses telles la migration, l'adhésion, la prolifération, la survie, la mort cellulaire...etc (figure 1.9). Plusieurs voies de transduction du signal impliquées dans l'invasion tumorale et métastasique, ont été mises en évidence. La voie des MAP kinases (Mitogen-activated protein kinases) figure parmi les systèmes de transduction les plus étudiés. Parmi les cascades impliquées dans cette voie, la cascade de ERK1/ERK2 (extracellular signal-regulated kinase 1 and 2) régule principalement la différenciation et la division cellulaire; les cascades JNK (c-Jun kinase) et p38 MAP kinase impliquées dans les réponses aux stress conduisent à l'apoptose et l'inflammation (Reddy *et coll.*, 2003). Les voies des GTPases (Ras, Rho, Rac) sont impliquées dans la migration, la prolifération, la différentiation cellulaires, l'organisation du cytosquelette, le transport nucléo-cytoplasmique, le trafic intracellulaire (Giehl, 2005), mais aussi d'autres voies impliquant le PKA, le PKC, le NF-kappaB...etc. Cependant, un seul stimulus peut déclencher plusieurs voies de signalisation menant à une seule réponse (figure 1.9).

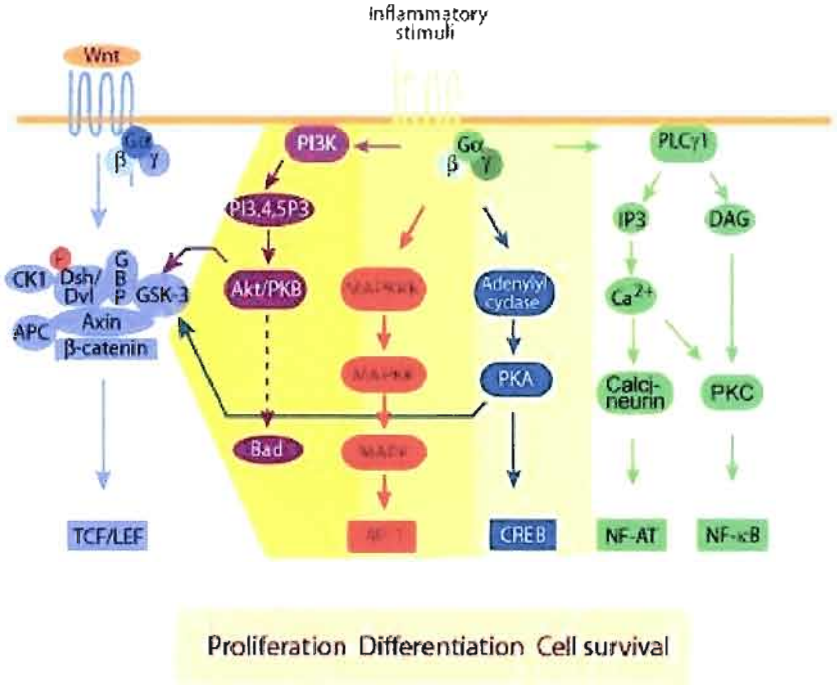

Figure 1.9 Voies de signalisation impliquées dans la prolifération, la différenciation et la survie cellulaire (Tor L, 2003).

1.4.2 Régulation de la MMP-9 par les voies intracellulaires

Plusieurs voies de transduction de signaux intracellulaires, peuvent être impliquées pour une même réponse des cellules par exemple, la cellule peut faire intervenir soit la voie des MAPKs, soit les RhoGTPases pour la régulation de l'expression de la MMP-9. Pour étudier les voies intracellulaires par lesquelles la cellule transduit son signal, des agents pharmacologiques sont utilisés pour stimuler ou inhiber la réponse cellulaire. Dans plusieurs études, nous voyons que les chercheurs ont démontré l'implication de différentes voies de transduction de signal pour réguler l'expression de la MMP-9 et cela dans plusieurs types cellulaires et par différents stimulis. C'est ainsi que l'implication des MAPKs a été démontrée dans la régulation de l'expression de la MMP-9. Dans les cellules endothéliales vasculaires, l'induction de l'expression de la MMP-9 *via* NF-kappaB par le facteur activateur des

plaquettes (PAF), est régulée par la voie intracellulaire du Ca^{2+}, PI3K et ERK (HM *et coll.,* 2005). Mais aussi dans des gliomes, l'inhibition de FAK, AP-1 et NF-kappaB et l'activation de ERK1/2 et Akt/PKB induiraient la sécrétion de la MMP-2 et -9 par l'émodine (Kim *et coll.,* 2005). Il a été démonté que la surexpression de l'intégrine $\alpha_3\beta_1$ induisait la sécrétion de MMP-9 dans les kératinocytes immortalisés de souris. D'une autre part, cette règulation induisait un phénotype immortalisé, suggérant un rôle possible de l'$\alpha_3\beta_1$ pour une conversion maligne. La stabilité de l'ARNm de la MMP-9 dans des kératinocytes immortalisés se fait *via* l'activation de MEK/ERK (Iyer *et coll.,* 2005). L'induction de l'expression de MMP-2 et MMP-9 par le TGF-ß impliquerait la voie des MAPK *via* p38, mais pas ERK dans les cellules épithéliales du sein (Kim *et coll.,* 2004). L'EGCg (épigallocatéchine gallate), un polyphénol du thé vert, ayant des propriétés anti-oxydants et anti-tumorales, bloque l'expression de la MMP-9 *via* la suppression de MAPK ERK1/2 et l'activation de AP-1 dans les cellules AGS gastriques humaines. (Kim *et coll.,* 2004). La suppression de ERK par l'EGCg réduit l'activité protéolytique de la MMP-9 dans les fibrosarcomes HT1080 (Maeda-Yamamoto *et coll.,* 2003).

D'autres voies intracellulaires sont impliquées. Il a été démontré que deux stimulus différents pouvait aboutir à une même réponse mais en implicant deux voies intracellulaires différentes par exemple, l'inhibition de l'expression génique de la MMP-9 par le curcumin lors de l'invasion des tissus sains par les cellules tumorales cérébrales se faisait *via* la voie de NF-kappaB et AP-1 (factor activator protein-1) (Woo *et coll.,* 2005). Par conséquent, son inhibition par l'ascochlorin impliquerait la transcription de AP-1 via ERK1/2. (Hong *et coll.,* 2005). Il a été aussi démontré dans le phénotype invasif des cellules épithéliales de sein, la régulation de l'expression de la MMP-2 et la MMP-9 impliquant Rac, Raf et PI3K (Shin *et coll.,* 2005).

L'ARN messager de la MMP-9 peut être sujet à la dégradation *via* son extrémité 3' UTRs riche en séquences AU (AU-rich elements: AREs). HuR est une protéine ubiquitaire nucléo-cytoplasmique appartenant à la famille des ELAV

(*embryonic lethal abnormal visual system)*, une famille de protéines qui lient les ARNm. Il a été démontré que la surexpression de HuR, augmente *in vivo* la stabilité des ARNm contenant des séquences ARE (Fan 2003). Dans les cellules mésengiales, l'interleukine-1-ß induit l'expression de MMP-9 *via* un recrutement de HuR. (Huwiler *et coll.,* 2003). Un autre facteur, l'oxide nitrique (NO), augmenterait la dégradation de l'ARNm de la MMP-9 en inhibant l'expression de HuR (Akool *et coll.,* 2003).

1.4.3 Régulation de la MT1-MMP par les voies intracellulaires

Il en est de même pour l'étude de la régulation de la MT1-MMP, les chercheurs utilisent des facteurs moléculaires externes pour stimuler ou inhiber son expression. Des travaux entrepris dans notre laboratoire ont montré que l'EGCg inhibe le clivage de CD44 médié par la MT1-MMP via la voie RhoA/ROK dans les glioblastomes (Annabi *et coll.,* 2005). De plus, l'inhibition de ERK bloque la migration médiée par la MT1-MMP (Gingras, 2001). Le cholestérol active la MT1-MMP *via* MEK-1 dans les fibrosarcomes HT1080 (Atkinson *et coll.,* 2004). L'adhésion des HT1080 au collagène de type I médiée par la MT1-MMP active la voie de ERK1/2 qui à son tour augmente l'expression de la MT1-MMP menant à l'augmentation de la migration cellulaire (Tetal, 2004). L'inhibition de la voie de ERK par le PD184352, son inhibiteur spécifique, diminue l'expression de la MMP-3, la MMP-9, la MMP-14 et CD44, inhibant ainsi l'invasion des cellules tumorales. (Tanimura *et coll.,* 2003). IGF-1 (Type-1-insulin-like growth factor) régule la synthèse de la MT1-MMP *via* la voie de PI 3-kinase/Akt. (Zhang et Brodt, 2003).

1.5 Approches thérapeutiques

1.5.1 Généralités

L'identification du tissu originaire du néoplasme primaire, le stade de la tumeur et l'état du malade sont cruciaux pour déterminer le type de traitement du cancer métastasé.

La chirurgie, la radiothérapie et la chimiothérapie restent des modalités standard du traitement du cancer. Leur but est d'obtenir une rémission de la maladie, c'est-à-dire une régression de la tumeur cancéreuse et, si possible, sa disparition clinique. Il existe deux types de traitement, les locaux et les systémiques ou les deux combinés. Les locaux telles la chirurgie et la radiothérapie sont privilégiés si une petite masse tumorale est localisée en une certaine partie d'un organe et qu'il n'y a pas de métastases distantes. La chirurgie, consiste à enlever la tumeur primaire. La radiothérapie consiste à exposer les malades à des rayonnements de haute énergie (rayons X, électrons, photons) destinés à détruire localement une masse tumorale. La sensibilité aux radiations varie selon les tissus et le type cellulaire. On l'emploie parfois avant la chirurgie pour réduire le volume de la tumeur ou après, pour empêcher les cellules cancéreuses de se développer à nouveau au même endroit (World Cancer Report, 2003).

L'expérience a démontré que dans la plupart des cas, le traitement local du cancer par chirurgie et/ou par radiothérapie ne réussit pas à supprimer complètement les cellules tumorales. En effet, même après le traitement local des métastases loin de la tumeur primaire peuvent subsistent. À ce moment-là, c'est la chimiothérapie, un traitement comportant l'administration de médicaments appelés agents antinéoplasiques qui sera le type de traitement. Selon leurs propriétés anticancéreuses (Allain, 2000), ils se divisent en plusieurs classes: les alcoylants qui provoquent une lésion biochimique au niveau du noyau et altèrent ainsi la survie de la cellule; les

antimétabolites qui trompent la cellule en imitant ses substances nutritives indispensables; les antimitotiques qui bloquent la division de la cellule (Allain, 2000).

Garcia-Carbonero, R et Paz-Ares, L ont publié ce mois ci (Avril 2006) une revue basée sur une recherche dans MEDLINE de 1966 à 2005 afin de démontrer le rôle d'un traitement systémique par chimiotherapie du mèsothéliome malin. En effet, le pemetrexed en combination avec le cisplatin, le premier régime de traitement, démontre une amélioration de survie des patients atteints du mèsothéliome pleural malin. Des études suggèrent l'efficacité anti-néoplastique semblable dans les patients avec le mèsothéliome d'origine péritonéale. D'autres agents ont montré la même efficacité incluant le vinorelbine et le gemcitabine, ou combinés avec des composés de platine (Garcia-Carbonero et Paz-Ares, 2006).

Contrairement à la chirurgie et à la radiothérapie, la chimiothérapie est un traitement systémique, c'est-à-dire qu'elle peut atteindre toutes les parties du corps cela explique les effets secondaires de ces médicaments. Elle détruit les cellules cancéreuses, même microscopiques, partout où elles se trouvent. De plus, elle peut exercer une action à long terme afin de contenir ou d'enrayer la croissance d'une tumeur. Le traitement par chimiothérapie peut être administré seul ou se combiner avec la chirurgie et la radiothérapie. Il existe deux formes de traitement systémique du cancer. L'hormonothérapie, utilisée dans le traitement des cancers hormono-dépendants, et de l'immunothérapie dont le rôle est de stimuler nos défenses immunitaires (notre système naturel de protection) contre le cancer. Elle utilise des stratégies axées sur l'interféron, les interleukines, les anticorps monoclonaux, les vaccins et la thérapie génique. Ces agents vont soit stimuler des réponses anti-tumorales, soit diminuer les mécanismes suppresseurs altérant les cellules tumorales ayant pour but d'augmenter leur potentiel immunogénique (les rendre reconnaissables par le système immunitaire) et augmenter la tolérance aux agents cytotoxiques et à la radiothérapie (Parvez, 2005).

1.5.2 Traitement des tumeurs hormono-dépendants: Le cancer de la prostate

L'hormonothérapie concerne essentiellement le traitement des tumeurs hormonosensibles. Les cancers du sein, de la prostate et de l'endomètre, sont les principaux cancers concernés par ces traitements. Le mode d'action de l'hormonothérapie consiste soit à supprimer la production naturelle d'hormones, soit à bloquer la fonction de l'hormone au niveau de son récepteur dans la cellule.

Le cancer de la prostate est plus fréquement rencontré chez les hommes agés de plus de 50 ans. Il représente la deuxième cause de décès par cancer chez l'homme dans le monde développé, après le cancer du poumon (World Cancer Report, 2003). En raison du dépistage rapide du cancer de la prostate utilisant des paramètres cliniques (examen d'une biopsie transrectal), pathologiques (balance de Gleason), et biochimiques (taux d'un antigène prostatique spécifique, le PSA, retrouvé dans le fluide séminal humain) (Gilliland *et coll.,* 1996), le cancer de la prostate est détecté dans le premier stade d'évolution du cancer où il n'aura atteint que la prostate et les vésicules séminales (Gilliland *et coll.,* 1996). Cependant, le cancer de la prostate peut envahir des tissus et des organes situés près d'elle ou atteindre d'autres organes distants (os, ganglions...).

Dans le cas où le cancer est détecté au stade où il ne dépasse pas la prostate, une prostatectomie est le mode de traitement le plus utilisé. Cette chirurgie consiste en une ablation de la prostate et des vésicules séminales. Environ 10 % des patients vont développer une récidive locale dans les 5 ans suivant la prostatectomie (World Cancer Report, 2003). La radiothérapie est utilisée pour traiter les cancers qui sont localisés à la prostate, ou qui ont atteint les tissus voisins. Elle consiste à placer des sources radioactives directement dans le tissu prostatique. Le cancer de la prostate est, comme tous les cancers glandulaires, un adénocarcinome et, dans la grande majorité des cas, est influencé par les hormones. Le traitement hormonal a pour but de diminuer la quantité d'hormones mâles (androgènes) au taux le plus faible (World Cancer Report, 2003). Chez l'homme, la testostérone est l'androgène principal. Elle

est, sécrétée par les testicules et stimule la prolifération des cellules cancéreuses. L'hormonothérapie bloquerait ainsi la prolifération des cellules cancéreuses. La chimiothérapie est utilisée lorsque le cancer de la prostate a évolué avec une extension extraprostatique et qu'il ne répond plus au traitement hormonal. (Ryan et Eisenberger, 2005)

1.5.3 Le PCK3145

L'American Cancer Society estime qu'il y a eu, en l'an 2004, 230 110 nouveaux cas de cancers de la prostate diagnostiqués et quelques 29 900 décès liés à cette maladie aux États-Unis seulement. Le cancer de la prostate est l'un des types de cancer les plus répandus chez les hommes et représente approximativement 30 % de tous les cas de cancers chez ces derniers.

La PSP94 (prostate secretory protein of 94 amino acids), une protéine sécrétoire de la prostate composée de 94 acides aminés, est l'une des trois principales protéines trouvées dans le fluide séminal humain avec l'antigène prostatique spécifique (PSA prostate-specific antigen) et la phosphatase prostatique acide (PAP prostatic acid phosphatase) (Lilja et Abrahamsson, 1988). La PSP94 est également retrouvée sous le nom de β-microséminoprotéine ou PIP (prostatic inhibin peptide).

Un certain nombre de ses fonctions, ont été postulées ou démontrées. La PSP94 serait un modulateur des niveaux de FSH dans circulation sanguine (hormone follicule-stimulante) (Sheth et coll., 1984), un inhibiteur de motilité des spermatozoïdes (Chao et coll., 1996). La PSP94 lie également des immunoglobulines dans la région reproductrice femelle (Hirano et coll., 1996). La PSP94 agit comme un régulateur de croissance des cellules épitéliales, un inducteur d'apoptose des cellules de cancer de prostate in vitro et in vivo (Garde et coll., 1999) et un régulateur des niveaux de calcium lors d'une hypercalcémie (Shukeir et coll., 2003).

L'utilité clinique de ces découvertes, fait actuellement l'objet d'études plus avancées. La PSP94 est utilisée comme biomarqueur du cancer de la prostate. En effet, les concentrations en PSP94 sériques des mâles normaux se trouvent entre 0-20 ng/ml, et les niveaux en PSP94 sériques chez les patients présantant un cancer de la prostate sont souvent fortement élevés (Huang et coll., 1993, Abrahamsson et coll., 1989, von der Kammer et coll., 1990).

Le PCK3145 est un peptide synthétique de 15 acides aminés avec un poid moléculaire de 1825 Da et un point isoélectrique de 3.5. Sa séquence est dérivée de la séquence native de la PSP94 de l'acide aminé 31 à l'acide aminé 45 : EWQTDNCETCTCYET. Plusieurs autres séquences ont été synthétisées à partir de la protéine native PSP94. Cependant, la séquence du PCK3145 a donné le meilleur résultat contre le cancer de la prostate. En effet, il a été rapporté lors du congrès 2004 de l'*American Society of Clinical Oncology* (ASCO) que le PCK3145, un agent thérapeutique dirigé contre le cancer de la prostate avancé et métastasé, a engendré des résultats positifs de l'essai clinique de phase IIa. Les résultats finaux confirment l'innocuité, la tolérabilité et l'efficacité préliminaire du PCK3145 pour l'ensemble des dosages évalués (Hawkins *et coll.,* 2005). Une fois l'étude complétée, sept patients présentaient une maladie stabilisée et une réponse partielle a été observée chez un patient après deux cycles de traitement. Les résultats les plus significatifs ont été observés au niveau de la normalisation des concentrations de la MMP-9 chez l'ensemble des patients qui présentaient des concentrations élevées avant le traitement, alors qu'aucun changement significatif n'a été observé après un cycle de traitement chez les patients présentant des concentrations normales avant le traitement (Hawkins, 2005).

In vitro, le PCK3145 réduirait la prolifération tumorale et métastasique (Shukeir *et coll.,* 2005). Aussi, en synérgie avec un modèle *in vivo* de cancer de prostate chez le rat, l'expression des cellules endothéliales marquées spécifiquement avec un

marqueur tumoral, le CD31, diminue de 43% dans les rats traités aux PCK3145. *In vitro*, dans les cellules endothéliales (HUVEC), le PCK3145 renverse l'induction de la phosphorylation de ERK par le VEGF ainsi que la phosphorylation du récepteur VEGFR-2. Ces effets anti-VEGF du PCK3145 sont identiques aux effets observés par des inhibiteurs pharmacologiques de ERK et de VEGFR respectivement le PD98059 et le PTK787, suggérant ainsi que le PCK3145 inhibe l'activité tyrosine kinase associée à VEGFR-2, ce qui empêche alternativement la signalisation intracellulaire via la cascade des MAPK. Il a été observé aussi que le PCK3145 inhibe la phosphorylation de récepteur du PDGF, le PDGFR dans les cellules musculaires lisses. De plus, le PCK3145 inhibe in vitro la formation de strctures tubulaires dans les cellules endothéliales et l'induction de la sécrétion de la MMP-2 par VEGF. Tous ces résultats suggèrent un potentiel important d'application du PCK3145 en tant qu'agent anti-angiogénique (Lamy *et coll.*, 2006).

1.6 Conclusion

Les mécanismes qui sont à l'origine du phénomène d'invasion tumorale et métastasique sont très complexes et encore trop peu connus. La compréhension de ces mécanismes et le développement de nouveaux agents anti-tumoraux sont l'axe de recherche le plus important de nombreux oncologues. Ce mémoire a permis de caractériser les principaux acteurs impliqués dans le développement tumoral et métastasique étudiés dans nos travaux *via* l'effet anti-métastasique du PCK3145.

CHAPITRE II

PRÉSENTATION DU PROJET

2.1 Introduction

Les travaux du laboratoire de médecine moléculaire du Dr. Richard Béliveau sont principalement axés sur la caractérisation des mécanismes cellulaires et moléculaires impliqués dans l'invasion et le développement du cancer telles la prolifération et la migration des cellules tumorales, la formation de tubes capillaires et l'angiogenèse associées à leur développement et particulièrement à l'implication des MMPs dans la progression tumorale. De plus, ce laboratoire s'intéresse aux effets et propriétés anticancéreuses d'une variété de composés provenant des aliments pouvant être utilisés pour la prévention ou le traitement des malades du cancer. Par exemple, nous avons montré que plusieurs catéchines du thé vert inhibent l'activité protéolytique de métalloprotéinases matricielles *in vitro*. Le laboratoire Procyon Biopharma inc. est une société de biotechnologie qui œuvre à la recherche et au développement de traitements novateurs contre le cancer jusqu'aux essais cliniques avancés. Le PCK3145, un peptide synthétique dérivé de la protéine native de la PSP94 est en phase clinique II pour le traitement du cancer de la prostate métastasique avancé. Il réduit la croissance des tumeurs prostatiques et les métastases en diminuant le niveau plasmatique de la MMP-9.

Nos travaux ont donc ciblé les mécanismes moléculaires régulant les fonctions de la MMP-9 dans les effets anti-métastasiques du PCK3145. Nous avons étudié en deux temps ces mécanismes moléculaires. Tout d'abord, nous avons dirigé nos

travaux sur la régulation de la MMP-9 à la surface de la cellule. La MMP-9 membranaire étant plus protéolytique; ayant un potentiel de dégradation des composants de la MEC plus important que celle retrouvée dans la MEC. Dans un second temps, nous nous sommes intéressés à élucider le mécanisme d'action du PCK3145 et analyser les voies de transduction activées par ce peptide pour inhiber la sécrétion de la MMP-9. L'objectif de ces travaux était donc de comprendre les mécanismes moléculaires impliqués dans le phénotype tumoral et métastasique en relation avec les effets anti-tumoraux du peptide PCK3145.

2.2 Régulations des fonctions de la MMP-9 par le PCK3145: Rôle de CD44 dans la diminution de l'interaction de la MMP-9 à la surface des cellules

La littérature, comme nous l'avons élaboré précédemment, rapporte que la MMP-9 est impliquée dans l'invasion tumorale et la formation des métastases *via* son potentiel de dégradation de la MEC. La MMP-9 peut se trouver aussi ancrée dans les membranes *via* sa liaison à des récepteurs membranaires. Il a été rapporté que la MMP-9 liée favoriserait davantage la migration des cellules cancéreuses que la MMP-9 soluble. L'une des protéines pouvant lier la MMP-9 est CD44, une glycoprotéine impliquée aussi dans l'invasion tumorale et la formation de métastases. CD44 peut être clivée par une autre protéine membranaire, la MT1-MMP. Il a été démontré dans notre laboratoire que ce clivage était dépendant de la voie RhoA/ROCK.

Le PCK3145, un peptide anti-métastasique réduit les niveaux plasmatiques de la MMP-9. Nous avons voulu savoir si, *in vitro*, le niveau de sécrétion de MMP-9 était inhibé par le PCK3145 et quel serait les mécanismes moléculaires impliqués dans la régulation de ses niveaux à la surface de la cellule. Nous nous sommes donc intéressés aux niveaux d'expression de la MMP-9 membranaire liée à CD 44 pouvant être régulés par le PCK3145.

Les résultats obtenus ont fait l'objet d'un article publié dans *Clinical Experimental Metastasis.* Volume 22(5):429-439. 2005, et sont présentés à la section Partie Expérimentale.

2.3 Régulations des fonctions de la MMP-9 par le PCK3145 : Implication de HuR dans l'inhibition de la sécrétion de la MMP-9.

Le PCK3145, dérivé de la protéine native PSP94 secrétée par la prostate, médie son action anti-métastasique en inhibant la sécrétion de la MMP-9; ce qui procure à ce peptide un potentiel thérapeutique unique. Nous avons donc tenté de caractériser un récepteur par lequel le PCK3145 module les fonctions de la MMP-9. L'un des agents thérapeutiques anti-tumoraux ayant un effet similaire d'inhibition d'expression et de sécrétion de la MMP-9 est l'EGCg, un polyphénol du thé vert. Il a été rapporté que l'EGCg transduit ses effets anticancéreux en se liant au récepteur de la laminine à 67 kDa. À la lumière de ses découvertes, nous avons fait un parallèle entre le PCK3145 et l'EGCg. Nous nous sommes aussi intéressés aux voies de transduction des signaux intracellulaires pouvant mener à l'inhibition de la MMP-9.

HuR, une protéine ubiquitaire, stabilise les ARNm contenant des séquences riches en séquences AU (AU-rich elements: AREs) afin d'inhiber leur dégradation cytoplasmique (Fan, X, 2003). Il a été démontré que l'oxide nitrique, augmenterait la dégradation de l'ARNm de la MMP-9 en inhibant l'expression de HuR (Akool el-S, *et coll.,* 2003). Finalement nous avons démontré le recrutement de HuR dans l'inhibition de la sécrétion de la MMP-9.

Les résultats obtenus ont fait l'objet d'un article publié dans *Anti-Cancer drugs.* 17: 429-438. 2006, et sont présentés à la section Partie Expérimentale.

DEUXIÈME PARTIE

PARTIE EXPÉRIMENTALE

CHAPITRE III

MANUSCRITS

3.1 "*A PSP-derived synthetic peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding : Implications in tumor metastasis* "

Annabi B., **Bouzeghrane M .,** Currie JC., Hawkins R., Dulude H., Daigneault L., Ruiz M., Wisniewski J., Garde S., Rabbani SA., Panchal C., Wu JJ. & Béliveau R. (2005)

Clin Exp Metastasis. 2005; 22:429-439.

La contribution de l'auteur du Mémoire pour la réalisation de cet article a été au niveau de l'expérimentation: toutes les expériences ont été réalisées par l'auteur et au niveau de l'écriture de l'article.

L'auteur du Mémoire est co-premier auteur dans cet article.

3.2 "*Inhibition of MMP-9 secretion by the anti-metastatic PSP94-derived peptide PCK3145 requires cell surface laminin receptors signalling* "

Annabi B, **Bouzeghrane M**, Currie JC, Dulude H, Daigneault L, Garde S, Rabbani SA, Panchal C, Wu JJ, Beliveau R.

Anti-Cancer drugs. 2006 Apr; 17: 429-438.

La contribution de l'auteur du Mémoire pour la réalisation de cet article a été au niveau de l'expérimentation: les 3/4 des expériences ont été réalisées par l'auteur et au niveau de l'écriture de l'article.

L'auteur du Mémoire est co-premier auteur dans cet article.

3.1 *''A PSP-derived synthetic peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding : Implications in tumor metastasis''*

Bouzegharne M., Annabi B., Currie JC., Hawkins R., Dulude H., Daigneault L., Ruiz M., Wisniewski J., Garde S., Rabbani SA., Panchal C., Wu JJ. & Béliveau R.

Clin Exp Metastasis. 2005; 22:429-439.

L'auteur du Mémoire est co-premier auteur dans cet article

PCK3145 un peptide derivé de la PSP94 inhibe la sécrétion de MMP-9 et clive CD44: Implication dans les métastases tumorales

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Le PCK3145 est un peptide synthétique de 15 acides aminés dérivé de la séquence native de la PSP94 31-45. *In vivo,* il réduit la croissance des tumeurs prostatiques et les métastases, en diminuant le niveau plasmatique de la métalloprotéinase matricielle 9 (MMP-9), un médiateur crucial lors de la dégradation de la matrice extracellulaire. Le mécanisme d'action de ce peptide n'est pas encore élucidé. *Méthodologie:* Des cellules de fibrosarcomes (HT-1080) ont été cultivées et traitées avec le PCK3145. Le lysat de cellules a servi pour une analyse par immunobuvardage de l'expression de RhoA, une petite GTPase et de la métalloprotéinase membranaire MT1-MMP. Le milieu conditionné a servi pour analyser l'activité gélatinolytique de MMP-9 par zymographie et l'expression de la protéine par immunobuvardage. L'expression génétique de RhoA, MT1-MMP, MMP-9, RECK et CD44 a été analysée par RT-PCR. L'analyse de l'expression de CD44 à la surface de la cellule et sa liaison à MMP-9 a été réalisée par cytométrie de flux. L'adhésion des cellules a été effectuée sur différentes protéines de la matrice extracellulaire, et leur migration spécifiquement sur l'acide hyaluronique (HA). *Résultats:* Le PCK3145 inhibe l'adhésion cellulaire des HT-1080 sur HA, laminine-1, et collagène de type-I suggérant l'implication du récepteur CD44. En effet, PCK3145 favorise le clivage de CD44 de la surface de la cellule vers le milieu extracellulaire. Le PCK3145 inhibe d'une part la sécrétion de MMP-9 et sa liaison à la surface de la cellule. Ce processus est corrélé à l'augmentation de l'expression génique et protéique de RhoA et MT1-MMP. *Conclusions:* Nos résultats suggèrent que le PCK3145 renverserait le processus métastasique des cellules tumorales en régulant les fonctions de MMP-9 en inhibant, d'une part sa sécrétion et d'autre part son potentiel à lier la surface de la cellule *via* son ancrage à la membrane par le récepteur CD44. Ce processus est dépendant de la signalisation médiée par RhoA et une augmentation de l'expression de MT1-MMP clivant CD44. En plus des effets cliniques positifs, ceci est la première démonstration de l'effet anti-métastasique du PCK3145 comme inhibiteur de sécrétion de MMP-9.

A PSP94-derived peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding: Implication in tumor metastasis

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Purpose: PCK3145 is a synthetic peptide corresponding to amino acids 31-45 of prostate secretory protein 94, which can reduce experimental skeletal metastases and prostate tumor growth *in vivo*. Part of its biological action involves the reduction of circulating plasma matrix metalloproteinase (MMP-9), a crucial mediator in extracellular matrix (ECM) degradation during tumor metastasis and cancer cell invasion. The antimetastatic mechanism of action of PCK3145 is however not understood. *Experimental design:* HT-1080 fibrosarcoma cells were treated with PCK3145, and cell lysates used for immunoblot analysis of small GTPase RhoA and membrane type (MT1-MMP) protein expression. Conditioned media was used to monitor soluble MMP-9 gelatinolytic activity by zymography and protein expression by immunoblotting. RT-PCR was used to assess RhoA, MT1-MMP, MMP-9, RECK, and CD44 gene expression. Flow cytometry was used to monitor cell surface expression of CD44 and of membrane-bound MMP-9. Cell adhesion was performed on different purified ECM proteins, while cell migration was specifically performed on hyaluronic acid (HA). *Results:* We found that PCK3145 inhibited HT-1080 cell adhesion onto HA, laminin-1, and type-I collagen suggesting the common implication of the cell surface receptor CD44. In fact, PCK3145 triggered the shedding of CD44 from the cell surface into the conditioned media. PCK3145 also inhibited MMP-9 secretion and binding to the cell surface. This effect was correlated to increased RhoA and MT1-MMP gene and protein expression. *Conclusions:* Our data suggest that PCK3145 may antagonize tumor cell metastatic processes by inhibiting both MMP-9 secretion and its potential binding to its cell surface docking receptor CD44. Such mechanism may involve RhoA signalling and increase in MT1-MMP-mediated CD44 shedding. Together with its beneficial effects in clinical trials, this is the first demonstration of PCK3145 acting as a MMP secretion inhibitor.

Running title : PCK3145 inhibits MMP-9-mediated metastasis

Key words : MMP-9, CD44, prostate cancer, metastasis, MT1-MMP, RhoA

The abbreviations used are : ECM, extracellular matrix; HRPC, hormone-refractory prostate cancer; HA, hyaluronan, hyaluronic acid; MMP-9, matrix metalloproteinase-9; MT1-MMP, membrane type-1 MMP; PMA, phorbol-myristate acetate; RECK, reversion inducing cysteine-rich protein with Kazal motifs; TGF-β, transforming growth factor- β; TNF, tumor necrosis factor

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in American males [1]. Androgen ablation as initial therapy for advanced prostate cancer provides high response rates but does not cure the disease, as nearly all men with metastases will eventually progress to hormone refractory prostate cancer (HRPC). It thus becomes crucial to develop new strategies to circumvent the progression of prostate cancer from localized growth to the invasion of surrounding tissues, and the development of distant bone and visceral organ metastasis. Prostate secretory protein 94 (PSP94), also known as prostatic inhibin or ß-microseminoprotein [2], is one of the three predominant naturally occurring proteins secreted by the prostate gland along with prostate-specific antigen and prostatic acid phosphatase [3]. In previous studies using the Dunning rat R-3327 MLL xenograft model, we have shown that PSP94 can reduce experimental skeletal metastases and prostate cancer growth *in vivo* [4], and that the amino acid 31–45 region of PSP94 (PCK3145) was sufficient to elicit PSP94-mediated anti-tumor effects [5]. More recently, a phase IIa clinical trial indicated that PCK3145 down-regulated the levels of plasma matrix metalloproteinase (MMP)-9 in patients with HRPC that had elevated levels (>100 μg/L) at baseline, while those with levels below 100 μg/L remained low [6, 7]. Such efficacy in reducing the levels of plasma MMP-9 in patients receiving PCK3145 suggests a biological effect possibly targeting the control of tumor-related ECM degradation and metastasis processes.

Prostate cancer metastasis, especially to the bone, is a multistep process that occurs at high frequency in patients with advanced disease causing significant morbidity and mortality [8]. A key mediator of this metastatic process is the hydrolysis of the surrounding extracellular matrix (ECM) by secreted soluble MMPs [9]. Among these, MMP-9 has been associated with tumor cell invasion and

metastasis and tumor-induced angiogenesis [10]. However, a major dilemma in our understanding of MMP-9 function is how the released protease is targeted to the right location at the pericellular space and how this regulates metastatic processes. Interestingly, recent functional characterization reveals that the cell surface CD44, a heavily glycosylated transmembrane protein that, as a consequence of extensive alternative splicing, exists in multiple variant forms, associates with MMP-9 in cultured murine mammary carcinoma and human melanoma cells [11, 12]. Such association of MMP-9 with CD44 has been suggested to link cell adhesion with pericellular proteolysis [11] and to promote tumor cell invasion in experimental metastasis assays [12]. CD44 cell surface function and regulation has been recently investigated by us in order to understand the highly infiltrative/metastatic phenotype of brain tumor-derived glioblastoma cells. We have shown that the caveolar localization of MT1-MMP, CD44, and RhoA at the leading edges of migrating glioma cells may initiate a functional crosstalk that would regulate the infiltrative phenotype of brain tumors [13]. Moreover, we have also provided evidence that cell surface shedding of CD44, in part, accounts for that infiltrative phenotype in U-87 glioblastoma cells [14].

In the present study, we addressed the possibility whether PCK3145 targets the above mentioned molecular pathways that regulate the metastatic phenotype of cancer cells. Our data provide new molecular evidence for the antimetastatic properties of PCK3145 as it triggers a RhoA/MT1-MMP-mediated CD44 cell surface shedding in HT-1080 fibrosarcoma cells. The use of such cellular model enabled us to simply ascertain the steps of the metastatic cascade that may further generate hypotheses that can be eventually evaluated in prostate cancer. We also show that PCK3145 exhibits a dual molecular control on MMP-9 functions as it inhibits both MMP-9 secretion and subsequent MMP-9 cell surface docking. Altogether, our data suggest that PCK3145 may antagonize tumor cell invasion processes by inhibiting a CD44/MMP-9 interaction that would lead to a decrease in tumor-associated ECM

degradation. The anticancer properties of this peptide will be highly beneficial and may potentially be exploited in targeting, not only the growth and spread of prostate cancer cells, but also in the metastasis processes of different other types of cancer.

MATERIALS AND METHODS

Materials. Agarose, sodium dodecylsulfate (SDS), gelatin, bovine serum albumin (BSA), phorbol-myristate acetate (PMA), tumor necrosis factor (TNF) and Triton X-100 were purchased from Sigma (Oakville, ON). TriZOL reagent was from Life Technologies (Gaithersburg, MD). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Type I collagen was extracted from rat tail tendon according to classical protocols [15]. The anti-CD44 R-phycoerythrin-conjugated mouse anti-human monoclonal antibody (G44-26) and mouse IgG2bκ (clone 27-35) were from BD Pharmingen (Franklin Lakes, NJ). The anti-MT1-MMP polyclonal antibody AB-815 was from Chemicon. Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The polyclonal antibodies against RhoA and CD44 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich Canada.

Cell culture and cDNA transfection method : The HT-1080 cell line was purchased from American Type Culture Collection and maintained in Dulbecco Minimum Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. The MT1-MMP cDNA was generated and validated by us, and encoded the full length MT1-MMP protein (Met_1–Val_{582}) [16]. HT-1080 cells were transiently transfected with cDNA using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed by western blotting. All experiments involving these cells were performed 36 hrs following transfection.

Mock transfections of HT-1080 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted from monolayers of cultured HT-1080 cells using the TriZOL reagent. One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON).

Primers:

MMP-9	forward: 5'-AAGATGCTGCTGTTCAGCGGG-3'
	reverse: 5'-GTCCTCAGGGCACTGCAGGAT-3' [17]
CD44s	forward: 5'-TTTGCCTCTTACAGTTGAGCCTG-3'
	reverse: 5'-GGTGCCATCACGGTTGACAATAG-3' [12]
RECK	forward: 5'-CCTCAGTGAGCACAGTTCAGA-3'
	reverse: 5'-GCAGCACACACACTGCTGTA-3') [18]
MT1-MMP	forward: 5'-ATTGATGCTGCTCTCTTCTGG-3'
	reverse: 5'-GTGAAGACTTCATCGCTGCC-3') [19]
RhoA	forward: 5'-CTGGTGATTGTTGGTGATGG -3'
	reverse: 5'-GCGATCATAATCTTCCTGCC -3') [20]

Primers were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of the amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal housekeeping gene control. PCR products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Gelatin zymography: To assess the extent of PMA- and TNF-induced extracellular MMP-9 activity, gelatin zymography was used as described previously [21]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE

in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in ddH_2O. Gels were further incubated at 37°C for 20 hrs in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were carried out with cells that had been serum-deprived by overnight incubation.

Immunoblotting procedures: Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution for MT1-MMP detection) or anti-mouse IgG (1/5,000 dilution for RhoA MMP-9 and CD44 detection) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

TCA/acetone precipitation: Trichloroactetic acid (TCA)/acetone precipitation of secreted soluble MMP-9 or shed CD44 was performed as follows. Equal volumes (300 µl) of conditioned media was mixed with 20% TCA/80% ice-cold acetone and incubated at -20°C for 1 hr. Protein pellet was precipitated following centrifugation at 11,500 rpm (18,000 x g) for 15 min at 4°C in a microcentrifuge. The supernatant was discarded, protein pellet washed with 1 ml ice-cold acetone and centrifuged as above. Acetone was drived off by heating the dry pellet at 95°C for 5-10 min.

Flow cytometry analysis: For assessment of cell surface CD44 and MMP-9 expression, cells were detached from plates, as previously described by us [13], and resuspended in 10% FBS/DMEM at a concentration of 10^6 cells/ml, washed 2 times and blocked for 15 min at room temperature in PBS containing 5% inactivated fetal calf serum (FCS/PBS). The cells were then incubated in 0.5% FCS/PBS with 0.5 μg/ml of the CD44 mAb, MMP-9 mAb or mouse IgG2bκ at room temperature for 30 min, washed once and resuspended in 0.5% FCS/PBS. Flow cytometry data was analyzed on a FACS Calibur flow cytometer with CellQuestPro software (BD Biosciences, Mississauga, ON).

Cell migration assay: Migration/invasion of cancer cells is a key event in tumor metastasis. *In vitro*, this process can be reconstituted by plating cells onto ECM-coated filters inserted in modified Boyden chemotactic chambers. Cells were dislodged after brief trypsinization, washed extensively and resuspended in MEM at a concentration of 10^6 cells/ml [13]. Cells ($5x10^4$) were then dispersed onto 1 mg/ml HA/PBS-coated chemotaxis filters (Costar; 8-μm pore size) within Boyden chamber inserts. Migration proceeded for 3 h at 37°C in 5% CO_2. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, coloured with 0.1% crystal violet/20% Methanol and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter using Northern Eclipse software. Data points indicate the mean obtained from three separate chambers within one representative experiment. The migration was quantified using computer-assisted imaging and data are expressed as the average density of migrated cells per four fields (magnification x 50).

Cell adhesion assays: Adhesion assays were performed as previously described [22]. Briefly, adhesion wells were coated with 10 μg/ml purified ECM

protein solutions for 2h at 37°C, then blocked by adding a solution of PBS/BSA 0.5%. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to precoated wells and allowed to adhere to the substrata for 4h at 37°C. After washing, adherent cells were stained with a solution of 0.1% crystal violet/20% (v/v) methanol and lysed with 1% SDS. Spectrophotometric absorbance was then measured at 600 nm.

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare the PCK3145 effect to vehicle treated cells. Probability values of less than .05 were considered significant, and an asterisk (*) identifies such significance in each figure.

RESULTS

PCK3145 inhibits MMP-9 secretion from HT-1080 cells. In the recent phase IIa clinical trial in HRPC, PCK3145 was shown to reduce the levels of circulating MMP-9 plasma levels ranging from 34 to 90% in patients having elevated level of MMP-9 (above 100 μg/L) at study entry [6, 7]. In order to investigate the effect of PCK3145 on MMP-9 secretion *in vitro*, we treated serum-starved HT-1080 fibrosarcoma cells with increasing doses of PCK3145 for 24 and 48 hrs. Doses of PCK3145 were found not to be cytotoxic as assessed by the measurement of the pro-apoptotic caspase-3 activity (not shown). MMP-9 extracellular levels were then assessed by western blotting and immunodetection. While MMP-9 extracellular levels continued to increase from 24 to 48 hrs in untreated cells, those from PCK3145-treatments decreased in a dose-dependent manner (Fig.1a). Interestingly, a 24 hours treatment with PCK3145 decreased MMP-9 by 20%, while a treatment of 48 hrs was needed to inhibit by approximately 80% the extracellular levels of MMP-9 (Fig.1b). This result confirms those observed in the phase IIa clinical trial on the efficacy of PCK to decrease MMP-9 levels up to 90%. Finally, no change in GAPDH and MMP-9 gene expression was observed upon treatment of the cells for 48 hrs with PCK3145 as assessed by RT-PCR (Fig.1c). Gene expression of two potential MMP-9 cell surface receptors, CD44 and RECK (reversion inducing cysteine-rich protein with Kazal motifs), was also found not to vary upon PCK3145 treatment (Fig.1c). This suggests that PCK3145 inhibits intracellular signal transduction mechanisms that regulate MMP-9 secretion.

PCK3145 inhibits PMA- and TNF-induced MMP-9 secretion from HT-1080 cells. In order to evaluate the capacity of PCK3145 to inhibit signal transduction leading to MMP-9 secretion, we have treated HT-1080 cells with two extremely potent MMP-9 inducers in the presence of PCK3145. Gelatin zymography confirmed

the inhibitory effect of PCK3145 on MMP-9 secretion (Fig.2a), and was used to monitor the effects of PCK3145 on phorbol-myristate-acetate (PMA)- and tumor necrosis factor (TNF)-induced MMP-9 secretion as described in the Methods section. This resulted in low levels of detectable basal MMP-9 gelatinolytic activity (Fig.2b). In contrast, while PCK3145 was able to inhibit basal MMP-9 secretion (Fig.1, Fig.2a), we show that PMA as well as TNF induced high MMP-9 secretion (Fig.2b). Interestingly, this PMA- and cytokine-mediated effect on MMP-9 was also significantly inhibited by PCK3145 (Fig.2c). This result suggests that PCK3145 may efficiently inhibit intracellular transduction mediated by serine/threonine protein kinase (PKC, PMA being an analogue of diacylglycerol) or that signalling triggered by cytokines.

PCK3145 specifically inhibits cell adhesion to laminin, type-I collagen and hyaluronic acid. ECM recognition is a crucial event in the cell adhesion process involved in tumor progression. This process is mediated and regulated through specialized cell surface receptors and integrins. Recent evidence suggests that a potential crosstalk between soluble MMP and cell surface integrins may regulate the cell's ability to recognize and adhere to its ECM environment [23]. We tested whether PCK3145 potentially downregulated HT-1080 cell adhesion. HT-1080 fibrosarcoma cells were treated with 300 μg/ml PCK3145 for 48 hrs, then dislodged and seeded onto purified ECM proteins-coated dishes as described in the methods section. We observed that cells pre-treated with PCK3145 had their adhesion significantly diminished by 31% on type-I collagen, 23% on laminin, and by 46% on hyaluronic acid (hyaluronan, HA) (Fig.3). Cell adhesion was unaffected on the other ECM proteins tested (fibronectin, vitronectin, elastin, and fibrin) (Fig.3). These results suggest that the expression or the function of specific cell surface receptors either from the integrin or non-integrin family is regulated by PCK3145. One common cell surface receptor that may recognize all three ECM proteins, that are

HA, laminin, and collagen, is CD44, which has recently been shown to also regulate prostate cell adhesion to extracellular HA [24-26].

PCK3145 regulates the expression of CD44 functional regulators RhoA and MT1-MMP, and inhibits cell migration on hyaluronic acid. Since CD44 gene expression was not affected by PCK3145 (Fig.1c), we sought to evaluate whether CD44 functional regulation would be affected by PCK3145. Recent evidence suggests that GTPase RhoA and MT1-MMP, two of the main CD44 functional regulators that also play an important role in number of processes related to metastasis, decreased cell migration and adhesion to HA [14]. Serum-starved HT-1080 cells were thus incubated with PCK3145 in conditions that significantly antagonize MMP-9 secretion (Fig.1a and b). Total RNA as well as cell lysates was isolated in order to specifically monitor RhoA and MT1-MMP respective gene and protein expression. We found that PCK3145-treated cells had their protein levels of RhoA and MT1-MMP significantly increased (Fig.4a, left panel). This increase was correlated with increased gene expression when RT-PCR was performed with specific RhoA and MT1-MMP primers (Fig.4a, right panel). GAPDH protein and gene expression were used as internal control and found not to vary upon PCK3145 treatment. Densitometry quantification of both protein (black bars) and gene (grey bars) expression of RhoA and MT1-MMP is provided in Fig.4b. These results highlight the potential role of RhoA/MT1-MMP signaling axis as being critical for CD44 functions in binding HA and that would be inhibited by PCK3145. Next, we transfected cells with the MT1-MMP cDNA and subjected them to PCK3145 treatment. Conditioned media from the respective conditions was TCA/acetone-precipitated and Western blotting followed by anti-CD44 immunodetection performed. We show that cells submitted to PCK3145 treatment induced endogenous MT1-MMP expression (Fig.4c), and that transfection with MT1-MMP resulted in the appearance of a recombinant immunoreactive MT1-MMP protein (Fig.4c). Total extracellular signal-regulated kinase (ERK) protein expression is used as an internal

loading control and shown not change in all conditions (Fig.4c). Interestingly, a proteolytic fragment of 75-kDa with CD44-immunoreactivity was detected in the conditioned media of both PCK3145-treated and MT1-MMP transfected cells (Fig.4c). This increase in CD44 shedding into the conditioned media was increased in PCK3145-treated MT1-MMP-transfected cells, but was not consistently induced suggesting a potential additive effect. Such effect has been already reported by many groups and is established as one of the MT1-MMP endpoint-mediated functions, together with the activation of proMMP-2, in the regulation of the ECM adhesion. The potential implication of decreased CD44 function thus prompted us to finally test the effect of PCK3145 on the migration of cells on top of HA-coated filters. The results show that PCK3145 pre-treatment decreased basal HT-1080 cell migration by approximately 50% (Fig.4d). As expected, decreased cell migration on HA was similarly observed in RhoA- and MT1-MMP-transfected cells (Fig.4d). Collectively, the inhibitory action of PCK3145 on HA recognition and subsequent cell adhesion/migration processes suggests that the expression of HA cell surface receptors such as those from the CD44 family could be targeted. Moreover, it is tempting to further suggest that this may also be a secondary regulation by PCK3145 of potential diminished cell surface docking of MMP-9 to CD44.

MMP-9 cell surface binding is downregulated in PCK3145-treated cells. In order to test the impact of the above forwarded hypothesis that PCK3145 triggers CD44 cell surface shedding, HT-1080 fibrosarcoma cells were treated with PCK3145. Cells were then subjected to immunophenotyping with either anti-CD44 or anti-MMP-9 antibodies as described in the method section. Flow cytometry was then performed to assess the respective cell surface levels of CD44 and membrane-bound MMP-9. In accordance with the capacity of PCK3145 to trigger the release of a CD44 immunoreactive fragment into the media, we show that the basal levels of CD44 and membrane-bound MMP-9 (Fig.5a, upper panels) were decreased in PCK3145-treated cells (Fig.5a, lower panels). This was quantified and shown to represent a 26%

decrease in CD44 cell surface expression, and a 52% decrease in membrane-bound MMP-9. This effect of PCK3145 on CD44 cell surface expression may in part explain the decreased adhesion to HA, collagen, and laminin which all represent ligands of CD44 extracellular domain [27]. More importantly, this also suggests a potential secondary level of regulation of PCK3145 on membrane-bound MMP-9 functions that could complement its primary inhibition on MMP-9 secretion.

DISCUSSION

Prostate cancer metastasis is a dynamic process involving adhesive interactions between cancer cells and ECM, and is an inevitable evolution in prostate carcinogenesis in HRPC patients [28]. New therapies that specifically target molecular entities regulating metastatic processes must be developed and mechanism of action understood. The discovery of PCK3145 as a novel potential anti-metastatic agent against HRPC provides a viable alternative among traditional cytotoxic therapies. In fact, the recently conducted phase IIa dose escalating clinical trial demonstrated that PCK3145 was safe and well tolerated in all doses (from 5 mg/m^2 to 80 mg/m^2) in patients with metastatic HRPC. A downregulation in levels of circulating plasma MMP-9 ranging from 34 to 90% in patients having an elevated level of MMP-9 (above 100 μg/L) at study entry was also observed after the treatment with PCK3145 [6, 7]. This *in vivo* PCK3145 activity suggests a biological effect possibly related to the control of MMP-9 mediated metastasis and prompted us to further explore the PCK3145 mechanism of action.

In the present study, we provide the first molecular basis for the action of PCK3145 against tumor growth and metastasis. The use of HT-1080 fibrosarcoma cells enabled us to model certain steps of the metastatic cascade that may relate to MMP-9-mediated ECM hydrolysis. Because we have tested a prostate secretory protein (PSP)94-derived peptide, our current data, in turn, generate hypotheses that can be eventually evaluated in prostate cancer. PCK3145 can indeed inhibit cell-ECM interaction, and more specifically interaction towards laminin, type-I collagen, and HA. Among these ECM proteins, insoluble laminin-rich ECM is one of the major component of the surrounding stroma from which the primary tumor site-derived prostate cancer cells will have to escape [28, 29]. Once cancer cells metastasize and reach the bone, they must then utilize the bone microenvironment to survive and propagate. Since the main component of bone ECM is type I collagen [30], the

PCK3145 inhibitory effect of cell adhesion to that particular ECM protein becomes extremely relevant and supports its use as a potential antimetastic agent. Interestingly, while binding to both laminin and type-I collagen can be mediated through integrin cell surface receptors such as α3β1 [31], recent evidence also suggests that another transmembrane protein, CD44, which acts as a cell surface receptor for HA and as a docking receptor for MMP-9 [11], potentiated the adherence of metastatic prostate cancer cells to bone marrow endothelial cells [24]. Accordingly, we show that PCK3145, in conjunction with its inhibitory effect on cell adhesion to type-I collagen and to laminin, also inhibited cell adhesion and migration onto HA. Moreover, we show that PCK3145 triggers CD44 cell surface shedding as a potential secondary mechanism of regulation of MMP-9 functions. PCK3145 may thus efficiently inhibit MMP-9-mediated metastatic processes through both MMP-9 secretion and its subsequent binding to the cell surface.

Association of MMP-9 with the cell surface is mediated by a distinct array of surface proteins that serve to regulate multiple aspects of the enzyme function including localization, inhibition and internalization [32]. Together with its inhibitory effect on MMP-9 secretion, our study specifically provides molecular evidence for an additional effect of PCK3145 on the cell surface association of MMP-9. Our observations suggest alternate new and unexpected conceptual considerations of this therapeutic peptide in targeting cancer cells metastasis processes. For instance, MMP-9 bound to the CD44 receptor is thought to process latent transforming growth factor (TGF)-β to its active form and to promote a degradative phenotype [11, 33, 34]. It becomes tempting to suggest that one of the PCK3145 biological consequences in inhibiting MMP-9 secretion and subsequent cell surface binding in metastatic processes may thus potentially impact on TGF-β ability to promote malignant progression and metastasis in inflammatory processes [35], bone metastatic tumor cells [36] and in prostate cancer cells [37].

The signal transducing events that lead to CD44 releasing process is also of particular interest in the PCK3145 action. Such proteolytic shedding from cells has been documented to involve intracellular signaling triggered by phorbol esters as well as cytokines [38, 39], two very potent inducers of MMP-9 in invading cells [40]. Interestingly, our results highlight the PCK3145 ability to efficiently inhibit PMA- and TNF-induced signaling. The latter pro-inflammatory cytokine being highly expressed in the serum of metastatic prostate cancer patients [41]. Moreover, while we show that PCK3145 was able to inhibit PMA- and TNF-induced MMP-9 secretion, PCK3145 also led to MT1-MMP activation and subsequent MT1-MMP-mediated CD44 proteolytic shedding. PCK3145-induced MT1-MMP-mediated CD44 shedding is supported by recent structure-function analysis demonstrating that the hemopexin-like domain of MT1-MMP was responsible for the binding and subsequent shedding of the standard hematopoietic form of CD44 [42]. This observation, however, suggests that several possible intracellular transduction pathways, besides that triggered by PMA and TNF, may be involved and regulated by PCK3145 action.

Interactions of prostate cancer cells within their microenvironment involve activation of cell surface receptors of integrin and non-integrin family, and this is reflected by the multiple downstream intracellular signaling triggered. Among these, intracellular Rho family GTPases play an important role in a number of processes related to metastasis, such as assembly of filopodia, lamellipodia and stress fibers [43]. Interestingly, RhoA-mediated signaling was also shown to regulate CD44 functions [44-46], and this mode of action is compatible with the induction of RhoA by PCK3145. Signaling that leads to the shedding of CD44 thus appears to regulate cell adhesion and migration involved in metastasis. Altogether, this published evidence further provides an interesting PCK3145-mediated intracellular crosstalk linking RhoA to the cell surface proteolytic activity and expression of MT1-MMP, which in turn regulates CD44 shedding and impairs MMP-9 cell surface docking.

Accordingly, a crucial RhoA/MT1-MMP signaling axis that regulated the cell surface shedding of CD44 was recently highlighted [14]. Noteworthy, cell fractionation data suggest that RhoA, CD44, MT1-MMP, and MMP-9 have the potential to co-localize at common cell surface Triton X-100-insoluble and cholesterol-enriched membrane domains termed caveolae [19, 47-49]. Interestingly, we show that PCK3145 targets all of these caveolae-associated proteins. Since caveolae also function as regulators of signal transduction in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis [50], it is tempting to suggest that PCK3145 acts as an inhibitor of caveolae-mediated intracellular transduction pathways that control MMP-9 secretion and cell–ECM interaction. As such, caveolin-1-mediated inhibition of invasion and metastasis was recently reported to occur partly through inhibition of MMP-2 and MMP-9 secretion [51], while genetic ablation of caveolin-1 delaysed advanced prostate tumor development [52]. Whether PCK3145 regulates caveolin expression and/or functions is currently under investigation.

In conclusion, our current study shows that PCK3145 may target MMP-9-mediated cancer cells metastatic processes through the suppression of MMP-9 secretion and binding to the cell surface. This process, may in part involve the proteolytic shedding of CD44 through a RhoA/MT1-MMP-mediated mechanism. Moreover, we show that PCK3145 can potentially inhibit cytokines- and PMA-induced MMP-9 secretion suggesting a role as a signal transduction inhibitor. The implications of such pleiotropic mode of action of PCK3145 on MMP-9 functions may impact, not only in cancer, but on pathological processes in which MMP-9 expression is upregulated such as inflammatory, degenerative, vascular, and infectious diseases.

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Fig.1

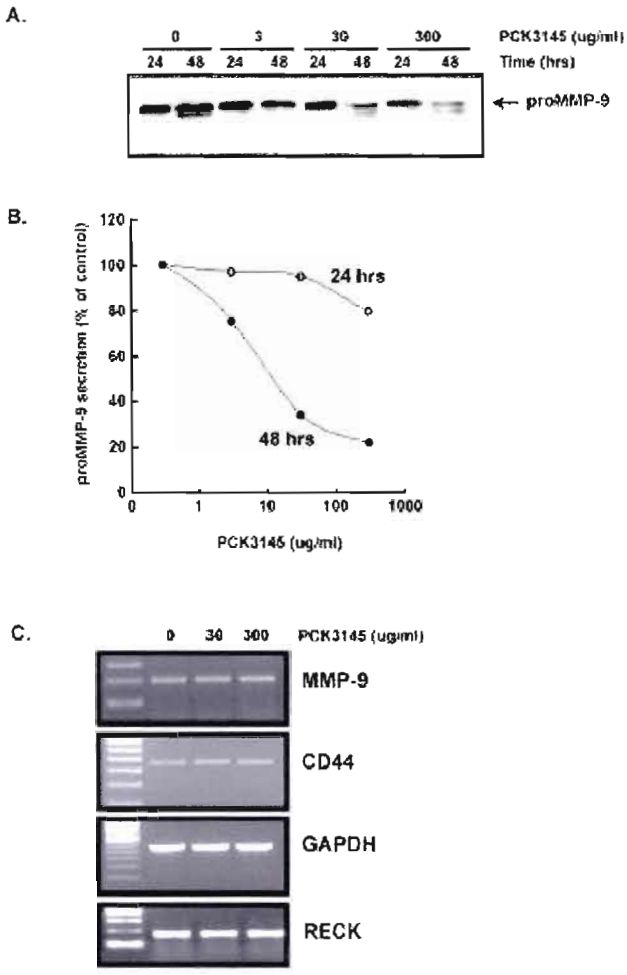

Fig.1: Extracellular MMP-9 levels are decreased in PCK3145-treated HT-1080 cells. (A) HT-1080 fibrosarcoma cells were treated with increasing PCK3145 concentrations for either 24 or 48 hrs in serum-free media. Equal volumes (300 μl) of the conditioned media were TCA/acetone-precipitated and MMP-9 protein levels assessed by Western blotting and immunodetection with anti-MMP-9 antibody. (B) Quantification of the combined latent proMMP-9 and active MMP-9 immunoreactive bands was performed by scan densitometry from a representative experiment. (C) Total RNA was isolated from 48 hrs PCK3145-treated cells and RT-PCR performed. Amplified cDNA fragments of MMP-9, CD44, RECK, and GAPDH were run on 1.8% agarose gels containing ethidium bromide.

Fig.2

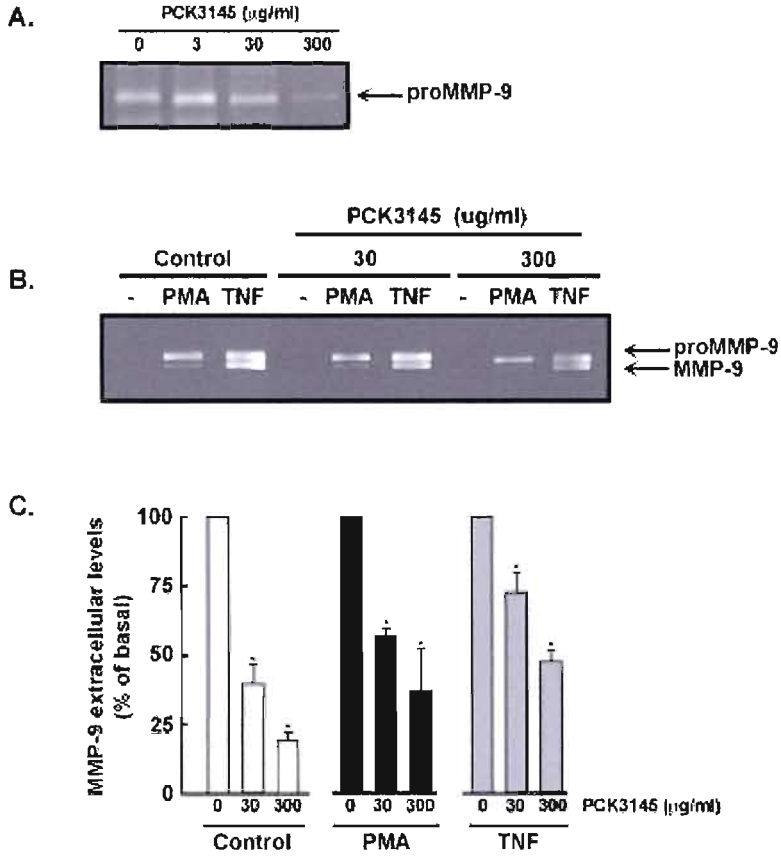

Fig.2: PCK3145 inhibits PMA- and TNF-induced MMP-9 secretion in HT-1080 cells. (A) Serum-starved HT-1080 fibrosarcoma cells were treated with increasing PCK3145 concentrations for 48 hrs in the absence or (B) presence of PMA (50 μg/ml) or TNF (10 μg/ml). Conditioned media was collected and 20 μL used to perform gelatin zymography as described in the Methods section. (C) Densitometric quantification of the total extracellular levels of MMP-9 (combined latent proMMP-9 and active MMP-9) was performed from three independent experiments. Values are expressed as the percent of either untreated cells (white bars, values taken from Fig.2a), PMA-treated cells (black bars, values taken from Fig.2b), or TNF-treated cells (grey bars, values taken from Fig.2b).

Fig.3

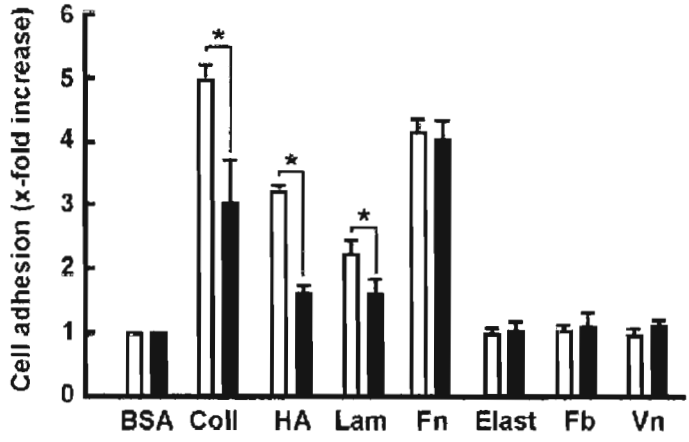

Fig.3: PCK3145 specifically inhibits HT-1080 cell adhesion to laminin, hyaluronic acid and to type-I collagen. HT-1080 fibrosarcoma cells were treated (black bars) or not (white bars) with 300 μg/ml PCK3145 for 48 hrs. Cells were then trypsinized and seeded on 10 μg/ml purified extracellular matrix proteins (BSA, bovine serum albumin; Coll, type-I collagen; Elast, elastin; Fb, fibrin; Fn, fibronectin; HA, hyaluronic acid; Lam, laminin-1; Vn, vitronectin) as described in the Methods section. Cell adhesion was left to proceed for 4 hours. Probability values of less than .05 were considered significant, and an asterisk (*) identifies such significance against the respective value of untreated cells that adhered to the specific ECM protein.

Fig.4

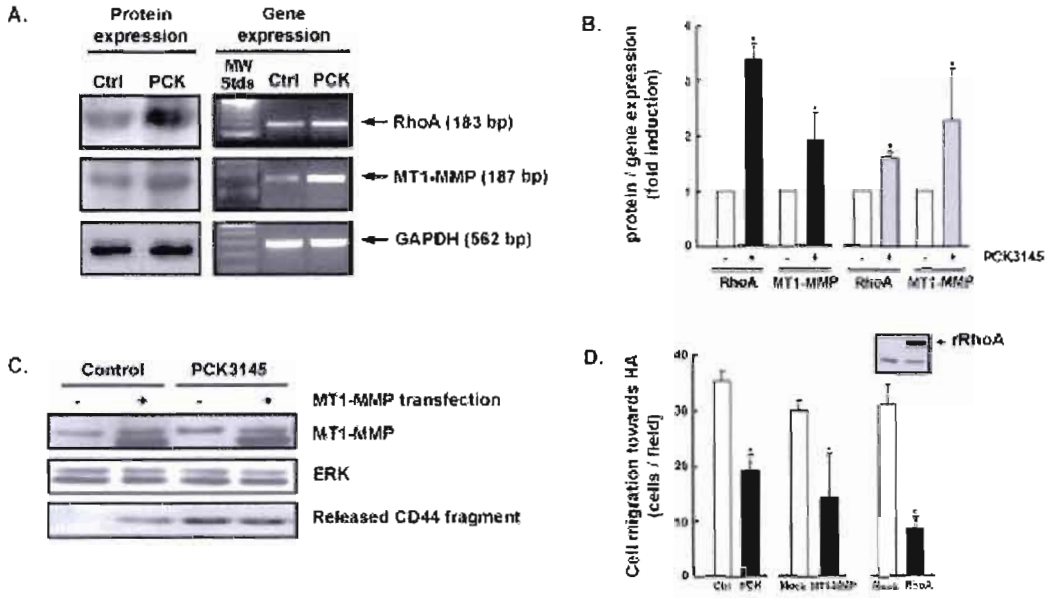

Fig.4: PCK3145 induces RhoA and MT1-MMP gene and protein expression that leads to CD44 cell surface shedding and decreased cell migration on hyaluronic acid. (A) HT-1080 fibrosarcoma cells were treated or not with 300 μg/ml PCK3145 for 48 hrs. Cell lysates and total RNA was isolated in order to perform Western blotting or RT-PCR as described in the Methods section. (B) Densitometry was performed in order to quantify the extent of protein (black bars) and gene expression (grey bars) induction by PCK3145 of RhoA and MT1-MMP. Data are expressed as the ratio of protein/gene expression over that of GAPDH. (C) HT-1080 cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or with a cDNA plasmid encoding MT1-MMP. Thirty-six hours post-transfection, cells were starved in serum-free media containing or not 300 μg/ml PCK3145 for 18 hours. Cell lysates were used to monitor MT1-MMP endogenous and recombinant MT1-MMP. Conditioned media was then collected and centrifuged to eliminate any floating cells. Equal volumes (600 μl) of the conditioned media were TCA/acetone-precipitated and subjected to Western blotting and immunodetection of CD44. (D) Alternatively, thirty-six hours post-transfection, cells were harvested by brief trypsinization and $5x10^4$ cells seeded on hyaluronic acid-coated filters. Migration was allowed to proceed as described in the Methods section. Probability values of less than .05 were considered significant, and an asterisk (*) identifies such significance against control (untreated or Mock cells). A representative immunodetection of the recombinant RhoA protein is shown.

Fig.5

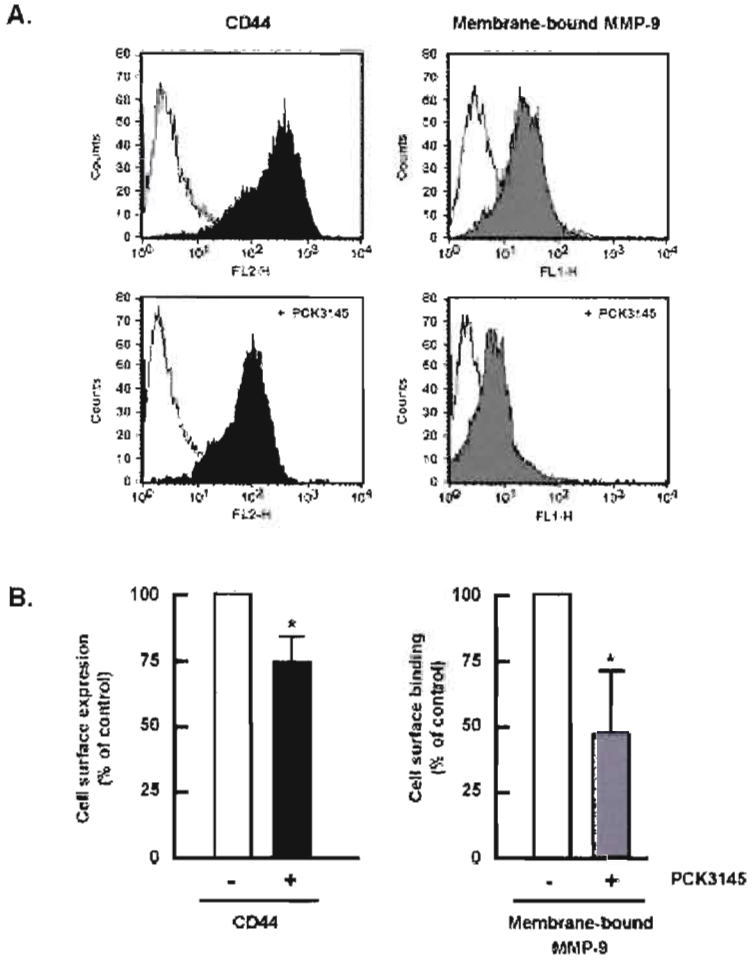

Fig.5: PCK3145-treated cells inhibits CD44 cell surface expression and MMP-9 cell surface binding. (A) HT-1080 fibrosarcoma cells were treated or not with 300 ug/ml PCK3145. Cells were then harvested and immunophenotyping performed for cell surface expression of CD44 (left panels) and MMP-9 (right panels). Flow cytometry was used to monitor CD44 cell surface protein expression in untreated (upper panels) or PCK3145-treated cells (lower panels). White tracings represent immunofluorescence using the isotype control IgG. (C) Flow cytometric results were quantified and the ratio of relative geometric mean values calculated. The effect of PCK3145 (black bars for CD44, grey bars for MMP-9) on the relative expression of cell surface CD44 or MMP-9 is expressed in percent of untreated cells (white bars) and are representative of three independent experiments.

3.2 "***Inhibition of MMP-9 secretion by the anti-metastatic PSP94-derived peptide PCK3145 requires cell surface laminin receptors signalling "***

Bouzegharne M., Annabi B, Bouzeghrane M, Currie JC, Dulude H, Daigneault L, Garde S, Rabbani SA, Panchal C, Wu JJ, Beliveau R.

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L'inhibition de la sécretion de MMP-9 par le PCK3145 requière un signal intracellulaire médié par le recepteur de la laminine

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Le PCK3145 est un peptide synthétique de 15 acides aminés (31 à 45) dérivé de la séquence native de la PSP94 31-45. *In vivo* il réduit la croissance des tumeurs prostatiques et des métastases. L'effet anti-métastasique et anti-tumoral du PCK3145 est partiellement expliqué *via* la suppression des niveaux de la métalloprotéinase MMP-9 extracellulaire *in vivo* et *in vitro.* Cependant aucun mécanisme d'action du PCK3145 n'a été élucidé. *Méthodologie:* La zymographie sur gélatine et l'immunobuvardage ont été utilisés pour analyser les niveaux de sécrétion de MMP-9 après traitement des HT-1080 au PCK3145. La cytométrie de flux a été utilisée pour analyser la liaison du PCK3145-FITC et de la laminine biotinylée à la surface des cellules HT-1080. Un test d'adhésion sur le PCK3145 a été effectué avec les HT-1080. Les cellules lysées on été utilisées pour analyser l'expression par immunobuvardage de HuR, ERK et phosphoERK. L'ARN a été isolé et une RT-PCR utilisée pour évaluer l'expression génique de HuR. *Résultats:* Le PCK3145 en liant les HT-1080, déclenche la phosphorylation de ERK menant à la réduction de MMP-9. La laminine inhibe la liaison du PCK3145 à la surface de la cellule et la phosphorylation de ERK. La surexpression du récepteur de la laminine de 67-kDa favorise la liaison du PCK3145 à la cellule. L'expression de HuR, une protéine liant l'ARNm de MMP-9, est diminuée par le PCK3145. L'inhibition de ERK par le PD98059, son inhibiteur spécifique, la protéine native de la laminine et le peptide derivé de la laminine le SIKVAV renversent la phosphorylation de ERK par le PCK3145. *Conclusions:* Le PCK3145 induit un signal intracellulaire rapidement *via* le récepteur de la laminine à 67kDa. Ce signal induit une diminution d'expression de HuR et déstabilise ainsi l'ARNm de MMP-9. Ce mécanisme expliquerait l'effet anti-métastasique du PCK3145 *via* l'inhibition de sécrétion de MMP-9.

The inhibition of MMP-9 secretion by the anti-metastatic PSP94-derived peptide PCK3145 requires cell surface laminin receptors signaling

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Purpose: PCK3145 is a synthetic peptide corresponding to amino acids 31-45 of prostate secretory protein 94 which can reduce experimental skeletal metastases, and prostate tumor growth. These antimetastatic and antitumoral effects of PCK3145 are partially explained by the *in vivo* and *in vitro* decrease in matrix metalloproteinase (MMP)-9 extracellular levels through, however, yet unidentified molecular mechanisms of action. *Experimental design:* Gelatin zymography and immunoblots were used to monitor the levels of secreted MMP-9 from HT-1080 cells. Flow cytometry was used to monitor HT-1080 cell surface binding of FITC-labeled PCK3145 and of biotin-labeled laminin. PCK3145-coated cell culture dishes were used to monitor cell adhesion. HT-1080 cell lysates were used for immunoblotting of HuR, ERK and phosphoERK. Total RNA was isolated and RT-PCR used to monitor HuR gene expression. *Results:* We found that PCK3145 bound to HT-1080 cell surface, and that this binding rapidly triggered ERK phosphorylation that, ultimately, led to a reduction of secreted MMP-9. Laminin inhibited both cell surface binding and ERK phosphorylation by PCK3145. Overexpression of the 67-kDa laminin receptor led to an increased binding of the cells to PCK3145. HuR, a protein that can bind to and stabilize MMP-9 mRNA, was found downregulated by PCK3145. The MEK inhibitor PD98059 as well as native laminin and SIKVAV laminin-derived peptide prevented that downregulation. *Conclusions:* Our data suggest that PCK3145 rapidly triggers intracellular signaling through cell surface laminin receptors. This leads to decreased HuR expression and subsequent destabilization of MMP-9 transcripts. This is the first molecular evidence demonstrating the intracellular signaling and antimetastatic mechanism of action of PCK3145 that leads to the inhibition of MMP-9 secretion.

Running title: PCK3145 binds to cell surface laminin receptors

Key words: ERK, laminin receptor, prostate cancer, metastasis, HuR, MMP-9

The abbreviations used are: ECM, extracellular matrix; EGCg, epigallocatechin-3-gallate; ERK, extracellular signal-regulated protein kinase; HRPC, hormone-refractory prostate cancer; 67LR, 67-kDa laminin receptor; MMP-9, matrix metalloproteinase-9

INTRODUCTION

Prostate cancer is frequently associated with bone metastases, which are in fact the main cause of morbidity and mortality for this tumor. It is also the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in American males [1]. Androgen ablation as initial therapy for advanced prostate cancer provides high response rates but does not cure disease, as nearly all men with metastases will eventually progress to hormone-refractory prostate cancer (HRPC). Currently no effective treatments exist for patients where hormone treatment has failed, and the management of HRPC is solely palliative. It thus becomes crucial to develop new strategies to circumvent the progression of prostate cancer from localized growth to the invasion of surrounding tissues, and the development of distant bone and visceral organ metastasis. Prostate secretory protein 94 (PSP94), also known as prostatic inhibin or ß-microseminoprotein [2], is a naturally occurring protein synthesized primarily in the prostate and found in large quantities in the seminal fluid [3]. Although the complete physiological role of PSP94 is not completely known, it is believed to be involved as a growth inhibitor and a promoter of cell death in the natural control of excessive and/or abnormal proliferation of epithelial cells both in normal prostate tissue as well as in malignant prostate tissues.

In previous studies, we have shown that PSP94 can reduce experimental skeletal metastases and prostate cancer growth *in vivo* [4], and that the amino acid 31–45 region of PSP94 (PCK3145) was sufficient to elicit PSP94-mediated anti-tumor effects [5]. More recently, a concluded phase IIa clinical trial indicated that PCK3145 down-regulated the levels of plasma matrix metalloproteinase (MMP)-9 in patients with HRPC that had elevated levels (>100 µg/L) at baseline [6]. Such therapeutic efficacy in reducing the levels of plasma MMP-9 in HRPC patients receiving PCK3145 suggests a biological effect possibly targeting the control of

tumor-related ECM degradation and metastasis without safety concerns nor adverse effects.

We have recently reported several molecular processes involved in tumor progression and which are targeted by PCK3145. Indeed, we demonstrated the potential antiangiogenic effect of PCK3145 *in vivo* and *in vitro* in endothelial cells as it inhibited VEGF signaling through the tyrosine kinase activity associated with the VEGF receptor [7]. Moreover, we also have provided evidence for an antimetastatic effect of PCK3145 targeting both MMP-9 secretion and subsequent cell surface docking to CD44 [8]. However, the complete mechanism of action of PCK3145, including the elucidation of the cell surface receptors and intracellular pathways that lead to reduced MMP-9, remained elusive. Our study shows for the first time that cell surface laminin receptors transduce PCK3145 intracellular signaling which involves a rapid and transient phosphorylation of ERK (Extracellular signal-Regulated protein Kinase). These effects of PCK3145 lead to the inhibition of the MMP-9 mRNA-binding and stabilizing protein HuR gene and protein expressions. The identification of the mechanism of action of PCK3145 as well as the potential involvement of cell surface laminin receptors allows to extend PCK3145 ability to target other types of cancer such as those expressing high levels of laminin receptors as observed in leukemia cells within bone marrow.

MATERIALS AND METHODS

Materials: Agarose, sodium dodecylsulfate (SDS), gelatin, bovine serum albumin (BSA), and Triton X-100 were purchased from Sigma (Oakville, ON). TriZOL reagent was from Life Technologies (Gaithersburg, MD). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Type-I collagen was extracted from rat tail tendon according to classical protocols [9]. Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The polyclonal antibodies against 67-kDa laminin receptor (67LR) and HuR were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-phospho-ERK was from Cell Signaling Technology (Beverly, MA) while the polyclonal anti-MMP-9 and ERK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). Fluorescein isothiocyanate (FITC)-N-terminal-labelled PCK3145 the laminin-1-derived peptide SIKVAV were synthesized by EZBiolab (Westfield, IN). Epigallocatechin-3-gallate (EGCg) and all other reagents were from Sigma-Aldrich Canada.

Cell culture and cDNA transfection method: The HT-1080 cell line was purchased from American Type Culture Collection and maintained in Dulbecco Minimum Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 □g/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. The human 67LR full-length cDNA was from OriGene Technologies (Rockville, MD). HT-1080 cells were transiently transfected with cDNA using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed by western blotting. All experiments involving

these cells were performed 36 hrs following transfection. Mock transfections of HT-1080 cultures with pcDNA (3.1^+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted from monolayers of cultured HT-1080 cells using the TriZOL reagent. One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON).
Primers for HuR (forward: 5'-TCGCAGCTGTACCACTCGCCAG-3', reverse: 5'-CCAAACATCTGCCAGAGGATC-3') [10] were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of the amplification. PCR products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Immunoblotting procedures: Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% BSA, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution for MMP-9, HuR, ERK, and phospho-ERK detection) or anti-mouse IgG (1/5,000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

Gelatin zymography: To assess the extent of MMP-9 activity, gelatin zymography was used as described previously [11]. Briefly, an aliquot (20 □l) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin.

The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37°C for 20 hrs in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background. All experiments were carried out with cells that had been serum-deprived by overnight incubation.

Cell adhesion assays: Adhesion assays were performed as previously described [12]. Briefly, adhesion wells were coated with increasing concentrations of PCK3145 for 2 hrs at 37°C, and then blocked by adding a solution of PBS/BSA 0.5%. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to pre-coated wells and allowed to adhere to the substrata for 4 hrs at 37°C. After washing, adherent cells were stained with a solution of 0.1% crystal violet/20% (v/v) methanol and lysed with 1% SDS. Spectrophotometric absorbance was then measured at 600 nm.

Flow cytometry analysis and fluorescein isothiocyanate-labeled PCK3145 binding assay : Fluorescein isothiocyanate (FITC) is currently the most commonly-used fluorescent dye for FACS analysis and was conjugated to the N-terminus of PCK3145. This enables us to follow cell binding of FITC-PCK3145 through the shift in fluorescence associated with cells that bind to it. 80-90% confluent HT-1080 cells were dislodged after brief PBS-citrate treatment, washed extensively, resuspended in 10% FBS/DMEM at a concentration of 10^6 cells/ml, washed once with 0.1% PBS/ 0.1% BSA and then incubated with 10 μg/ml FITC-PCK3145 for 1 hr on ice (4°C). After washing with PBS/BSA, the cells were suspended in 1 ml PBS/BSA, and analyzed on a FACS Calibur flow cytometer with the CellQuestPro software (BD Biosciences, Mississauga, ON). Results are expressed as the ratio of relative

geometric mean values from the PCK3145-treated cells to their untreated controls and are representative of three independent experiments.

PCK3145 biotinylation and HPLC analyses: PCK3145 was biotin labeled according to Pierce and purified by FPLC-chromatography using Akta-explorer with a resource RPC-30ml column (Amersham Bioscience QC). The biotin-labeled peptide was eluted with a gradient from 20% CH_3CN to 80% CH_3CN (+0.05%TFA) at a flow rate of 4 ml/min. Products were monitored at different wavelengths (205, 229 and 254 nm) with a UV-900 cell-10.

Gene reporter assay: The Great EscAPe SEAP reporter system (CLONTECH), in which a secretory alkaline phosphatase (SEAP) form is fused to promoters activated by different responsive elements (described in the legend of Fig.5), was used to monitor the effect of PCK3145 on different response elements. Cells were transfected with the various constructs and aliquots of the conditioned media were collected at different times. SEAP activity was measured by the hydrolysis of p-nitrophenylphosphate [13].

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare the PCK3145 effect to vehicle treated cells. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

RESULTS

PCK3145 inhibits MMP-9 secretion from HT-1080 cells. PCK3145 was shown to reduce the levels of circulating MMP-9 plasma levels in phase IIa clinical trial [6]. In order to investigate and confirm the effect of PCK3145 on MMP-9 secretion *in vitro*, we treated serum-starved HT-1080 fibrosarcoma cells with increasing doses of PCK3145 for 48 hrs [8]. Doses of PCK3145 were found not to be cytotoxic as assessed by the measurement of the pro-apoptotic caspase-3 activity (not shown). MMP-9 extracellular levels were then assessed by western blotting and immunodetection. As expected from the clinical data, MMP-9 extracellular levels decreased significantly in a dose-dependent manner in PCK3145-treated cells (Fig.1a). A maximum inhibition of approximately 80% of extracellular levels of MMP-9 was observed at 300 ug/ml (Fig.1b). This *in vitro* result confirms those observed in the phase IIa clinical trial on the efficacy of PCK3145 to decrease MMP-9 levels.

PCK3145 binding to the cell surface of HT-1080 cells is inhibited by laminin. In order to assess whether the inhibitory action of PCK3145 on MMP-9 secretion involves any cell surface receptors-mediated signaling, we first need to know if PCK3145 binds to the cell surface of HT-1080 fibrosarcoma cells. N-terminal fluorescein isothiocyanate (FITC)-labelled PCK3145 was synthesized and incubated with HT-1080 cells for 30 minutes at 4°C in order to minimize intracellular uptake of the labelled-peptide [14], and cell-associated fluorescence measured by flow cytometry as described previously [15]. As shown by the significant shift in fluorescence, we demonstrate that FITC-PCK3145 effectively bound to the HT-1080 cell surface in a dose-dependent manner with a plateau reached around 10 μg/ml (Fig.2a). Furthermore, because we had previously shown that PCK3145 inhibited cell adhesion to laminin [8] and that this may inhibit metastasis processes, we also

performed a competition experiment by incubating the cells with FITC-labelled PCK3145 (Fig.2B, upper panel, shaded plot) or with both laminin (30 μg/ml) and FITC-labelled PCK3145 (Fig.2B, lower panel). Interestingly, our results show a complete inhibition of PCK3145 cell surface binding in the presence of excess laminin (Fig.2b). This latter observation is the first evidence that cell surface receptors with laminin-binding activities might trigger the subsequent intracellular signaling by PCK3145.

PCK3145 inhibits cell surface binding of laminin. In an attempt to characterize the potential laminin receptor involved in PCK3145 binding to the cells, we decided to label laminin with biotin and to analyze cell surface binding by flow cytometry. Moreover, the effect of epigallocatechin-3-gallate (EGCg), a green tea-derived molecule which shares the ability with PCK3145 to inhibit MMP secretion [16, 17] and which receptor was identified as the non-integrin 67-kDa laminin receptor (67LR) [18] was also tested. We show that labelled-laminin bound to the cell surface of HT-1080 cells by the shift in fluorescence (Fig.3a, control). Interestingly, while excess non-labelled laminin (Fig.3a, Laminin) and PCK3145 (Fig.3a, PCK3145) effectively competed for respectively 30% and 45% inhibition (Fig.3b) with that binding of labelled-laminin, we found that EGCg was a very potent agent to inhibit biotin-labeled laminin cell surface binding by 80% (Fig.3a, EGCg). The observed partial inhibitory effect of PCK3145 may be due to the fact that laminin can bind to multiple cell surface receptors from integrins and non-integrins family, while PCK3145 may rather bind to a more specific cell surface laminin receptor. Whether the 67LR is involved in recognizing PCK3145 was next investigated.

Cell adhesion to PCK3145 is induced in 67-kDa laminin receptor-transfected cells. In order to characterize the potential involvement of the 67LR protein in interacting with PCK3145, we designed an adhesion assay to PCK3145-coated dishes. Native HT-1080 cells were dislodged by trypsinization and seeded ontop of

culture dishes that were coated with increasing concentrations of PCK3145. We found that HT-1080 cells recognized and adhered to PCK3145-coated dishes with a maximal adhesion found at 10 μg/ml of PCK3145 (Fig.4a). Interestingly, although higher concentrations of PCK3145 still triggered significant cell adhesion, it was however gradually decreasing. Whether this binding to PCK3145 involved the 67LR was next investigated by transiently transfecting HT-1080 cells with the 67LR plasmid cDNA. Using mock versus 67LR-transfected cells, we observed that while basal cell adhesion remained unaffected, an increase in adhesion to PCK3145 occurred when cells overexpressed the recombinant 67LR protein (Fig.4b). Immunoblotting was used to show that efficient expression of the recombinant 67LR protein was generated (Fig.4c, insert), and increase in cell adhesion monitored in time (Fig.4c).

PCK3145 induces a rapid but transient phosphorylation of ERK. Ligand binding to laminin receptors induces the activation of many intracellular pathways [19]. We next tested which potential intracellular pathways are activated by PCK3145 potential binding to the cell surface. We performed a gene-reporter assay as described in the methods section in order to monitor the activity of eukaryotic promoters and enhancers that are triggered by PCK3145. We show that PCK3145 significantly triggers two pathways: the MAPK/ERK pathway (SRE) and the NFkB pathway (Fig.5a, black bars). The 6.6-fold increase in the MAPK/ERK pathway is extremely strong as compared to that of the NFkB pathway (3.5-fold). The latter however potentially suggests the involvement of pro-apoptotic pathways that would be triggered by PCK3145 and that we previously reported in our *in vivo* model [5]. PCK3145 induction of the MAPK pathway is further confirmed by the rapid and transient induction of ERK phosphorylation (Fig.5b) peaking at 1 minute while total ERK levels remained unaffected (Fig.5b and 5c). Finally, the effects of PCK3145 were also compared to those of a scrambled peptide which was unable to induce ERK

phosphorylation as PCK3145 did (not shown). This rapid and transient effect of PCK3145 suggests that a "hit-and-run" mechanism of action be envisioned.

Laminin and laminin-derived peptide SIKVAV antagonize PCK3145-induced ERK phosphorylation and inhibitory action on MMP-9 secretion. In order to confirm the transduction mechanism involved in PCK3145 inhibitory action on MMP-9 secretion, we treated HT-1080 cells with either the native laminin-1 protein or with a laminin-1-derived peptide SIKVAV. The latter is known for its capacity to induce MMP-9 secretion [20]. Confluent serum-starved cells were treated with these agents in the presence or not of 300 μg/ml PCK3145 for 24 hrs. Conditioned media was isolated and gelatin zymography performed to monitor the extent of MMP-9 extracellular levels. Results show that PCK3145 was indeed able to inhibit by 50% MMP-9 extracellular levels (Fig.6a, control). Interestingly, SIKVAV-induced MMP-9 secretion was also antagonized by PCK3145 (Fig.6b), while native laminin-1 inhibited PCK3145 inhibitory action on MMP-9 secretion (Fig.6b). Finally, PCK3145-induced ERK phosphorylation was also monitored in the presence of laminin-1 and laminin-1-derived peptide SIKVAV. We show that ERK phosphorylation is completely inhibited by the presence of native laminin or SIKVAV (Fig.6c). Collectively, these results are strong evidence that laminin and laminin-derived peptides interfere with PCK3145 intracellular signaling and subsequent MMP-9 secretion inhibitory actions.

PCK3145 inhibits the gene and protein expression of the MMP-9 mRNA stabilizing factor HuR. A missing link between laminin and MMP-9 expression still hampers any elucidation of the potential mechanism of action of PCK3145. Recent studies suggested that MMP-9 expression be induced through a stabilizing nuclear factor HuR [21], and that $\alpha_3\beta_1$ integrin, an integrin known to bind laminin, regulated MMP-9 mRNA [22]. These published evidence link Hur, an mRNA stabilizing factor that is ubiquitously expressed and that has the ability to bind to AU-rich elements

(AREs) and prevent mRNA degradation. Interestingly, AREs are expressed in the 3'-untranslated region of MMP-9 [21, 23]. Whether PCK3145 regulates HuR expression was tested. Serum-starved cells were treated with PCK3145 in the presence or not of laminin or SIKVAV, and cells harvested for either RNA extraction or cell homogenates. RT-PCR revealed that HuR gene expression was indeed downregulated by PCK3145 and that both laminin and laminin-derived peptide SIKVAV antagonized PCK3145's inhibitory effect (Fig.7a). This was further confirmed independently with specific anti-HuR antibody and immunodetection (Fig.7b). Furthermore, treatment of the cells with PD98059, a MEK inhibitor, completely reversed the inhibition of HuR expression by PCK3145 (Fig.7c). This effect of PCK3145 on HuR may partly explain the inhibition of MMP-9 expression and subsequent diminished extracellular levels.

DISCUSSION

MMP-9 is a matrix metalloproteinase involved in prostate cancer progression and which expression can be regulated at several levels. Although most published studies have focused on transcriptional control of MMP-9, there is increasing evidence that its expression can also be regulated at the steps of mRNA stability, translation and protein secretion. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion and metastasis, when tumor cells need to induce or maintain MMP-9 levels in response to changing environmental cues. While PCK3145, a 15-mer peptide derived from PSP94, does not affect MMP-9 enzymatic activity *per se*, we show that it however significantly reduces its gene expression which, consequently, leads to decreased extracellular MMP-9 secreted levels [24, this study]. We believe that we are now able to integrate the recently obtained experimental data on the mechanism of action of PCK3145 into a cohesive model linking cell surface laminin binding activities, CD44 cell surface expression modulation and MMP-9 secretion all linked to the metastatic process (Fig.8).

The novel laminin receptor–mediated regulation of MMP-9 expression mechanism of action of PCK3145 provides solid foundation to our hypothesis that PCK3145 acts through receptor-mediated signaling. Further investigations into the molecular basis of laminin receptor-mediated tumor progression may help unravel new targets for PCK3145. For instance, increased expression of the 67LR has been reported in a variety of human carcinomas (colon, breast, stomach, liver, and ovary) and directly correlates with a higher proliferation rate of malignant cells and tendency to metastasize [25]. In addition, the 67LR is detectable in anaplastic large cell lymphomas and in small subsets of high-grade B-cell non-Hodgkin's or Hodgkin's lymphomas [26], and the detection of the 67LR in prostate cancer biopsies has been proposed to act as a predictor of recurrence after radical prostatectomy [27].

Moreover, expression of the 67LR has been found to mediate acute myeloid leukemia cell adhesion to laminin and to frequently be associated with monocytic differentiation [28]. In light of these documented expression of the 67LR, it is thus tempting to suggest that alternate cancers, or cancers at specific stage of development, such as monocytic-oriented acute myeloid leukemia, could potentially be efficiently targeted by PCK3145.

Our data suggest that intracellular signaling by PCK3145, that leads to the inhibition of MMP-9 extracellular levels, is mediated through cell surface laminin receptor-like activity such as that transduced by the 67LR. This is re-enforced by the unexpected observation that PCK3145 shares, to some extent, homology with different structural chain precursors of laminin including alpha-2, alpha-5, and beta-1 chains (not shown), which characteristic suggests that PCK3145 may also share the potential to interact with common cell surface receptors. Interestingly, EGCg, a green tea catechin that similarly to PCK3145 inhibits MMP-9 secretion, is also a 67LR ligand that antagonized cell binding to PCK3145. This confirms that such cell surface receptors may regulate PCK3145 effects. The partial inhibition of the cell surface laminin binding by PCK3145 however suggests the potential involvement of other cell surface laminin receptors. Undoubtedly, our data support that PCK3145 cell signaling, at least through ERK phosphorylation, involves laminin receptors.

We have identified HuR, a MMP-9 mRNA stabilizing factor, to be targeted by PCK3145. Indeed, both of its gene and protein expression were downregulated by PCK3145, and this dowregulation was reversed by laminin receptor ligands. PCK3145 can thus be viewed as a laminin receptor-mediated signal transduction inhibitor. Whether alternate intracellular signaling are involved in PCK3145 actions still remains to be investigated although RhoA- and ERK-induced pathways have been highlighted [8, this study]. As HuR also binds to the AU-rich elements of RNAs encoding genes for cytokines, growth factors, tumor suppressor genes, proto-

oncogene, and cell cycle regulators, one can envision that downregulation of HuR by PCK3145 may also inhibit cell proliferation or induce apoptosis. These alternate cellular processes are currently under investigation.

PCK3145 may transduce its intracellular HuR inhibitory effects through the binding of specific cell surface receptors of non-integrin origin such as the 67LR. Such PCK3145 intracellular transducing events involve ERK and previous studies have interestingly demonstrated similar cooperativity between integrins and growth factor receptors in the regulation of MAPK signaling pathways. For example, growth factor-dependent induction of ERK signaling in NIH 3T3 cells is strongly dependent on integrin-mediated cell adhesion where the activation of MEK/ERK signaling was identified as an adhesion-dependent event. Our model (Fig.8) suggests a distinct novel mechanism whereby cell surface laminin receptor-like activities can cooperate with ECM protein stimuli to induce MEK/ERK expression that could potentially be targeted by PCK3145.

The recently conducted phase IIa clinical trial with PCK3145 confirmed its therapeutic safety and tolerability for HRPC [6]. Interestingly, this effect was in part correlated to a marked reduction in the high levels of plasma MMP-9 (values above 100 μg/ml), suggesting a biological effect possibly related to control metastasis. Although we have already characterized some of the potential antiangiogenic and antimetastatic properties of PCK3145 [7, 8], the present study provides the first molecular clue for the potential mechanism of action of PCK3145 against prostate cancer tumor growth and metastasis. The identification of the cell surface laminin receptor activities involved in the transduction of PCK3145 intracellular actions may now help extend the future applications of that therapeutic peptide against other types of cancer in which high levels of MMP-9 and/or laminin receptor expression is associated.

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Fig.1

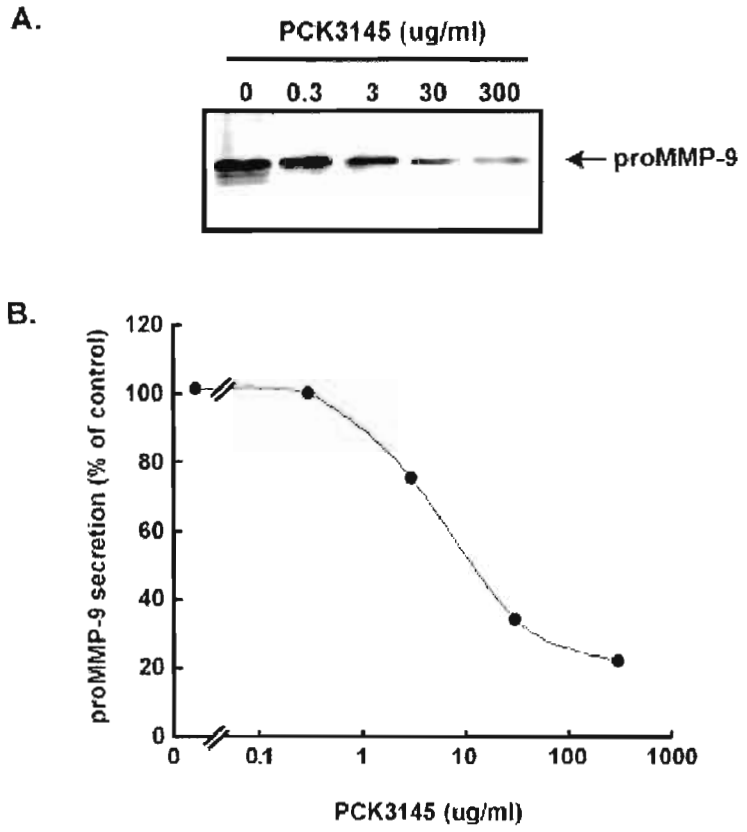

Fig.1: Extracellular MMP-9 levels are decreased in PCK3145-treated HT-1080 cells. (A) HT-1080 fibrosarcoma cells were treated with increasing PCK3145 concentrations for 48 hrs in serum-free media. Equal volumes (300 µl) of the conditioned media were TCA-precipitated and MMP-9 levels assessed by Western blotting and immunodetection with anti-MMP-9 antibody. (B) Quantification of a representative experiment is presented and was performed by scan densitometry.

Fig.2

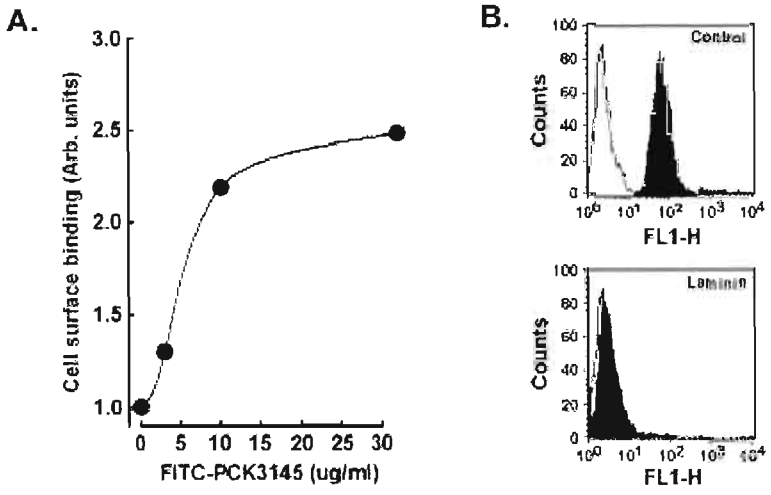

Fig.2: Cell surface binding of PCK3145 is inhibited by laminin. (A) HT-1080 fibrosarcoma cells were trypsinized and 10^6 cells incubated with increasing concentrations of N-terminal FITC-labelled PCK3145 for 1 hr at 4°C. Cell-associated fluorescence was measured by flow cytometry. (B) HT-1080 cells were incubated with 10 μg/ml of FITC-labeled PCK3145 alone (control) or in the presence of 30 μg/ml laminin (laminin) and cell–associated fluorescence measured in the same conditions as above.

Fig.3

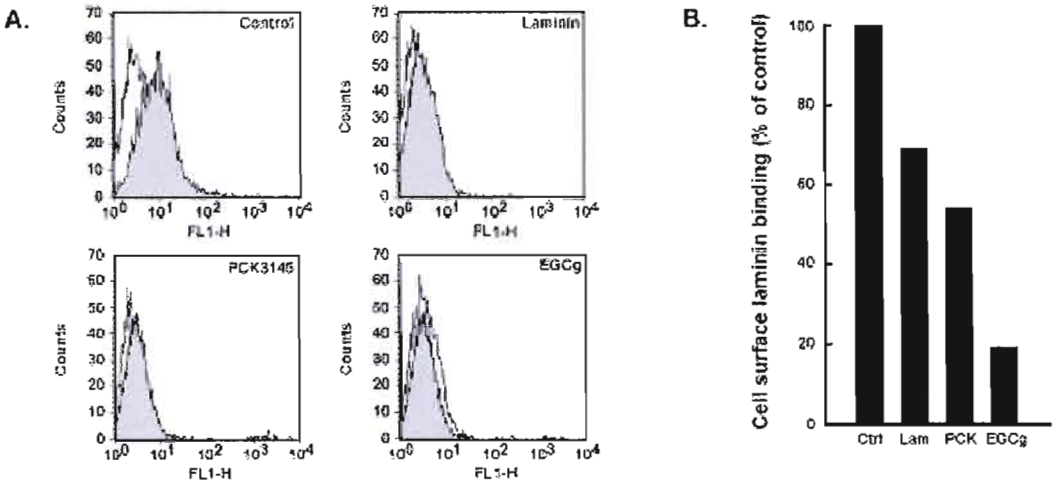

Fig.3: Cell surface binding of laminin is inhibited by PCK and by the green tea catechin EGCg. (A) HT-1080 fibrosarcoma cells were trypsinized and 10^6 cells incubated with 1 μg/ml biotin-labelled laminin (shaded plots) for 1 hr at 4°C. Cell-associated fluorescence was measured by flow cytometry as described in the methods section in the presence of 30 μg/ml laminin (laminin), 30 μg/ml PCK3145 (PCK3145), or in the presence of 30 μM EGCg (EGCg). A representative experiment is presented and (B) quantification performed as described in the methods section.

Fig.4

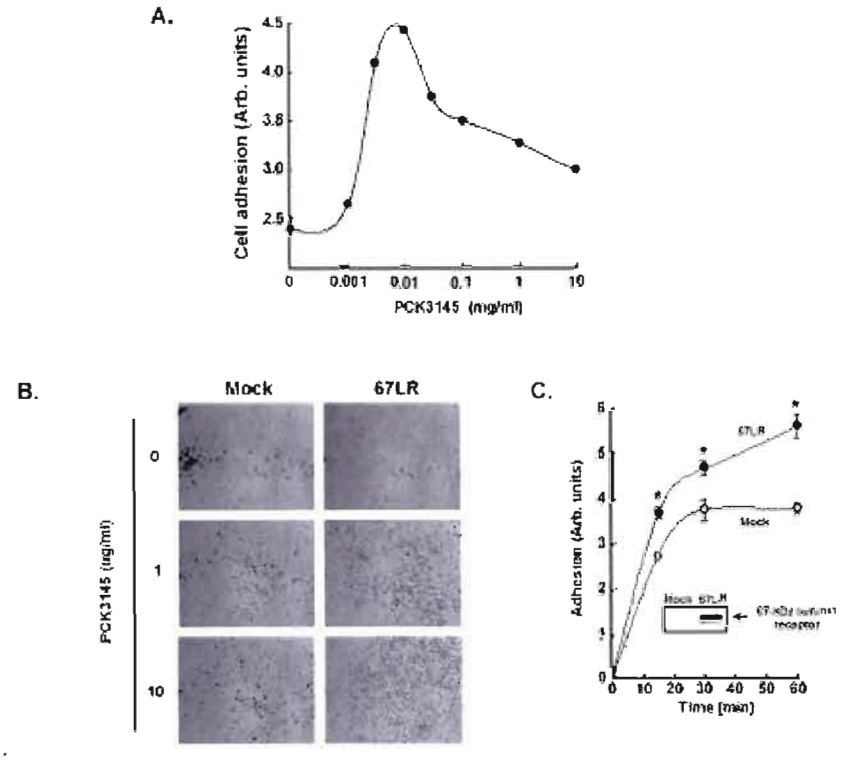

Fig.4: Cell adhesion to PCK3145 is increased in 67-kDa laminin receptor transfected cells. (A) HT-1080 fibrosarcoma ($25x10^4$ cells / well) were seeded on top of 1 μg/ml PCK3145-coated dishes. Cell adhesion was left to proceed for 1 hr and was quantified as described in the methods section. (B) Control (Mock) or 67LR-transfected HT-1080 cells were seeded on PCK3145-coated dishes and left to adhere. Representative pictures are taken and show increased adhesion to PCK3145 when the 67LR is overexpressed. (C) A time course of cell adhesion to 1 μg/ml PCK3145 shows a rapid and significant increase in 67LR-transfected cells. A representative immunodetection of the 67LR precursor is shown (insert).

Fig.5

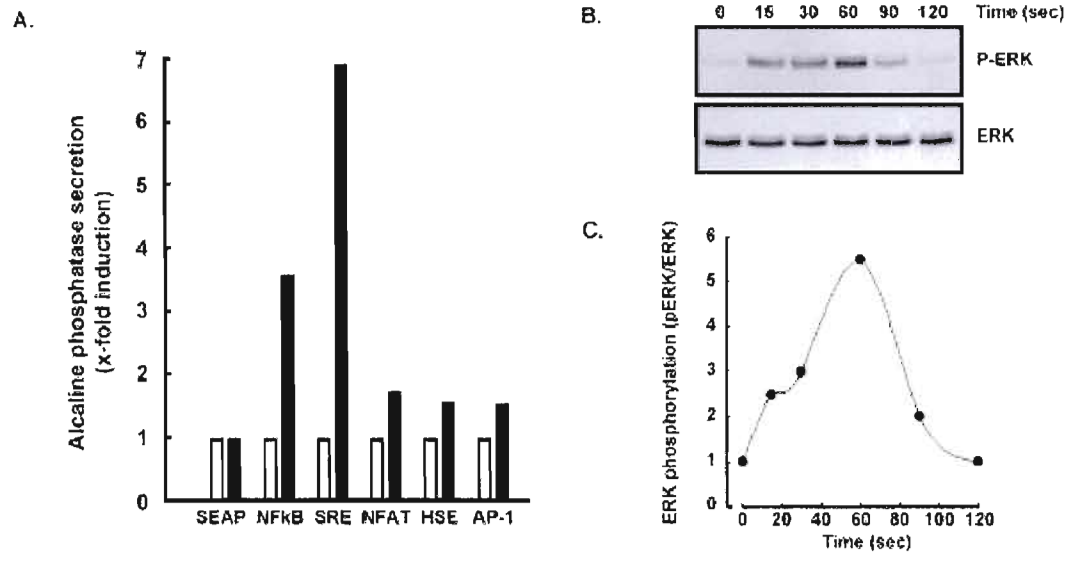

Fig.5: PCK3145 transduces its intracellular effects through the MAPK pathway. (A) HT-1080 cells were transfected with cDNA plasmids encoding the following transcription factors binding elements : nuclear factor of activated T-cells (NFAT), nuclear factor of kB-cells (NFkB), serum responsive element (SRE), calcineurin/protein kinase C element (NFAT), heat shock element (HSE) and activator protein-1 (AP-1). Cells were then treated with 300 μg/ml of PCK3145 for 24 hrs, and secreted alkaline phosphatase assessed in the media as described in the methods section. (B) Overnight serum-starved quiescent HT-1080 cells were incubated with vehicle (phosphate-buffered saline, PBS) or 300 μg/ml PCK3145 for up to 2 min at 37°C. The cells were scraped from the culture dishes in PBS containing NaF/Na_3VO_4 and incubated in the same medium buffer for 1 hr at 4°C. The resulting lysates were further clarified by centrifugation. Western blotting and immunodetection using anti-phospho-ERK and anti-ERK antibodies was then performed. (C) The extent of ERK phosphorylation in a representative experiment was assessed by scanning densitometry.

Fig.6

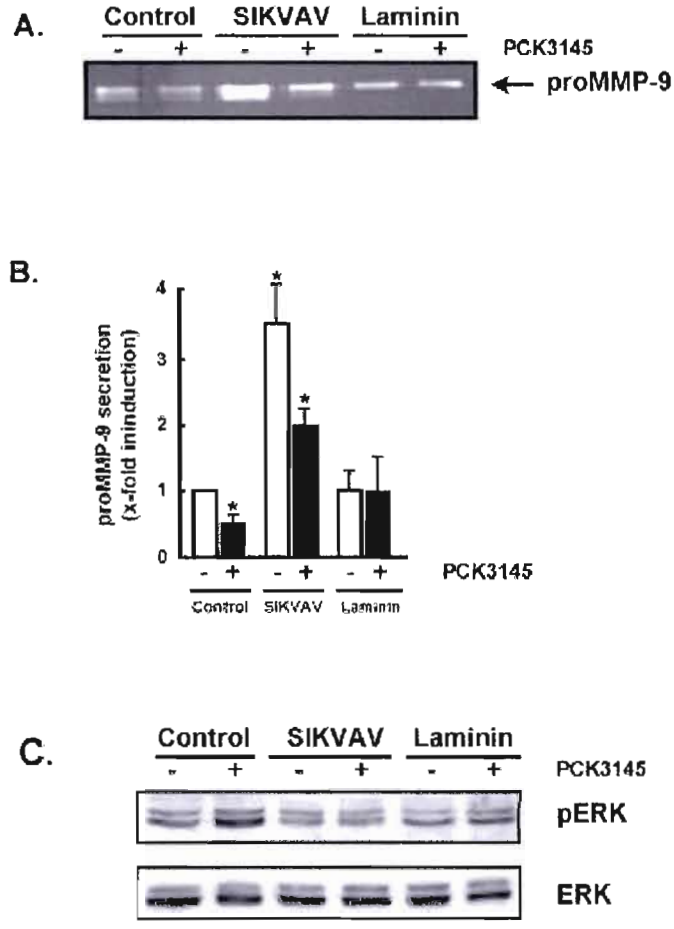

Fig.6: Laminin and laminin-derived peptide SIKVAV antagonize PCK3145-induced ERK phosphorylation and inhibitory action on MMP-9 secretion. (A) Serum-starved HT-1080 cells were treated with either 300 μg/ml PCK3145, SIKVAV, laminin, or an equimolar combination of SIKVAV and laminin. ProMMP-9 levels in the conditioned media were assessed by gelatin zymography as described in the methods section. (B) A mean of three experiments was quantified by scanning densitometry in non-treated (white bars) and PCK3145-treated (black bars) cells. (C) The extent of ERK phosphorylation was monitored at 1 minute of incubation as described in the legend of Fig.5.

Fig.7

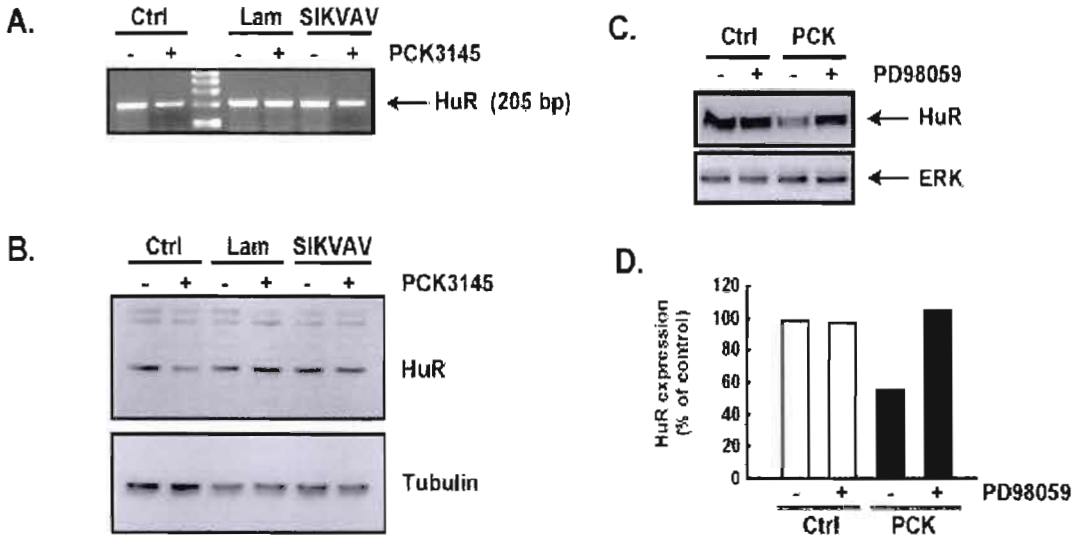

Fig.7: Laminin and laminin-derived peptide SIKVAV antagonize PCK3145 effect on HuR expression. (A) HT-1080 cells were treated with 300 μg/ml PCK3145 in the presence or not of laminin or laminin-derived peptide SIKVAV for 24 hrs. Total RNA was extracted and RT-PCR performed in order to assess HuR gene expression as described in the methods section. (B) Cell lysates from treated cells were used to monitor HuR protein expression by westernblotting. Tubulin expression was used as an internal control. (C) Cells were treated with PCK3145 in the presence or not of PD98059, a MEK kinase pharmacological inhibitor. HuR and ERK protein expression was assessed by western blotting as above. (D) Densitometric quantification of HuR protein expression was performed in order to assess the extent of PCK3145 effect (black bars) in the presence or not of PD98059.

Fig.8

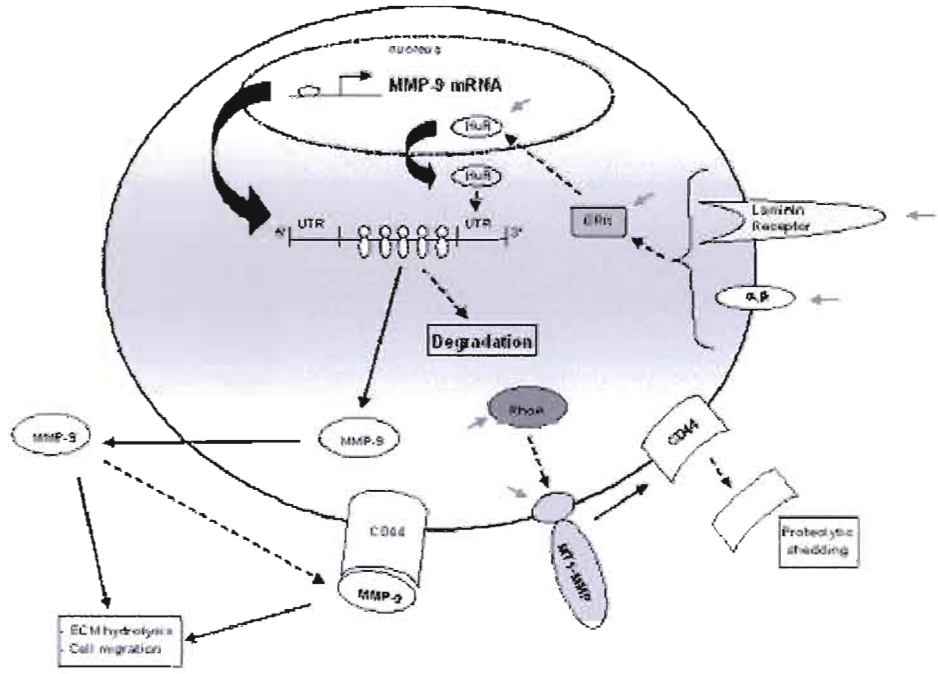

Fig.8: Integrative model of the combined anti-metastatic effects of PCK3145 on cancer cells. In light of our results, we propose that PCK3145 transduces its inhibitory effect on MMP-9 secretion, and subsequent downregulation of HuR gene and protein expression, through some laminin cell surface binding activity. We have characterized the implication of the 67LR as one of the potential cell surface receptors for PCK3145. However, we can not exclude the implication of alternate laminin binding activities such as that of $\square_3\square_1$ integrin which was recently also reported to regulate HuR expression [22]. HuR downregulation by PCK3145 will favor the degradation of MMP-9 mRNA levels. We also provide evidence for a rapid and transient activation of ERK by PCK3145 that relay the signaling regulating HuR expression and subsequent MMP-9 extracellular levels. A secondary regulation mechanism of MMP-9 functions was also recently reported by us [8], whereas CD44, the docking receptor for extracellular MMP-9, was proteolytically shed from the cell surface through a RhoA/MT1-MMP mechanism [8]. Collectively, as indicated by the arrows, we have highlighted the multilevel molecular mechanism of action of PCK3145 that we believe are involved in its anti-metastatic effects.

CONCLUSION

DISCUSSION ET PERSPECTIVES

Les travaux présentés dans ce mémoire donnent une approche sur la complexité des mécanismes régulant l'invasion tumorale et métastasique. Cette compléxité est due au nombre de réactions moléculaires et cellulaires qui intervient au cours de ce processus. Ces mécanismes sont déclenchés dans le corps humain par un facteur extérieur de risque de cancer (cité à la section 1.1.1) ou un stimulus moléculaire lors des expériences scientifiques *in vivo* ou *in vitro*. Ce facteur ou stimulus est considéré comme premier messager, qui se lie à la cellule *via* un récepteur membranaire, induit ainsi une cascade d'événements intracellulaires faisant intervenir un second messager intracellulaire pour se traduire en une réponse cellulaire telle que la migration, la prolifération, la différentiation, la mort cellulaire. Tout au long de nos projets, nous avons tenté de mieux comprendre ces mécanismes moléculaires complexes à travers les effets anticancéreux d'un peptide thérapeutique dirigé contre le cancer de la prostate avancé et métastasé.

Le cancer de la prostate, hormono-dépendant, se développe et sa progression est en grande partie influencée par les hormones: HRPC (hormone-refractory prostate cancer). Le traitement le plus utilisé est la prostatectomie radicale lorsque celui-ci est localisé. L'évolution de la carcinogenèse prostatique induit très rapidement des métastases impliquant des interactions entre les cellules tumorales et la matrice extracellulaire. Les modes d'action des agents thérapeutiques anti-cancéreux tels la cisplatin, le vinorelbine et le gemcitabine, sont peu ou pas connus. La découverte du PCK3145, un peptide synthétique ayant des propriétés anti-métastasique contre le cancer de la prostate, confirme l'innocuité et la tolérabilité chez les patients atteints de HRPC métastasé lors d'essais cliniques de phase IIa pour l'ensemble des dosages

évalués (de 5 à 80 mg/m^2). Le niveau de la MMP-9 plasmatique de ces patients ayant un taux de 100 µg/l de la MMP-9 circulant a baissé de 34 à 90% (Hawkins *et coll.*, 2005). Ces résultats positifs enregistrés *in vivo* implicant l'inhibition de l'activité de la MMP-9 au niveau de l'évolution des métastases, suggèrent un effet biologique anti-cancéreux du PCK3145.

Les études présentées dans ce mémoire ont fait l'objet de deux publications. L'une d'elle, publiée dans *Clinical & Experimental Metastasis*, a permis d'établir pour la première fois les mécanismes moléculaires de l'action anti-tumorale et anti-métastasique du PCK3145. Dans un modèle cellulaire de fibrosarcome HT-1080, le PCK3145 inhibe les niveaux de sécrétion de la MMP-9. L'induction de la sécrétion de la MMP-9 par le PMA et le TNFα est inhibée par le PCK3145, ceci suggère que le PCK3145 partage les mêmes voies de signalisations intracellulaires que celles transduites par le PMA et le TNFα. Le PCK3145 interfère aussi au niveau des interactions cellule-MEC, plus spécifiquement, il inhibe la liaison de la cellule à une matrice de laminine, de collagène de type-I et de HA. La lamine constitue un des composants majeurs du stroma environnant à partir duquel la cellule cancéreuse issue de la tumeur primaire se propagerait (Tantivejkul *et coll.*, 2004; Hauschka *et coll.*, 1986). Lorsqu'elle envahit le tissu osseux, elle utilisera cet environnement pour survivre et se multiplier. Étant donné que le composant majeur de la matrice osseuse est le collagène de type-I (Hauschka *et coll.*, 1986), l'inhibition de l'adhésion par le PCK3145 sur une matrice de collagène lui confère un effet potentiel anti-métastasique important.

Il est intéressant de noter que la liaison à ces deux types de matrice se fait non seulement par l'intermédiaire d'un récepteur d'adhésion, l'intégrine $\alpha_3\beta_1$ (Iyer *et coll.*, 2005), mais fait aussi intervenir une autre protéine transmembranaire, le CD44, récepteur de surface spécifique à HA liant à la surface la MMP-9 (Yu *et coll.*, 1999), ce qui a pour but de renforcer l'adhésion des cellules cancéreuses aux cellules

endothéliales dérivées de la moelle osseuse (Draffin *et coll.*, 2004). Nos résultats sont en parfait accord avec cette notion. En effet, nous montrons que le PCK3145 en plus d'inhiber l'adhésion à la laminine, le collagène et HA, il induit une inhibition de la migration des fibrosarcomes sur une matrice de HA. De plus, nous mettons en évidence le rôle du PCK3145 dans le déclenchement du clivage de CD44 empêchant la migration des cellules sur la matrice de HA et constituant ainsi un mécanisme secondaire potentiel de la régulation de l'activité de la MMP-9. Il apparaît à la lumière de ces résultats, que l'effet inhibiteur du PCK3145 sur le processus métastasique impliquant la régulation des fonctions de la MMP-9, se situerait à deux niveaux : au niveau de la sécrétion de la MMP-9 et de sa liaison subséquente à la surface de la cellule. L'association de la MMP-9 à la surface de la cellule peut être assurée par de nombreuses protéines de surface régulant ainsi sa fonction incluant la localisation à la surface cellulaire, l'inhibition et l'internalisation (Fridman *et coll.*, 2003). En plus de son action inhibitrice de la sécrétion de la MMP-9, notre étude a révélé une évidence moléculaire de l'effet du PCK3145 sur l'association de la MMP-9 à la surface cellulaire. En effet, de nombreuses études ont établi que la liaison de la MMP-9 à CD44 entraînait l'activation du TGF-β et favorisait la dégradation de la MEC (Yu *et coll.*, 1999; Yu *et coll.*, 2000; Daniel *et coll.*, 2005). Nous pouvons suggérer alors que les effets anti-métastasiques du PCK3145 dans l'inhibition de la sécrétion de la MMP-9 et son ancrage subséquent dans la membrane cellulaire s'opposerait à la progression tumorale et métastasique induite par le TGF-β dans les processus d'inflammation (Wahl *et coll.*, 1993), dans les cellules métastasiques osseuses (Duivenvoorden *et coll.*, 1999) et dans les cellules cancéreuses prostatiques (Sehgal *et coll.*, 1999).

L'étude des voies de transduction du signal menant au clivage de CD44 par le PCK3145 est particulièrement intéressante. Il a été démontré qu'un tel clivage protéolytique impliquerait une voie de signalisation déclenchée par des esters de phorbol et des cytokines, deux inducteurs potentiels de la sécrétion de la MMP-9 dans

l'invasion cellulaire (Lohi *et coll.,* 1995). Cependant, nos résultats mettent en évidence l'habilité du PCK3145 à inhiber la signalisation intracellulaire déclenchée par le PMA et le TNF. Il est par ailleurs connu que les patients atteints de cancer métastasique de la prostate ont des taux sériques élevés de ces cytokines pro-inflammatoires (Michalaki *et coll.,* 2004). Il est important de souligner que nos travaux ont démontré que le PCK3145 entraîne également l'activation de la MT1-MMP et le clivage protéolytique de CD44 par la MT1-MMP. Par conséquent l'augmenation de l'activité de la MT1-MMP est connue pour être corrélée avec le potentiel invasif des cellules cancéreuses mais n'est pas le seul responsable de ce caractère, alors cette augmentation pourrait ne pas influer sur le potentiel anti-métastasique du PCK3145. Dans une étude future, il serait important d'analyser la modulation de MT1-MMP par le PCK3145 sur la migration et la prolifération cellulaire. Par analyse des relations entre la structure et la fonction, il a été démontré que le domaine hémopexine de la MT1-MMP est responsable de la liaison et du clivage de la forme standard hématopoïétique de CD44 (Suenaga *et coll.,* 2005). Il apparaît donc que plusieurs voies de signalisation, dont celle déclenchée par le PMA et le TNF, semblent impliquées et régulées par le PCK3145. Parmi ces voies de transduction du signal, celles impliquant les GTPases intracellulaires de la famille des Rho jouent un rôle important dans la progression tumorale et la formation de métastases, telle que l'assemblage des filipodes, des lamellipodes et des fibres de stress (Sahai *et coll.,* 2002). Il est intéressant de noter l'implication de la signalisation médiée par RhoA dans la régulation des fonctions de CD44 (Bourguignon *et coll.,* 2001; Shi *et coll.,* 2001; Kawano *et coll.,* 2000).

L'ensemble de ces données nous a incité à exploiter les voies impliquant RhoA. En effet, ce mode d'action est compatible avec l'induction de RhoA par le PCK3145. Cette signalisation menant au clivage de CD44 contrôlerait l'adhésion et la migration sur une matrice de HA. L'ensemble de nos résultats renforce le lien entre l'induction par le PCK3145 de la voie de signalisation intracellulaire impliquant RhoA et l'augmentation d'expression de la MT1-MMP et son activité protéolytique.

Ce dernier à son tour régule le clivage de CD44 empêchant ainsi l'ancrage de la MMP-9. En effet, dans ce contexte, une étude récente entreprise dans notre laboratoire a souligné l'implication de RhoA et la MT1-MMT dans le clivage de CD44 (Annabi *et coll.,* 2005). Il a été démonté que RhoA, CD44, MT1-MMP, et MMP-9 co-localisent dans le domaine cavéolaire riche en cholestérols (Abecassis *et coll.,* 2002; Perschl *et coll.,* 1995; Gingras *et coll.,* 1998). Conformément nous avons montré que le PCK3145 cible l'ensemble de ces protéines associées aux cavéoles. D'autant plus que, les cavéoles régulent les voies de transduction menant à la transformation oncogénique des cellules, à la cancérogenèse et aux métastases (Williams *et coll.,* 2005). À la lumière de ces résultats, nous pourrons suggérer un potentiel inhibiteur du PCK3145 impliquant des voies de transduction intracellulaires médiées par les cavéoles, lesquelles régulent la sécrétion de la MMP-9 et les interactions les cellules et la MEC. En effet, l'inhibition de l'invasion et la formation de métastases par l'intermédiaire des cavéoles a lieu travers l'inhibition de sécrétion de la MMP-2 et la MMP-9; (Williams *et coll.,* 2004), alors que la suppression de la cavéoline-1 inhibe le développement du cancer de la prostate (Williams *et coll.,* 2005). Cependant, la régulation de l'expression ou des fonctions des cavéoles par le PCK3145 est présentement sous investigation.

En conclusion pour cette première étude, nous démontrons clairement l'implication anti-métastasique du PCK3145 à deux niveaux : -au niveau de la sécrétion de la MMP-9 impliquant les voies de signalisation intracellulaire menant à l'induction de la MMP-9 par les cytokines et le PMA, - au niveau de l'ancrage de la MMP-9 à la surface de la cellule impliquant le clivage de CD44 *via* RhoA/MT1-MMP. À la lumière de ces résultats, le mode d'action pléiotrope du PCK3145 sur la régulation de la MMP-9 peut engendrer un impact non seulement sur le cancer de la prostate et autres cancers mais également sur d'autres pathologies reliées à une augmentation de l'expression de la MMP-9 telles les maladies inflammatoires, dégénératives, vasculaires et infectieuses.

Nous nous sommes intéressés, dans une seconde étude (article publié dans *Anti-Cancer Drugs*), après la caractérisation des mécanismes moléculaires du PCK3145 régulant l'interaction de la MMP-9 à la surface de la cellule, à déterminer le mode d'action et les voies de signalisation intracellulaires par lesquelles le PCK3145 transduit son action pour inhiber la sécrétion de la MMP-9. Celle-ci, impliquée dans la progression du cancer, peut être régulée *via* des voies de signalisation intracellulaires à différents niveaux, au niveau de l'expression génique mais aussi au niveau post-traductionnel. L'inhibition de la sécrétion de la MMP-9 par le PCK3145, n'agit pas au niveau de l'expression du gène (Garde *et coll.,* 2005; Annabi *et Coll.*, 2006). D'après les résultats expérimentaux obtenus, nous pouvons ajouter au mécanisme d'action du PCK3145 sa liaison à un récepteur non-intégrine: le récepteur à la laminine de 67 kDa (67RL). En effet, il a été démonté que le PCK3145 inhibait la liaison des cellules à une matrice de laminine (Annabi *et coll.,* 2005). D'après les résultats obtenus dans cette seconde étude, l'implication du 67RL dans la régulation de la sécrétion de la MMP-9 par le PCK3145 est fortement soutenue. Cependant, ce qui nous a aussi dirigé vers cette hypothèse, c'est l'effet similaire d'inhibition de sécrétion de la MMP-9 par l'EGCg, une catéchine du thé vert, un agent anti-métastasique qui transduit ses effets aussi à travers sa liaison au 67LR.

L'implication du 67RL est retrouvée aussi dans plusieurs types de cancer humain (colon, sein, estomac, foie et ovaire). En effet, l'augmentation de son expression serait corrélée avec la prolifération des cellules (Menard *et coll.,* 1998). De plus, une détection du 67LR dans une biopsie du cancer de la prostate a été proposée comme prédicateur d'une rechute après une prostatectomie (Waltregny *et coll.,* 2001). Par ailleurs, l'expression de ce récepteur favorise l'adhésion des cellules leucémiques myéloïdes sur une matrice de laminine associée à la différentiation des monocytes (Montuori *et coll.,* 1999). L'ensemble de ces données suggère que le PCK3145 peut cibler d'autres cancers que celui de la prostate telle que la leucémie myéloïde où le taux de 67RL est élevé. Nos résultats indiquent que l'inhibition de la

sécrétion de la MMP-9 requiert ce récepteur de la laminine à 67 kDa et est dépendante de l'activation de la voie des MAPKinases. Le PCK3145 entraine la diminution de la sécrétion de la MMP-9 *via* HuR, protéine intracytoplasmique qui stabilise l'ARNm de la MMP-9. Par conséquent aucune évidence directe dans cette étude ne démontre que le PCK3145 lie le récepteur à 67 kDa. Dans une étude future, il serait envisagable de faire une expérience de chromatographie d'affinité, en liant le PCK3145 biotinylé ou marqué à l'iode 125 à des billes de Sépharose et faire passer dans la colonne un lysat de cellule. Les fractions éluées seront annalysées sur gel SDS-PAGE, et les bandes seront révélées par un anti-corps anti-biotine. Les bandes seront alors découpées et séquencées afin de confirmer la présence du récepteur à 67 kDa.

Nous nous sommes ensuite intéressés aux voies de transduction intracellulaires induites par le PCK3145. Nos résultats montrent que la transduction du signal par le PCK3145 implique la phosphorylation de ERK1/2 via le récepteur de 67kDa. Cette phosphorylation est contrée par des ligands de ce récepteur tels la laminine et le SIKVAV, un dérivé de la laminine. L'implication d'autres voies intracellulaires induites par le PCK3145 autres que RhoA et ERK1/2 restent à déterminer (Annabi *et coll.*, 2005; Annabi *et coll.*, 2006).

HuR, une protéine intracytoplasmique qui stabilise l'ARNm de la MMP-9 en se liant à sa séquence riche en AU, a été identifiée comme un facteur ciblé par le PCK3145. Son expression génique et protéique est inhibée et cette inhibition est annulée par les ligands du récepteur à la laminine. Comme HuR peut également lier les séquences riches en AU d'autres gènes codant pour les cytokines, les facteurs de croissances, les gènes suppresseurs tumoraux, les proto-oncogènes, les facteurs de régulation du cycle cellulaire, on peut enviser qu'une régulation à la baisse de HuR par PCK3145 peut également empêcher la prolifération de cellules ou induire l'apoptose. Ces processus cellulaires sont une perspective à envisager dans les travaux futurs. Le PCK3145 transduit donc son signal intracellulaire inhibant l'expression de

HuR *via* sa liaison au récepteur à la laminine à 67 kDa ou à l'intégrine $\alpha_3\beta_1$. D'autres études ont démontré l'implication des intégrines dans la régulation des voies intracellulaires impliquant les MAPK telle la régulation de l'adhésion cellulaire des NIH 3T3 impliquant la phosphorylation de MEK/ERK par les facteurs de croissance EGF, impliquant aussi les intégrines (Aplin *et coll.*, 2001).

Les résultats récents de recherche clinique de phase II du PCK3145 confirme sa thérapie anticancéreuse tolérable et non toxique pour les malades du cancer de la prostate hormono-dépendant HRPC (Hawkins *et coll.*, 2005). Il réduit leur niveau de la MMP-9 plasmatique, suggérant un effet biologique anti-métastasique. Une première étude a confirmé la pharmacocinétique et l'efficacité du PCK3145 anti-métastases. L'impact du PCK3145 en clinique est très important, suggérant un effet biologique relatif au control du développement métastasique. De ce faite, le PCK3145 peut être utilisé contre plusieurs types de cancer, il agit sur les HT-1080, une lignée cellulaire de fibrosarcomes où même le 67kDa n'est pas surexprimé cela suggèrerait l'existence d'un autre mécanisme moléculaire où le PCK3145peut agir. Il pourrait agir aussi sur une cellule normale mais ce résultat n'a pas été observé. Il ouvre aussi une nouvelle approche anti-métastasique dirigée contre d'autres types de cancer où l'expression de récepteur de laminine à 67 kDa est importante telle la leucémie. D'ailleurs, une nouvelle étude est en court de développement utilisant un autre type cellulaire que les HT-1080, les HL-60, une lignée cellulaire de leucémie, afin de mettre en évidance le récepteur de la laminine à 67 kDa comme étant le récepteur spécifique du PCK3145.

À la lumière de nos travaux faisant le fruit de deux articles (publiés dans *Clinical & Experimental Metastasis* et dans *Anti-Cancer Drugs*) nous pouvons maintenant caractériser le mode d'action moléculaire du potentiel anti-angiogénique et anti-métastasique du PCK3145 (Lamy *et coll.*, 2006, Annabi *et coll.*, 2005; Annabi

et coll., 2006). Un modèle proposé du mode d'action du PCK3145 est illustré dans la figure 8 publié dans *Clinical & Experimental Metastasis*.

L'implication du récepteur à la laminine de 67 kDa et celle de l'intégrine $\alpha_3\beta_1$ ont été suggérées comme récepteurs par lequel le PCK3145 transduit un signal intracellulaire impliquant les voies de RhoA et de MEK/ERK. Ces voies de transduction vont réguler les fonctions de la MMP-9 soit en empêchant son ancrage à la surface de la cellule en clivant son récepteur CD44 *via* l'augmentation de la MT1-MMP soit en réduisant sa sécrétion par destabilisation de son ARNm *via* HuR. La caractérisation de ces modes d'action donne au PCK3145 un plus large effet thérapeutique ciblant d'autres types de cancers et d'autres maladies caractérisées par des taux élevés de la MMP-9 et/ou du récepteur à la laminine.

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ANNEXE

AUTRES MANUSCRITS RÉALISÉS

1. *''Probing the infiltrating character of brain tumors : Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg''*

Annabi B., **Bouzeghrane M.,** Moumdjian R., Moghrabi A. & Béliveau R.

J Neurochem. 2005 Aug; 94: 906-16

L'auteur du Mémoire est second auteur dans cet article

2. *''The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate''*

McLaughlin N., Annabi B., **Bouzegharne M.**, Temme A. Bahary J.P., Moumdjian R. & Béliveau R.

Brain Research. 2006 jan, 1071:1-9

L'auteur du Mémoire est troisième auteur dans cet article.

1. ***''Probing the infiltrating character of brain tumors : Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg''***

Annabi B., **Bouzeghrane M.**, Moumdjian R., Moghrabi A. & Béliveau R.

J Neurochem. 2005 Aug;94(4):906-16. Epub 2005 Jun 30.

L'Inhibition du clivage de CD44 de la surface cellulaire des gliomes par la catéchine du thé vert l'EGCg est dépendante de RhoA/ROK: Une implication thérapeutique pour le caractère infiltrant des tumeures cérébrales

Borhane Annabi, **Mounia Bouzeghrane**, Robert Moumdjian, Albert Moghrabi et Richard Béliveau

L'acide hyaluronique (HA) est le constituant majeur de la matrice extracellulaire (MEC) du cerveau. La liaison des gliomes à la MEC est reconnue pour être regulée par un complexe composé de la métalloprotéinase membranaire de type-1 (MT1-MMP), CD44 et la cavéoline localisée dans les invaginations membranaires des cellules.
Dans ce travail, les voies de signalisations intracellualires impliquées dans la reconnaissance des cellules du gliome à son ligant, le HA, sont étudiées.
Nous démontrons qu'une surexpression de la GTPase RhoA régule à la hausse l'expression de la MT1-MMP et cible le clivage de CD44 de la surface des glioblastomes, les U-87. Ce clivage est aussi observé lors d'une surexpression de la forme sauvage d'un recombinant MT1-MMP, alors que ce processus est inversé lors de la surexpression d'un mutant tronqué de sa partie cytoplasmique. Cela suggère que la partie cytoplasmique de MT1-MMP est responsable de la transduction du signal intracellulaire. En effet nous démontrons que cette partie est responsable de la transduction du signal intracellulaire dépendant de RhoA et menant au clivage de CD44. De plus, un traitement des glioblastomes avec un inhibiteur de Rho-kinase (ROK), le Y27632, ou avec l'EGCg, une catéchine du thé vert avec des propriétés anti-MMP et anti-angiogénique, antagonise le clivage de CD44 induit par RhoA et MT1-MMP. Cependant, la surexpression du recombinant ROK stimule le clivage de CD44. Nos résultats suggèrent en conclusion que la voie intracellulaire dépendante de RhoA/ROK régule le clivage de CD44 médié par la MT1-MMP. Ce processus moléculaire pourrait expliquer partiellement le caractère infiltrant des tumeurs cérébrales et peut donc cibler une approche thérapeutique anti-cancéreuse.

Cet article nous a aidé a mieux comprendre les mécanismes intracellulaires menant au clivage de CD44 par l'EGCg médié par MT1-MMP et a contribué dans l'élaboration d'un second article en mettant en évidance les effets du PCK3145 sur le clivage de CD44.

Probing the infiltrating character of brain tumors: Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg

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The abbreviations used are : ECM, extracellular matrix; EGCg, epigallocatechin-(3)-gallate; HA, hyaluronic acid, hyaluronate, hyaluronan; MMP, matrix metalloproteinase; MT1-MMP membrane type-1 MMP; ROK, Rho-associated kinase

ABSTRACT

Glioma cell surface binding to hyaluronan (HA), a major constituent of the brain extracellular matrix (ECM) environment, is regulated through a complex membrane type-1 matrix metalloproteinase (MT1-MMP)/CD44/caveolin interaction that takes place at the leading edges of invading cells. In the present study, intracellular transduction pathways required for the HA-mediated recognition by infiltrating glioma cells in brain was investigated. We show that the overexpression of the GTPase RhoA upregulated MT1-MMP expression and triggered CD44 shedding from the U-87 glioma cell surface. This potential implication in cerebral metastatic processes was also observed in cells overexpressing the full-length recombinant MT1-MMP, while the overexpression of a cytoplasmic domain-truncated from of MT1-MMP failed to do so. This suggests that the cytoplasmic domain of MT1-MMP transduces intracellular signalling leading to RhoA-mediated CD44 shedding. Treatment of glioma cells with the Rho-kinase (ROK) inhibitor Y27632, or with EGCg, a green tea catechin with anti-MMP and antiangiogenesis activities, antagonized both RhoA- and MT1-MMP-induced CD44 shedding. Conversely, overexpression of recombinant ROK stimulated CD44 release. Taken together, our results suggest that RhoA/ROK intracellular signalling regulates MT1-MMP-mediated CD44 recognition of HA. These molecular processes may partly explain the diffuse brain-infiltrating character of glioma cells within the surrounding parenchyma and thus be a target for new approaches to antitumor therapy.

Key words: human glioma metastasis, CD44, RhoA, MT1-MMP, hyaluronan, green tea

Running title: RhoA triggers CD44 cell surface shedding

INTRODUCTION

Although intrinsic brain tumors fail to metastasize, they do exhibit diffuse infiltration of the surrounding brain parenchyma (Bolteus *et coll.,* 2001). Local diffuse invasion is poorly understood, but is thought to be a multi-faceted biological phenomenon of interactive mechanisms involving cell motility, adhesion and enzymatic remodeling of the extracellular matrix (ECM) components. These features of the infiltrating tumor cells presently preclude successful therapy regardless of the histological type or grade of malignancy (Pilkington 1997). A better understanding of the molecular mechanisms controlling tumor astrocyte detachment from initial brain tumor sites is thus needed to combat these tumors.

The principal ECM molecules that have been identified in the normal brain parenchyma are hyaluronic acid (HA) and chondroitin sulfate (Bignami *et coll.,* 1992). Of these two, small HA polymers are known to efficiently promote tumor cell migration (Sugahara *et coll.,* 2003). HA is in fact the principal, but by no means the only, ligand of CD44, a membrane glycoprotein belonging to the superfamily of immunoglobulin receptors. CD44 is also implicated in the promotion of tumor growth, invasiveness, and metastatic potential in experimental and human cancers (Gunthert *et coll.,* 1998). Recent findings suggest that CD44 provides a docking site for matrix metalloproteinase (MMP)-9 on the surface of melanoma and carcinoma cells and can thus indirectly contribute to pericellular proteolysis of types IV and V collagen (Yu *et coll.,* 1999). Although several other cell surface receptors for HA have been reported, recent works have demonstrated that gliomas express significant levels of CD44 and that such expression could be relevant in determining their highly invasive behavior (Akiyama *et coll.,*2001; Ranuncolo *et coll.,* 2002).

Several studies have revealed different molecular and cellular mechanisms regulating CD44-mediated processes (Gal *et coll.,* 2003; Xu *et coll.,* 2003). Among these, the role of intracellular Rho-mediated signalling, leading to cytokine production and breast tumor progression was reported (Bourguignon *et coll.,* 2003). Interestingly, quantitative RT-PCR analysis in U-87 glioma cells showed that levels of the small RhoA GTPase, a potent modulator of actin polymerization/depolymerization dynamics triggering assembly of filopodia, lamellipodia and stress fibers, increased by 75% after the addition of galectin-1, an ECM glycoprotein that is synthesised by tumor astrocytes and which favours their migration (Camby *et coll.,* 2002). Important molecular processes regulating cell migration, tumor invasion and metastasis have also recently been highlighted by the common cell surface localization of CD44 with a membrane type (MT)-1 MMP at the leading lamellipodia edge of motile cells (Kajita *et coll.,* 2001; Mori *et coll.,* 2002). These studies demonstrated that CD44 directed MT1-MMP to lamellipodia by associating with its hemopexin-like domain, and that cell surface MT1-MMP-mediated cleavage of CD44 subsequently played a critical role in promoting tumor cell migration. Lamellipodia formation is also known to be, at least partly, orchestrated by the small GTPase of the Rho family (Ridley *et coll.,* 2003). Noteworthy, one other common feature between RhoA, MT1-MMP and CD44 is their partial localization within Triton X-100-insoluble and cholesterol-enriched membrane domains termed caveolae (Perschl *et coll.,* 1995; Gingras *et coll.,* 1998; Annabi *et coll.,* 2001). Interestingly, the caveolar location of MT1-MMP was recently suggested to provide a regulatory mechanism in glioma (Annabi *et coll.,* 2004) and breast carcinoma cell invasion (Rozanov *et coll.,* 2004).

In light of the common caveolar localization of MT1-MMP, CD44, and RhoA at the leading edges of migrating glioma cells, we hypothesise that a crosstalk between these players may regulate the infiltrating phenotype of brain tumors. In the present study, we have investigated the mechanisms involved in the regulation of CD44

functions in cells derived from a highly infiltrating and vascularized brain tumor glioblastoma. Specifically, we addressed the intracellular signalling pathways that lead to CD44-mediated detachment of U-87 glioma cells from HA. Collectively, our results provide the first evidence for a cell surface functional cross-talk between MT1-MMP/RhoA/ROK that impacts on the ability of gliomas to bind HA through CD44, and that may be efficiently targeted by the anti-cancer properties of the green tea polyphenol epigallocatechin-(3)-gallate EGCg (Annabi *et coll.,* 2002; Demeule *et coll.,* 2002).

MATERIALS AND METHODS

Materials: Agarose, (-)-epigallocatechin 3-gallate (EGCg), sodium dodecylsulfate (SDS), gelatin, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). TriZOL reagent was from Life Technologies (Gaithersburg, MD). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). The anti-CD44 R-phycoerythrin-conjugated mouse anti-human monoclonal antibody (G44-26) and mouse IgG2bκ (clone 27-35) were from BD Pharmingen (Franklin Lakes, NJ). The anti-MT1-MMP polyclonal antibody AB-815 and the anti-Erk antibody were from Chemicon (Temecula, CA). The anti-Myc and anti-RhoA antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and cDNA transfection method: The U-87 glioma cell line was purchased from American Type Culture Collection and maintained in Eagle's Minimum essential medium (MEM) containing 10% (v/v) bovine calf serum (BCS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. The MT1-MMP cDNA constructs have been previously generated and validated by us (Annabi *et coll.,* 2001) as follows: Wt encodes the full length MT1-MMP protein (Met^{1}–Val^{582}); Δ1 encodes a protein which lacks the entire C-terminal 20 amino acid cytoplasmic domain (Met^{1}–Phe^{562}). The cDNA encoding the Wt Myc-tagged RhoA and constitutively active Myc-tagged RhoA-associated kinase (ROK) were generously provided by Dr Allan Hall (University College London, London, UK) and Dr Salhia Bodour (The Hospital for Sick Children, Toronto, ON) respectively. U-87 cells were transiently transfected with cDNA constructs using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed with a cDNA plasmid encoding Green Fluorescent Protein (GFP) that was cloned in the same plasmid backbone

(pcDNA3.1+). Fluorescent microscopy visualisation confirmed cell transfection by the presence of green fluorescent cells that were routinely found to represent 8-15% of total cells transfected (not shown). All experiments involving these cells were performed 36 hrs following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted from monolayers of cultured U-87 cells using the TriZOL reagent. One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON).

Primers:

CD44s	forward: 5'-TTTGCCTCTTACAGTTGAGCCTG-3'
	reverse: 5'-GGTGCCATCACGGTTGACAATAG-3'
	(Annabi *et coll.*,2004)
MT1-MMP	forward: 5'-ATTGATGCTGCTCTCTTCTGG-3'
	reverse: 5'-GTGAAGACTTCATCGCTGCC-3')
	(Annabi *et coll.*,2001)
RhoA	forward: 5'-CTGGTGATTGTTGGTGATGG -3'
	reverse: 5'-GCGATCATAATCTTCCTGCC -3')
	(Turcotte *et coll.*, 2003)

Primers were derived from human sequences and PCR conditions were optimized so that the gene products were at the exponential phase of the amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal housekeeping gene control. PCR products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Immunoblotting procedures: Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution for MT1-MMP detection) or anti-mouse IgG (1/5,000 dilution for RhoA and CD44 detection) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

Flow cytometry analysis: For assessment of cell surface CD44 expression, cells were detached from plates, as previously described by us (Annabi *et coll.,* 2004), and resuspended in 10% FBS/DMEM at a concentration of 10^6 cells/ml, washed 2 times and blocked for 15 min at room temperature in PBS containing 5% inactivated fetal calf serum (FCS/PBS). The cells were then incubated in 0.5% FCS/PBS with 0.5 μg/ml of the CD44 mAb or mouse IgG2bκ at room temperature for 30 min, washed once and resuspended in 0.5% FCS/PBS. Flow cytometry data was analyzed on a FACS Calibur flow cytometer with CellQuestPro software (BD Biosciences, Mississauga, ON).

Cell migration assay: Cells were dislodged after brief trypsinization, washed extensively and resuspended in MEM at a concentration of 10^6 cells/ml (Annabi *et coll.,* 2004). Cells ($5x10^4$) were then dispersed onto 1 mg/ml HA/PBS-coated chemotaxis filters (Costar; 8-μm pore size) within Boyden chamber inserts. Migration proceeded for 3 h at 37°C in 5% CO_2. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, coloured with 0.1% crystal

violet/20% Methanol and counted by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter using Northern Eclipse software. Data points indicate the mean obtained from three separate chambers within one representative experiment.

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare the relative RhoA- or MT1-MMP-induced effects on HA cell adherence, CD44 cell surface expression, or migration on HA to untreated (Mock or control) U-87 cells. Probability values of less than .05 were considered significant, and an asterisk (*) identifies such significance in each figure.

RESULTS

Overexpression of recombinant RhoA or MT1-MMP decreases U-87 glioma cells adhesion to HA. RhoA is thought to regulate several processes involved in cancer invasion and it was recently found to regulate CD44 binding to HA (Ito *et coll.,* 2004). We have shown that the function of the HA receptor CD44 was also regulated through a complex interplay involving MT1-MMP (Annabi *et coll.,* 2004), which is of particular importance in brain tumor development. We decided to analyze the roles of RhoA and MT1-MMP in cell-HA interaction. Cells were cultured on plastic and transiently transfected with cDNA plasmids encoding the full-length (Wt) MT1-MMP and RhoA recombinant proteins, as well as plasmid encoding a truncated cytoplasmic form (Δ1) of MT1-MMP that anchors to the plasma membrane but fails to transduce any intracellular signalling (Gingras *et coll.,* 2001). Transfected cells were then trypsinized, seeded on HA-coated dishes, and adhesion left to proceed for 2 hours. We found that overexpression of RhoA or Wt-MT1-MMP triggered a loss of approximately 60-65% in cell-HA adhesion, while Mock or Δ1-MT1-MMP-transfected cells had their respective adhesion to HA unaffected (Fig.1a and 1b). Generation and selection of stably transfected U-87 cells with the respective cDNA plasmids is currently underway in order to confirm and further characterize the new RhoA- and MT1-MMP-mediated phenotype observed. Interestingly, a bi-directional crosstalk was seen between RhoA and MT1-MMP expression. Indeed, overexpression of recombinant RhoA triggered an increase in endogenous MT1-MMP expression, while Wt-MT1-MMP overexpression induced RhoA expression in U-87 cells (Fig.1c). Moreover, the cytoplasmic domain of MT1-MMP appears to be essential for that induction since Δ1-MT1-MMP did not trigger RhoA expression in U-87 cells. This suggests that the cytoplasmic domain of MT1-MMP regulates crucial intracellular signalling leading to the induction of RhoA.

RhoA and MT1-MMP overexpression trigger CD44 cell surface shedding from U-87 glioma cells. We have previously reported that MT1-MMP overexpression antagonized functional recognition and binding of HA by reducing CD44 cell surface expression (Annabi *et coll.*, 2004). Whether CD44 was shed from the cell surface of glioma cells remained to be evaluated. We transfected U-87 cells grown on plastic dishes with Wt-MT1-MMP, Δ1-MT1-MMP and RhoA cDNAs and assessed the presence of CD44 in the conditioned media by western blotting. We observed a 75 kDa CD44-immunoreactive protein that appeared in the conditioned media isolated from RhoA and Wt-MT1-MMP-expressing cells (Fig.2a). Interestingly, no CD44 shedding was triggered by the overexpression of a cytoplasmic-deleted recombinant form of MT1-MMP. Furthermore, immunophenotyping of CD44 cell surface expression of the transfected cells was performed using flow cytometry. Accordingly, a significant shift to lower fluorescence levels was observed in RhoA- and MT1-MMP-transfected cells (Fig.2b), suggesting that less CD44 remained at the cell surface (Fig.2c) and supporting the assumption that CD44 is released in the culture media.

Cell migration on hyaluronic acid is inhibited in RhoA- and MT1-MMP-transfected U-87 glioma cells. Overexpression of recombinant RhoA and MT1-MMP was achieved by transfecting U-87 cells grown on plastic. Cells were then briefly trypsinized and seeded on HA- or gelatin-coated filters inserted into Boyden Chambers. We found that overexpression of either RhoA or MT1-MMP inhibited U-87 cell migration on HA by 72% and 67%, respectively (Fig.3, grey bars). This was an expected result as the binding of the cells to HA was already shown to be reduced due to lower CD44 cell surface expression, as demonstrated by the increased CD44 shedding induced by both RhoA and MT1-MMP in transfected cells. In contrast, cell migration on gelatin was only found significantly increased in Wt-MT1-MMP

transfected cells (Fig.3, black bars). This suggests that the cell surface receptors that are involved in HA recognition are specifically decreased.

The actions of green tea catechin EGCg and of ROK inhibition reverse RhoA- and MT1-MMP-induced shedding of CD44. We have demonstrated that treatment of U-87 glioma cells with EGCg, a naturally occurring green tea catechin for which we have reported anti-MMP and anti-angiogenic activity (Demeule *et coll.,* 2002), also inhibited HA binding to CD44 in Type-I collagen-treated U-87 glioma cells (Annabi *et coll.,* 2004), while it had no influence on basal cell migration on HA (not shown). Whether EGCg antagonized RhoA-mediated CD44 cell surface shedding was assessed in parallel with functional inhibition of RhoA functions by the Rho-associated kinase (ROK) inhibitor Y27632. Interestingly, ROK was recently suggested to regulate CD44 expression in osteoclasts (Chellaiah *et coll.,* 2003), while its inhibition led to a decreased interaction of CD44 with a Na^+-H^+ exchanger suggesting a potential role for RhoA in transducing CD44 signalling (Bourguignon *et coll.,* 2004). Inhibition of ROK, as well as treatment of U-87 cells with EGCg, significantly inhibited both RhoA- and MT1-MMP-mediated CD44 shedding into the conditioned media (Fig.4a). CD44 cell surface expression was also measured by flow cytometry and this was quantified in Fig.4b. Moreover, the observed EGCg inhibition of RhoA- and MT1-MMP-mediated shedding was primarily due to a downregulation in RhoA and MT1-MMP mRNA levels (Fig.4c) (21), while the functional inhibition of downstream signalling from RhoA with the ROK inhibitor did not affect significantly the expression of either gene.

The green tea catechin EGCg, but not functional inhibition of ROK, reverses RhoA-induced expression of MT1-MMP. Whether RhoA-induced MT1-MMP protein expression could be regulated by EGCg or functional inhibition of ROK was next assessed. U-87 glioma cells were cultured on plastic and subsequently transfected with empty vector (Mock) or RhoA cDNA. Thirty-six hours post-transfection cells

were treated or not for 18 hours with either 20 μM EGCg or 5 μM Y27632. Western blotting followed by MT1-MMP immunodetection revealed that the only significant inhibition of MT1-MMP protein expression was observed in EGCg-treated U-87 cells (Fig.5a). Protein expression of basal and of RhoA-induced MT1-MMP was unaffected in U-87 cells treated with the ROK inhibitor Y27632 (Fig.5b). This suggests that functional ROK inhibition cannot overcome the MT1-MMP induction by constitutive expression of recombinant Wt-RhoA, or that MT1-MMP induction was mediated by another RhoA effector. Alternate yet unidentified targets of EGCg affecting RhoA functions may also be assumed. Importantly, although EGCg reduced basal MT1-MMP protein levels, it also significantly diminished the RhoA-induced increase in MT1-MMP protein levels.

Rho-associated kinase-induced CD44 shedding is inhibited by green tea catechin EGCg. Several different enzymes have been identified as possible downstream targets for RhoA signalling. One such enzyme is Rho-associated kinase (ROK), which is a serine-threonine kinase known to interact with Rho in a GTP-dependent manner (Manser *et coll.,* 1998). U-87 glioma cells were transfected with a cDNA encoding a constitutively active ROK, and CD44 shedding was assessed in the conditioned media. The overexpression of a recombinant constitutively active form of ROK was found to induce CD44 shedding from the cell surface (Fig.6a) in agreement with the effect of the ROK inhibitor (Fig.4a), while CD44 shedding was undetectable in Mock-transfected cells (Fig.6a). Interestingly, the green tea catechin EGCg reduced ROK-induced CD44 shedding, suggesting that a crucial RhoA/ROK-mediated intracellular signalling pathway is involved in the shedding of CD44 in glioma cells. This effect of EGCg was correlated to the expression of CD44 at the cell surface (Fig.6b) as assessed by flow cytometry. CD44 levels (shaded plots on right side) decreased in RhoA- and ROK-transfected cells as seen by the shift of signal intensity to the left, while EGCg treatment (dotted lines) antagonized both of the RhoA- and ROK-mediated effects on CD44.

DISCUSSION

Tumor astrocytes migrate into the normal brain parenchyma along preferred routes such as blood vessel walls situated in the grey matter (Giese *et coll.,* 1994) or myelin tract in the white matter (Giese *et coll.,* 1996). The fact that transformed astrocytes can detach themselves from initial tumor sites is the major reason why any type of therapy remains ineffective today against astrocytic tumors (Sehgal *et coll.,* 1998). We have previously shown that the interaction between CD44 and hyaluronan in glioma cells is a complex process that involves specialized plasma membrane microdomains and that can mediate both cell–cell and cell–ECM interactions (Annabi *et coll.,* 2004). In the present study, we provide evidence regarding the mechanism by which shedding of CD44 is thought to stimulate brain tumor cell motility. Elucidation of such mechanism may impact on a variety of physiological and pathophysiological processes including tumor metastasis, wound healing and leukocyte extravasation at sites of inflammation.

Moreover, we illuminate the potential to reduce that infiltrating character of brain tumors by the use of EGCg, a green tea catechin that has anticancer properties (Demeule *et coll.,* 2002). Previous studies have shown that EGCg possessed the property to inhibit MT1-MMP-mediated ECM degradation and migration of glioma cells (Annabi *et coll.,* 2002) and to inhibit MT1-MMP-mediated angiogenesis (Oku *et coll.* ,2003). We now exhibit a new effect of EGCg inhibiting the MT1-MMP-induced signaling leading to CD44 cell surface shedding. This CD44 downregulation from the cell surface via receptor shedding may be required for cells to detach from the brain ECM and facilitate movement. This infiltrating/metastatic character of glioma cells likely involves a RhoA/ROK-regulated release of a soluble extracellular fragment of CD44, which also involves intracellular signaling through MT1-MMP (see Fig.7 for summarized scheme). In support to our observations, intravenous administration of EGCg was recently reported to target MT1-MMP-mediated *in vivo*

tumor angiogenesis (Yamakawa *et coll.,* 2004). Unfortunately, the levels of circulating CD44 were not assessed in that report. However, it is tempting to suggest that the circulating soluble CD44 increases could be considered as markers for tumor dissemination and could potentially also interfere competitively with the ability of membrane-bound CD44 to interact with HA (Ahrens *et coll.,* 2001). Whether alternate receptors for HA (such as RHAMM) may also be regulated at the cell surface remains to be investigated. Finally, and although not yet clearly established, ADAM10 was recently reported to shed CD44 from the cell surface through a similar mechanism to that of MT1-MMP (Nakamura *et coll.,* 2004). Whether RhoA, which is known to regulate the functions of ADAM12 (Thodeti *et coll.,* 2003), also regulates ADAM10 has yet to be determined. Altogether, these numerous effects of RhoA indeed highlight potential alternate molecular and cellular processes besides those involving MT1-MMP.

Of particular interest is the fact that several of the documented CD44 releasing processes from cells involve phorbol esters, a calcium ionophore ionomycin or cytokines (DeGrendele *et coll.,* 1997; Ristamaki *et coll.,* 1997), which are also known as MT1-MMP inducers in invading cells (Yu *et coll.,*1997; Park *et coll.,*2000). More recently, structure-function analysis has shown the hemopexin-like domain of MT1-MMP to be responsible for the binding and subsequent shedding of the standard hematopoietic form of CD44 (Suenaga *et coll.,* 2005). Concomitant with CD44 shedding, cytoskeletal reorganization occurs. Pharmacological disruption of actin assembly reduced CD44 shedding, whereas activation of Rho family GTPases, which regulate actin filament assembly, enhanced CD44 cleavage (Shi *et coll.,* 2001). Shedding of CD44 has also been reported to be induced by Ras, an oncoprotein involved in cell motility and migration. The effect of Ras on CD44 processing appears to be mediated by members of the Rho family of GTPases (Kawano *et coll.,* 2000). Taken together, these data suggest that shedding of CD44 is controlled by Ras and Rho GTPases (Cdc42 and Rac1), possibly via regulation of the actin cytoskeleton.

We now provide additional intracellular crosstalk linking RhoA to the cell surface proteolytic activity and expression of MT1-MMP. The specific contribution of MT1-MMP to cytoskeleton changes remains to be determined. Whether the intracellular domain of MT1-MMP transduces any RhoA-mediated cell morphology changes is also under investigation. Finally, in agreement with our data in glioma cells, transcriptional regulation of the MT1-MMP gene was also demonstrated in a study which showed that RhoA and functional inhibition of ROK restored MT1-MMP mRNA that was inhibited by LPA in human osteosarcoma cells (Matsumoto *et coll.*, 2001).

Rho family GTPases play an important role in a number of processes related to metastasis. It is thus not surprising that the overexpression of certain Rho GTPases in human tumors often correlates with poor prognosis (Fritz *et coll.*, 1999). In particular, survival prediction in human gliomas based on proteome analysis has recently identified the increased RhoA levels as potential biomarkers for anti-glioma therapy (Iwadate *et coll.*, 2004). Indeed, the high level of intracellular expression of RhoA facilitates its translocation to the membrane where it is activated, resulting in stimulation of the RhoA-ROK-actomyosin system, and leading to migration (Itoh *et coll.*, 1999). ROK has been shown to phosphorylate the cytoplasmic domain of the $CD44v_{3,\ 8-10}$ isoform and to up-regulate the interaction between the $CD44v_{3,\ 8-10}$ isoform and the cytoskeletal protein ankyrin during HA/CD44-regulated tumor cell migration (Bourguignon *et coll.*, 1999). Thus, ROK is clearly one of the important signaling molecules required for membrane-cytoskeleton interaction, Ca^{2+} regulation, and HA/CD44-mediated cell function. Moreover, several cellular proteins, including the cytoplasmic domain of CD44 and IP_3 receptors, have been identified as ROK-specific cellular substrates during HA-CD44 signaling (Singleton *et coll.*, 2002). Whether MT1-MMP's intracellular domain may also be a substrate for ROK-mediated phosphorylation remains to be established. Coordinated mechanisms involving RhoA/MT1-MMP crosstalk in cell motility and cell surface proteolysis that

could also possibly influence glioma cells invasiveness have been reported. For instance, glioblastoma cell growth and proliferation *in vitro* was recently shown to be regulated by the chemokine stromal cell-derived factor (SDF-1α) that is expressed in several human glioblastoma multiforme tumor tissues (Barbero *et coll.,* 2003). Interestingly, SDF-1α was also reported to promote invasion and to trigger the activation of the GTPase RhoA-dependent signaling in the highly metastatic BLM melanoma cell line, leading to the control of MT1-MMP expression (Bartolome *et coll.,* 2004). Altogether, these data indicate that SDF-1α through RhoA regulation could play important roles during glioma cell invasion and directional migration for basement membrane and brain parenchyma infiltration.

In the present study, we propose a RhoA/ROK-mediated CD44 cell surface shedding mechanism that may regulate glioma infiltration in brain parenchyma. We also provide *in vitro* evidence for a new cellular action of green tea catechin EGCg that could help optimize current therapeutic approaches for brain tumors treatments. We have already demonstrated that EGCg can be efficiently used in conjunction with radiotherapeutic modalities to efficiently target those tumor-derived endothelial cells that escaped ionizing radiation-induced apoptosis (Annabi *et coll.,* 2003). Even more exciting is the fact that a receptor for EGCg has recently been identified as the 67-kDa laminin receptor (Tachibana *et coll.,* 2004). Such laminin receptors have been shown to regulate the invasion of malignant glioma cells potentially through Rho GTPases intracellular signaling (Fukushima *et coll.,* 1998). Whether EGCg can directly interact and inhibit intracellular RhoA functions remains to be investigated. We now propose that the infiltrating character of brain tumors may also be efficiently targeted by the anti-cancerous properties of green tea catechin EGCg, which could then be used in synergy with currently employed therapeutic modalities.

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Fig.1

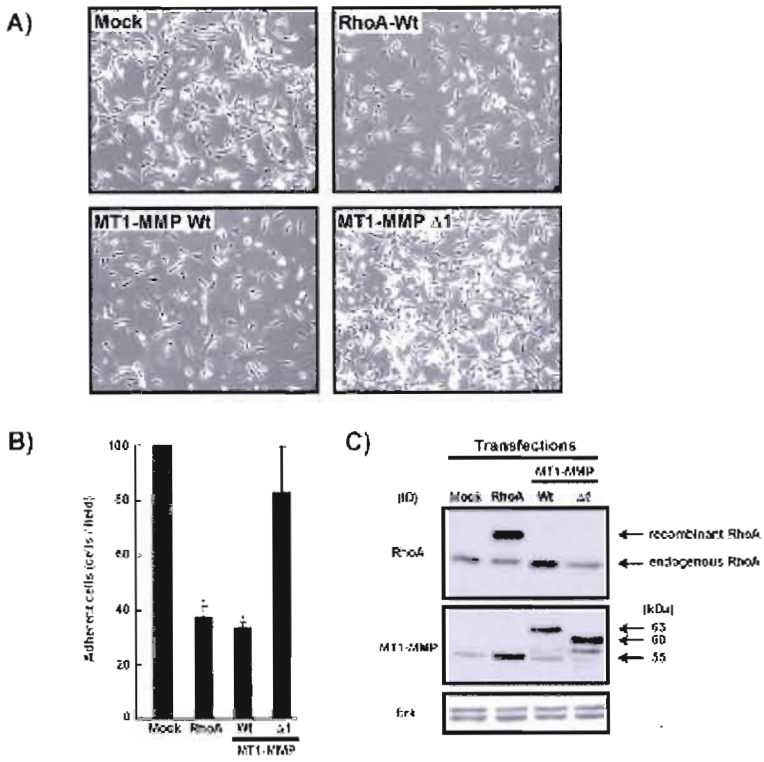

Fig.1: Recombinant RhoA and MT1-MMP overexpression induces loss of cell-HA adhesion in U-87 glioma cells. U-87 glioma cells were cultured on plastic dishes until they reached approximately 60% confluency. Cells were then transfected with cDNA plasmids as follows: Mock (pcDNA3.1^{+}), Wt-RhoA, Wt-MT1-MMP or Δ1-MT1-MMP, as described in the Methods section. Thirty-six hours post-transfection, cells were trypsinised and seeded on HA-coated dishes (1 mg/ml). (A) Pictures of the adherent cells were taken 2 hours after seeding and quantified (B). Western blotting of the cell lysates generated from the respective cell transfections (20 µg / well) was performed on 12% SDS-PAGE for RhoA and ERK, and 9% SDS-PAGE for MT1-MMP. Immunodetection was performed as described in the Methods section (C). The 63-, 60-, and 55-kDa immunoreactive bands observed for MT1-MMP represent respectively the full-length-proMT1-MMP form, the cytoplasmic (Δ1)-truncated form, and the mature 55 kDa processed form of the endogenous MT1-MMP. Data are representative of three independent experiments.

Fig.2

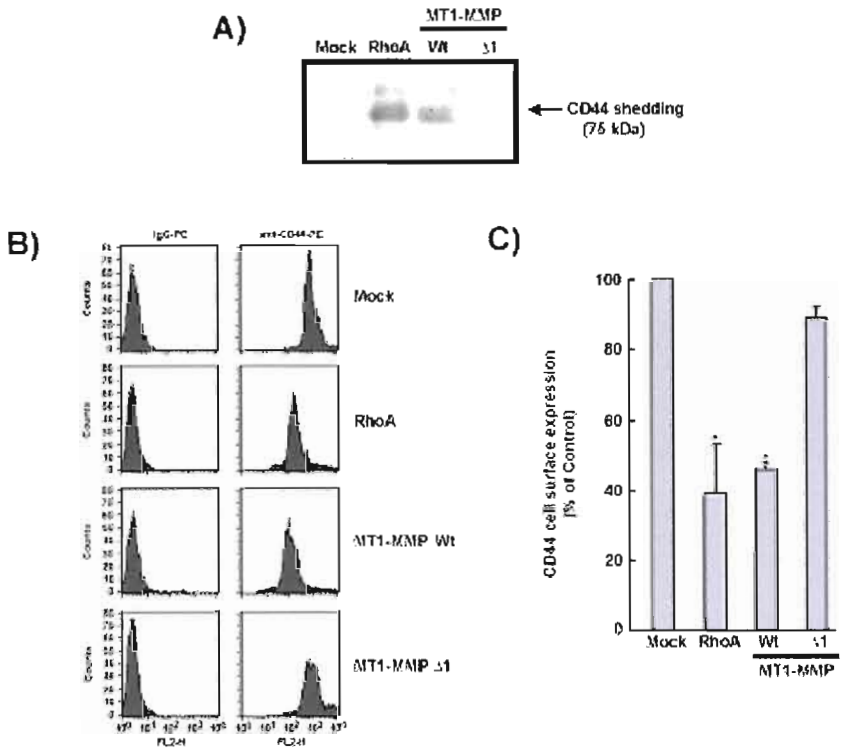

Fig.2: RhoA and MT1-MMP overexpression trigger CD44 cell surface shedding from U-87 glioma cells and inhibit cell migration on hyaluronic acid. (A) U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or with cDNA plasmids encoding RhoA, Wt-MT1-MMP, or Δ1-MT1-MMP. Thirty-six hours post-transfection, cells were starved in serum-free media for 18 hours. Conditioned media was then collected and centrifuged to eliminate any floating cells. Equal volumes (600 µl) of the conditioned media were TCA-precipitated and subjected to Western blotting and immunodetection of CD44. (B) Flow cytometry was used to monitor CD44 cell surface protein expression as described in the Methods section. (C) Flow cytometric results were quantified and expressed as the ratio of relative geometric mean values from the transfected cells to their mock (controls) and are representative of three independent experiments.

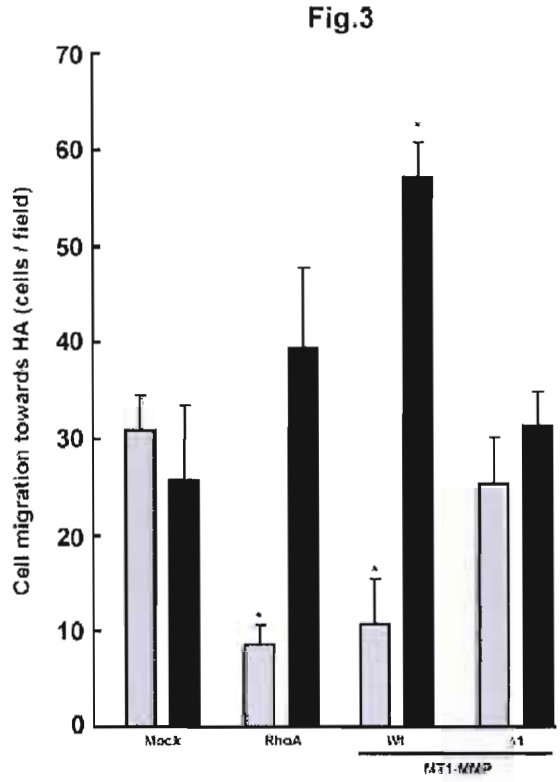

Fig.3: Cell migration on hyaluronic acid is inhibited in RhoA- and MT1-MMP-transfected U-87 glioma cells. U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or cDNA plasmids encoding RhoA, Wt-MT1-MMP, or Δ1-MT1-MMP. Thirty-six hours post-transfection, cells ($5x10^4$) were harvested by brief trypsinization and seeded on hyaluronic acid- (grey boxes) or gelatin- (black boxes) coated filters. Migration was allowed to proceed as described in the Methods section.

Fig.4

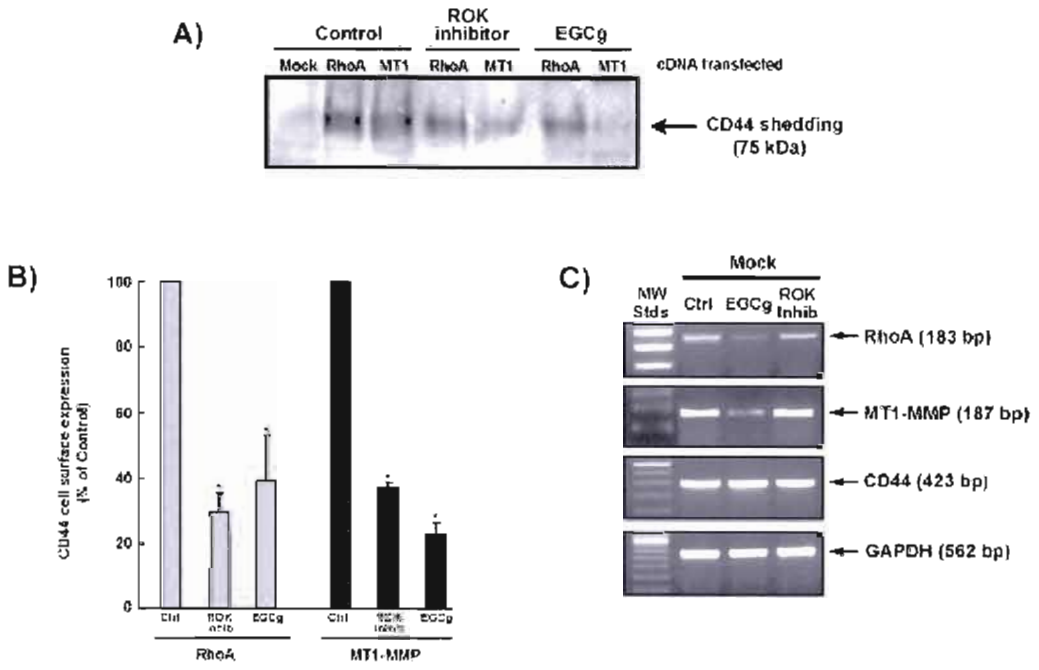

Fig.4: Functional inhibition of ROK and the green tea catechin EGCg reverse RhoA- and MT1-MMP-induced shedding of CD44. (A) U-87 glioma cells were cultured on plastic dishes and subsequently transfected with empty vector (Mock) or cDNA plasmids encoding RhoA or Wt-MT1-MMP. Thirty-six hours post-transfection, cells ($5x10^5$) were starved in serum-free media supplemented (or not) with 20 μM EGCg or 5 μM Y27632 (a Rho-kinase inhibitor) for 18 hours. The conditioned media were then assessed for CD44 as described in the legend to Fig.2. (B) Quantification of the CD44 cell surface expression was performed by flow cytometry as described in the legend of Fig.2c for the RhoA- and MT1-MMP-transfected cells. (C) The effects of EGCg and of the ROK inhibitor were also assessed on RhoA, MT1-MMP, and CD44 gene expression by RT-PCR as described in the Methods section.

Fig.5

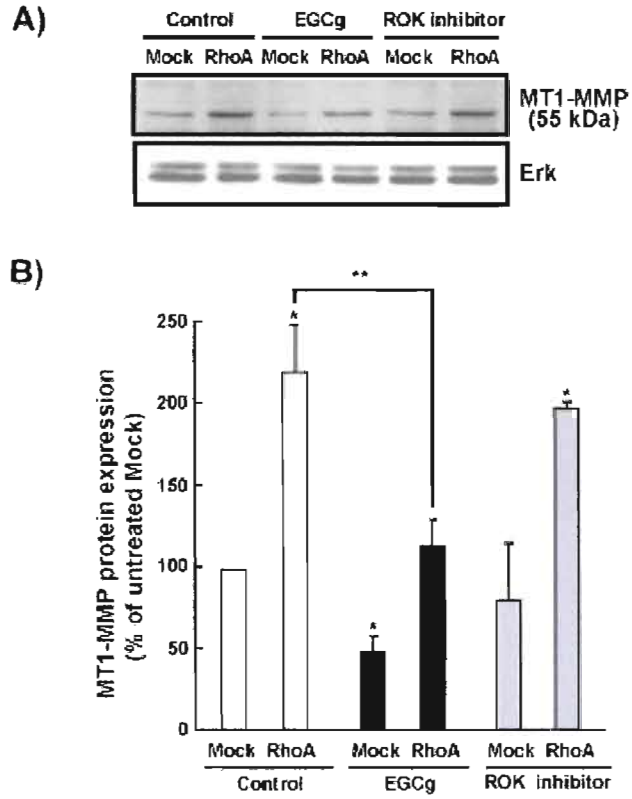

Fig.5: The green tea catechin EGCg and ROK inhibition reverse RhoA-induced expression of MT1-MMP. (A) U-87 glioma cells were cultured on plastic and subsequently transfected with empty vector (Mock) or RhoA cDNA. Thirty-six hours post-transfection, cells were treated (or not) for 18 hours with either 20 μM EGCg or 5 μM Y27603 (ROK inhibitor). Cells were then lysed and subjected to 9% SDS-PAGE, followed by an immunodetection of MT1-MMP or ERK. (B) Densitometric quantification was performed on three independent experiments. Probability values of less than .05 were considered significant, and an asterisk (*) identifies such significance against control (Mock-transfected cells), while (**) identifies significant difference between RhoA-transfected cells and RhoA-transfected cells followed by EGCg treatment.

Fig.6

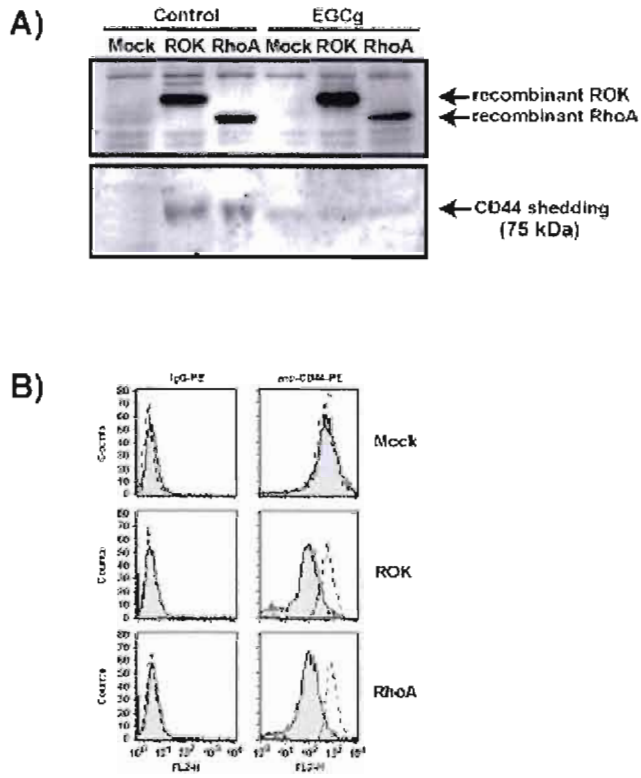

Fig.6: **ROK-induced CD44 shedding is inhibited by the green tea catechin EGCg.** U-87 glioma cells were cultured on plastic and subsequently transfected with either an empty vector (Mock), a cDNA encoding a constitutively active ROK or a cDNA encoding Wt RhoA. Thirty-six hours post-transfection, cells were treated (or not) for 18 hours with 20 μM EGCg. (A) Cells were then lysed and subjected to 9% SDS-PAGE, followed by immunodetection of Myc in order to detect the recombinant myc-tagged ROK and myc-tagged RhoA. The conditioned media from Mock-, ROK-, and RhoA-transfected cells was also assessed for CD44 content as described in the legend to Fig.2. (B) Flow cytometry was used to monitor CD44 cell surface protein expression as described in the Methods section. Shaded plots represent the untreated cells, while the dotted plots represent the EGCg-treated cells. A representative experiment out of two independent treatments is shown.

Fig.7

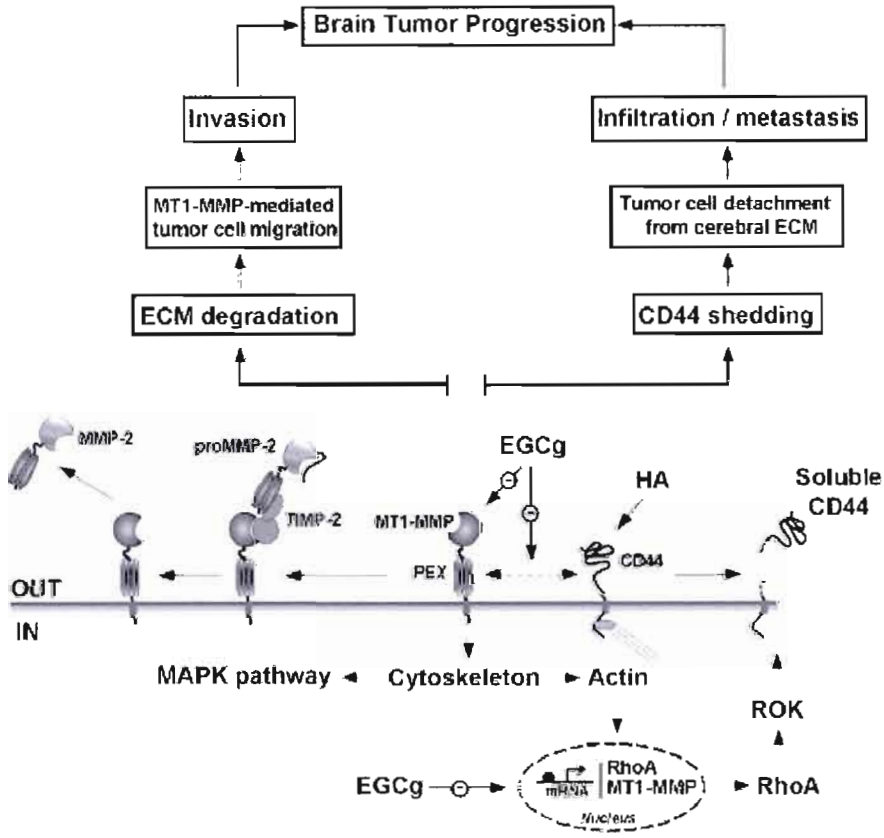

Fig.7: Scheme of the proposed regulatory mechanism of RhoA/ROK-mediated CD44 cell surface shedding leading to glioma infiltration in brain parenchyma. Brain tumor progression is characterized by the invasive and infiltrating character of the invading cells. These properties are, in part, mediated by MT1-MMP which regulates the degradation of the ECM through the formation of a trimolecular complex with TIMP-2 and the latent proMMP-2 form. This complex eventually leads to the release of an active MMP-2 form which regulates tumor invasion. On the other hand, MT1-MMP is also thought to be a multifunctional protein which regulates several pericellular processes at the cell surface of glioma cells that may reflect the infiltrating character of the brain tumor. In the basal state, MT1-MMP regulates CD44 cell surface expression and hyaluronic acid (HA) binding through a MAPK-dependent pathway (Annabi *et*

coll., 2004), and this is antagonized by inhibition of MT1-MMP functions by EGCg, a green tea catechin with anticancer and antiangiogenic properties. The profound cytoskeletal reorganisation induced by MT1-MMP's intracellular domain may also regulate CD44 cell surface functional expression through the upregulation of both RhoA and MT1-MMP gene expression. EGCg can also antagonize this event by downregulating their gene expression levels. Overall, RhoA/ROK intracellular signalling is an important step that regulates the mechanisms leading to infiltrating/metastatic processes involved in the interaction of glioma cells with their brain ECM environment and that could be efficiently targeted by the green tea catechin EGCg.

2. ***''The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate''***

McLaughlin N., Annabi B., **BOUZEGHRANE M.**, Temme A. Bahary J.P., Moumdjian R. & Béliveau R.

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La régulation d'expression de la survivine dans le phénotype radiorésistant des glioblastomes est stimulée par RhoA et inhibée par le polyphénol du thé vert l'épigallocatéchine-3-gallate

Nancy McLaughlin[1,5], Borhane Annabi[2], **Mounia Bouzeghrane[1]**, Achim Temme[3], Jean-Paul Bahary[4], Robert Moumdjian[5], Richard Béliveau[1*]

L'aggressivité des glioblastomes multiformes (GBM) est renforcée dans les cellules tumorales radiorésistantes. La combinaison de la radiothérapie et la chimiothérapie a été envisagée comme approche thérapeutique pour GBM. Le but de cette étude est de déterminer la modulation de la réponse cellulaire à la radiation ionisante (IR) par l'épigallocatéchine-3-gallate (EGCg), un polyphénol du thé vert ayant des propriétés anti-cancéreuses et l'implication de médiateurs de la signalisation intracellulaire et d'inhibiteurs des protéines d'apoptose dans ce processus. Des GBM humaines, les U-87, ont été cultivées et transféctées par un recombinant de la survivine, RhoA et de la cavéoline-1. Les cellules transféctées ou non ont été irradiées par une seule dose subléthale. La prolifération cellulaire est analysée par compte nucléaire des cellules et l'apoptose détéctée par l'essai fluorimétrique de la caspase-3. L'expression des protéines est analysée par immunobuvardage. L'IR (10 Gy) réduit alors la prolifération des U-87 de 40% *via* un mécanisme indépendent de la caspase-3. La surexpression de la survivine induit un effet cytoprotectif contre IR tandis que la surexpression de RhoA conférerait un effet de sensibilisation à l'IR. Les U-87 traitées préalablement avec EGCg ont montré une diminution du taux de prolifération dépendante de la dose de l'IR. L'effet inhibiteur de EGCg sur la croissance des U-87 n'a pas été renversé par la surexpression de la survivine. Cependant, les cellules transfectées avec la survivine et prétraitées avec l'EGCg sont devenues sensibles à IR et l'expression de RhoA endogène de ces cellules était diminuée. Un potentiel thérapeutique de l'EGCg visant les voies intracellulaires anti-apoptotiques des cellules cancéreuses est suggérée pour agir en synergie avec l'IR.
La radiorésistance des GBM est probablement médiée par un mécanisme dépendant de la survivine en conjonction avec RhoA. La combinaison de molécules naturelles anti-cancéreuses comme l'EGCg avec la radiothérapie pourrait être suggérée pour améliorer l'efficacité des traitements.

The survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-) epigallocatechin-3-gallate

Nancy McLaughlin[1,5], Borhane Annabi[2], **Mounia Bouzeghrane[1]**, Achim Temme[3], Jean-Paul Bahary[4], Robert Moumdjian[5], Richard Béliveau[1*]

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ABSTRACT

Introduction: Glioblastoma multiforme's (GBM) aggressiveness is potentiated in radioresistant tumor cells. The combination of radiotherapy and chemotherapy has been envisioned as a therapeutic approach for GBM. The goal of this study is to determine if epigallocatechin-3-gallate (EGCg), a green tea-derived anti-cancer molecule, can modulate GBM's response to ionizing radiation (IR) and whether this involves mediators of intracellular signaling and inhibitors of apoptosis proteins.
Material and Methods: U-87 human GBM cells were cultured and transfected with cDNAs encoding for Survivin, RhoA or Caveolin-1. Mock and transfected cells were irradiated at sublethal single doses. Cell proliferation was analyzed by nuclear cell counting. Apoptosis was detected using a fluorimetric caspase-3 assay. Analysis of protein expression was accomplished by Western immunobloting.
Results: IR (10 Gy) reduced control U-87 cell proliferation by 40% through a caspase-independent mechanism. The overexpression of Survivin induced a cytoprotective effect against IR while the overexpression of RhoA conferred a cytosensitizing effect upon IR. Control U-87 cells pretreated with EGCg exhibited a dose-dependent decrease in their proliferation rate. The growth inhibitory effect of EGCg was not antagonized by overexpressed Survivin. However, Survivin-transfected cells pretreated with EGCg became sensitive to IR and their RhoA expression was downregulated. A potential therapeutic effect of EGCg targeting the prosurvival intracellular pathways of cancer cells is suggested to act synergistically with IR.
Conclusion: The radioresistance of GBM is possibly mediated by a mechanism dependent on Survivin in conjunction with RhoA. The combination of natural anti-cancerous molecules such as EGCg with radiotherapy could improve the efficacy of IR treatments.

Theme: Disorders of the nervous system

Topic: Neuro-oncology

Keywords: Human glioma, Radiotherapy, Survivin, RhoA, EGCg, Green tea

Abbreviations:
EGCg: Epigallocatechin-3-gallate
GBM: Glioblastoma multiforme
IR: Ionizing radiation

INTRODUCTION

Glioblastoma multiforme (GBM) represents the most aggressive and invasive primary cerebral neoplasm in the adult population. Median length of survival without further therapy is usually less than one year from the time of diagnosis [18, 28]. When surgical excision is considered, the goal should be gross total removal to prolong quality survival [32]. However, the effect of surgical resection on the time to tumor progression and the median length of survival can only be optimized when combined with other therapies. For instance, conventional local field radiotherapy has been shown by itself to prolong median survival for 6-8 months [23]. Unfortunately, 90% of patients receiving radiation therapy following GBM resection still develop tumor recurrence in the proximity of the primary site [30]. Neither whole brain irradiation nor high focal radiation doses can decrease the incidence or change the location of recurrences [45]. Ionizing radiation (IR)-induced alterations of the malignant behavior are not unique to astrocytic tumors and are still poorly understood [40]. Recent studies have reported that GBMs' recurrence following IR is partially mediated by an enhanced invasive character of radioresistant tumor cells, which makes them more difficult to treat [17, 59].

Chemotherapy is thus used either as an adjuvant, concurrent, or pre-irradiation treatment along with radiotherapy for malignant primary tumors. However, only modest benefits in survival have been reported [23, 34, 44]. More recently the combination of chemotherapeutic drugs such as temozolomide with molecules exhibiting an anti-angiogenic activity have been shown to be safe and more effective with respect to survival than the administration of the chemotherapeutic agent alone [9, 55]. Also attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding or reversing the multi-stage carcinogenesis process. Recent reports have proposed that some phytochemicals can function as sensitizers, augmenting the effectiveness of conventional radiotherapy [15, 25]. Epigallocatechin-

3-gallate (EGCg), a major anti-oxydative green tea polyphenol, has been recognized for its anti-mutagenic and anti-carcinogenic properties [20, 48]. More recently, we have shown that EGCg also possessed anti-angiogenic properties as it suppressed vascular endothelial growth factor receptors functions in endothelial cells [33]. We have also demonstrated that EGCg efficiently targeted endothelial cells that escaped IR-induced apoptosis [6]. Whether this natural polyphenol can be used to target pro-survival pathways involved in GBM radioresistant phenotype is unknown.

The identification of the molecular mechanisms underlying GBM radioresistance thus becomes essential for the development of combination therapies against this lethal condition. Survivin, along with other markers, has been proposed as a major factor for radioresistance in glioblastoma [13]. Survivin, belonging to the family of inhibitor of apoptosis proteins, is involved in the modulation of apoptosis [3, 16, 36, 46] in the regulation of cell growth [3, 49, 52], in the regulation of mitotic events such as chromosomal segregation and cytokinesis [54, 57], and in the process of angiogenesis [12, 39]. Its expression has been associated with enhanced malignant potential of gliomas, increased cell viability after IR exposure and adverse clinical prognosis [14, 31]. Rho proteins, which belong to a family of small GTPases, are also involved in the control of key cellular processes such as modulation of the cytoskeleton, receptor internalization, or cell adhesion [7, 22, 58]. More specifically, farnesylated RhoB pathway has been suggested as a key factor in glioblastoma resistance to IR. Indeed, overexpression of RhoB in radiosensitive cells increased cell survival after IR [2]. Conversely, inhibition of RhoB led to the appearance of multinucleated cells and induced a post-mitotic cell death that led to decreased glioma cell survival [1]. Interestingly, the same effect was observed in glioma cells transduced with a $p34^{cdc2}$ phosphorylation-defective SurvivinT34A, suggesting a link between Survivin and Rho proteins [53]. Although studies have shown that the expression of RhoA and RhoB were similar in brain tumors of grades II to IV [24],

the specific role of RhoA, in conjunction with Survivin, in glioma radioresistance remains to be investigated. Caveolin-1, a protein associating RhoA to caveolae-enriched membrane domains [26, 42], is proposed to participate in cell survival and angiogenesis [37, 41]. The molecular implication of Caveolin-1 in glioma radioresistance has also not yet been investigated. Hence, the goal of this study is to determine whether EGCg can sensitize GBMs' response to radiation and whether specific molecular markers are involved.

MATERIALS AND METHODS

Materials: Agarose, (-)-epigallocatechin 3-gallate (EGCg), sodium dodecylsulfate (SDS), gelatin, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Mouse anti-Survivin monoclonal antibody was from Cell Signaling Technology (Beverly, MA), mouse anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-Caveolin-1 monoclonal antibody was from BD Pharmingen (Mississauga, ON) and mouse β-actin monoclonal antibody was from Sigma. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, Il) and enhanced chemiluminescence (ECL)-Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were bought from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes from Boehringer Mannheim. Trypsin was from INVITROGEN (Burlington, ON).

Cell culture and cDNA transfection: The U-118, U-138, and U-87 human glioblastoma cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. U-87 cells were transiently transfected with cDNA constructs using the non-liposomal FUGENE-6 transfection reagent. The EGFP-tagged WT-Survivin cDNA construct has been described previously [54]. The Myc-tagged WT-RhoA cDNA construct was provided

by Dr Allan Hall (London, UK). GFP-tagged WT-Caveolin-1 was provided by Dr Sung-Soo Yoon (Sung Kyon Kwan University, Korea). Transfection efficiency was analysed by Western blotting and fluorescent microscopy. All experiments involving these cells were performed 36 hrs following transfection. Mock transfection of U-87 cultures with the empty pcDNA (3.1+) expression vector was used as controls.

EGCg and irradiation treatment: Cells were treated in serum-free MEM supplemented (or not) with EGCg (3-30 μM) for 6 hours and were overlaid with sufficient medium to subsequently provide efficient build up doses. Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. After irradiation, MEM containing 20% FBS was added and cultures were left to recuperate for 18 hours. Non-irradiated control cells were handled similarly to the cells which were subjected to IR.

Cell proliferation assay: The extent of cell proliferation was assessed 18 hours after irradiation. Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each probe, 150 μl homogenate was saved for nuclear cell counting using an automatic nuclear counter and its specific reagents (New Brunswick Scientific Co., Edison, NJ) and for cell number determination using Trypan blue for exclusion of dead cells.

Fluorimetric caspase-3 activity assay: U-87 cells were grown to about 60% confluence and treated with EGGg or IR doses. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for one hour at 4°C and the lysates were clarified by centrifugation at 16,000g for 20 minutes. Caspase-3 activity was determined by

incubation with 50 μM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 minutes at 37°C on a fluorescence plate reader (Molecular Dynamics) (λ_{ex}= 400nm, λ_{em}=505nm).

Immunoblotting procedures: Total protein lysates from control cells and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution) in TBST containing 5% non-fat dry milk. The secondary antibodies were visualized by enhanced chemiluminescence and quantified by densitometry.

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare the relative proliferation rates. Probability values of less than .05 were considered significant. In each figure statistically significant differences are identified by an asterisk (*) for ECGg or IR treatment compared to control, while double asterisk (**) is to show significance between combined EGCg/IR treatment and either EGCg or IR treatment alone.

RESULTS

Sublethal, low, single dose IR partially inhibits U-87 and DAOY cell proliferation. We examined the proliferation rates of several human high grade astrocytoma (U-138, U-118, U-87) and medulloblastoma (DAOY) cell lines in response to increasing doses of IR (Fig.1). We observed a dose-dependent decrease of the cell proliferation rate at increasing IR doses, up to 30 Gy. DAOY was the most radiosensitive cell line, as the proliferation rate was decreased by 70% after 10 Gy and by 95% after 30 Gy exposures. U-87 cells were the most radioresistant cell line when compared to the other astrocytoma cell lines; their proliferation rate was decreased by only 40% after 10 Gy and by 50% after 30 Gy exposures.

Sublethal, low, single dose IR inhibits U-87 cell proliferation by a caspase-independent mechanism. In order to assess whether the decrease in cell proliferation was due to IR-induced caspase-mediated apoptosis, we measured caspase-3 activity. In all human astrocytoma cell lines analysed, radiation exposure up to 10 Gy did not induce caspase-3 activity (Fig.2). However, at 30 Gy, the caspase-3 activity was twofold increased in U-138 and U-118 cells, whereas in U-87 cells no significant increase in caspase-3 activity was detectable. In contrast, IR induced significant caspase-3 activity in DAOY cells even at doses as low as 3 Gy. These results prompted us to further investigate the possible apoptosis-independent mechanisms underlying the observed radioresistance of U-87 cells.

Low dose IR induces the expression of prosurvival proteins in U-87 cells. We investigated the protein expression of Survivin, RhoA, and Caveolin-1 in irradiated U-87 cells. We selected 10 Gy as the delivered IR dose since it was associated with a significant decrease in cell proliferation and absence of caspase-3 activity (Fig.1 and 2). After exposure to IR, Survivin expression in U-87 control cells increased 2-fold

over basal expression, while that of RhoA increased 4-fold (Fig.3a and 3b). Interestingly, the expression of Caveolin-1, a protein regulating RhoA functions, also increased by 4.5-fold in irradiated U-87 cells. □-actin and total Erk protein levels were not affected by IR (Fig.3a).

Overexpression of Survivin and RhoA exhibits antagonistic effects on the radioresistant phenotype of U-87 cells. U-87 cells were cultured to 50% confluence and transfected with cDNA constructs encoding Survivin, RhoA, or Caveolin-1 proteins. Transfection efficiencies were assessed by the presence of GFP positive cells in Survivin and Caveolin-1 transfectants (Fig.4a) and by immunodetection for ectopic RhoA protein in RhoA-transfected cells (Fig.4b). Surprisingly, ectopic expression of Survivin induced RhoA, whereas recombinant Caveolin-1 did not (Fig.4b). Mock-transfected controls and cells either expressing Survivin, RhoA or Caveolin-1, were then exposed to increasing doses of IR. In mock-transfected U-87 cells, we observed a dose-dependent decrease in the cell proliferation rate (open circles) when cells were irradiated with doses up to 30 Gy. Interestingly, the proliferation rate of U-87 cells overexpressing Survivin (closed circles) did not decrease upon IR treatment, and maintained a proliferation rate similar to non-irradiated cells (Fig.4c). This clearly demonstrates that Survivin confers resistance to irradiation. Strikingly, cells overexpressing RhoA (closed squares) exhibited a significantly decreased cell proliferation rate in comparison to mock U-87 cells (Fig.4c). Overexpression of RhoA thus seems to render U-87 cells more sensitive to IR resulting in a decreased proliferation capacity. Overexpression of Caveolin-1 (open squares) had no influence on the U-87 cell proliferation rate (Fig 4c).

Survivin overexpression does not antagonize EGCg's antiproliferative effect. Recent studies have demonstrated that EGCg, a green tea-derived polyphenol, inhibits the growth of various human cancer cell lines [8, 43, 51], particularly human

glioblastoma cells [61]. We further investigated the effect of EGCg on U-87 glioma cell proliferation. We show that EGCg indeed inhibited cell proliferation in a dose-dependent manner with an optimal inhibitory effect at 25 μM of more than 70% (Fig.5a). Caspase-3 activity was measured in parallel and found not to be significantly induced by EGCg (Fig.5a) suggesting that the inhibition in cell proliferation was not due to apoptosis. Cells were cultured to 50% confluence and transfected with cDNAs encoding for Survivin, RhoA, or Caveolin-1, treated with EGCg, and left to recuperate overnight. Maximal inhibition of proliferation (35-40% of untreated cells) was achieved with 25 μM EGCg (Fig.5b). The growth of U-87 cells overexpressing RhoA, Survivin, and Caveolin-1 was also decreased by EGCg, similarly to the mock U-87 cells. We conclude that neither RhoA, Survivin, nor Caveolin-1 were able to fully reverse EGCg's antiproliferative effect. Individual, maximally inhibitory treatments with EGCg (25 μM) and IR (10 Gy) were also compared to show that only Survivin was able to reverse the inhibitory effect that IR has on cell proliferation.

Effect of combined EGCg and low dose IR on U-87 glioma cells proliferation. We studied the effect of a combined approach consisting of pre-treating U-87 cells with EGCg followed by exposure to sublethal single IR doses on the protein expression levels of Survivin, RhoA, and Caveolin-1. EGCg did not affect the steady state protein levels of Survivin, RhoA and Caveolin-1 in non-irradiated cells (Fig.6a). Interestingly, while IR induced the expression of Survivin, RhoA and Caveolin-1, EGCg pre-treated cells showed a dose-dependent decrease in the IR-induced protein expression of Survivin and of RhoA (Fig.6a). The protein expression levels of Caveolin-1 were increased in cells treated with both EGCg and irradiation. The effect of this combined treatment was further investigated with respect to RhoA expression. U-87 cells were transfected with the Survivin cDNA, which induced RhoA expression (Fig.4b and Fig.6b). Interestingly, this effect was reversed when Survivin-

transfected cells were treated with EGCg but not when they were exposed to IR (Fig.6b). Consequently, the cytoprotective effect that Survivin has towards IR may, in part, be caused by RhoA signalling, which is significantly abrogated when cells are treated with EGCg. Most importantly, cell proliferation assays showed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments (Fig.6c, left panel). Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to reverse the cytoprotective effect that Survivin had towards IR (Fig.6c, right panel). Prosurvival intracellular pathways might thus be targeted by EGCg, a phytochemical that could be efficiently used as an adjunct to radiotherapy.

DISCUSSION

Glioblastomas multiforme (GBM) are highly invasive primary tumors of the adult central nervous system. Although radiotherapy is routinely prescribed in the management of high grade gliomas, its efficacy remains limited because of tumor cell radioresistance, enhanced invasive character following radiation and resultant tumor recurrence [17, 59]. In our study, we first analyzed the sensitivity of various human high-grade astrocytoma- and medulloblastoma-derived cell lines to ionizing radiation (IR). U-87 glioma cells were found to be the most radioresistant cell line, while DAOY medulloblastoma cells were the most radiosensitive cell line tested. This is in accordance with the reported radioresistance of glioblastoma cells [13, 17] and radiosensibility of medulloblastoma cells [35]. Studies have indeed shown that p53-mediated apoptosis is an important response of medulloblastomas to radiotherapy [19]. In contrast, p53 mutations are present in as much as 40-60% of GBMs [60], suggesting that either pro-survival mechanisms or p53-independent events may regulate their therapeutic response to IR [29].

In light of the emergence of several markers of glioma malignant progression, the molecular characterization of GBMs' radioresistance was next investigated. Among these markers, Survivin has been recently related to the malignant phenotype of gliomas as its expression is associated with glioma progression from low- to high-grade [14, 31]. Furthermore, Survivin expression is well recognized as a predictor of adverse clinical prognosis for patients with gliomas [14], as it increases survival of primary GBM cell lines through its capacity to suppress caspase-mediated apoptosis [13] by directly binding to caspase-9 or by interacting with smac-DIABLO [38, 47]. The effects of Survivin in our study support the lack of IR-induced growth inhibition that we observed in GBM. Furthermore, Survivin is also implicated in the regulation of cell division [3, 49] as it is localized in multiple components of the mitotic

apparatus and centrosomes and participates in cell cytokinesis [54, 57], a process that involves RhoA [4]. Novel functions of Survivin have been proposed to emerge following IR such as double-strand DNA break repair and enhancement of tumor cell metabolism potentially mediating radioresistance [13]. In addition, some studies have suggested that overexpression of Survivin may facilitate evasion from checkpoint mechanisms of growth arrest and promote resistance to chemotherapeutic regiments targeting the mitotic spindle [27]. Whether these mechanisms also regulate the radioresistant phenotype of GBM remains to be confirmed.

Our study shows that IR exposure increases the basal expression of Survivin in U-87 glioma cells, possibly through RhoA-mediated intracellular signaling, and that this overexpression subsequently confers radioprotection against IR. Rho GTPases function as molecular switches that modulate the activation of several enzymes involved in different biological processes related to tumor progression, such as cell growth, transcriptional regulation, and apoptosis [7, 22, 58]. Interestingly, RhoA-induced apoptosis is independent of p53 but dependent on modulation of anti-apoptotic proteins [21]. In our study, whether RhoA expression was induced by IR or through cDNA cell transfection, a cytosensitizing effect was observed in U-87 cells which resulted in a significant decrease in cell proliferation following IR. On the other hand, although the overexpression of recombinant Survivin also induced RhoA expression, the resultant was rather a cytoprotective effect on cell proliferation following IR. This paradoxal effect might be explained by post-transcriptional modifications that would be induced by IR and that may alter RhoA's function or cellular distribution to specialized membrane domains. We previously reported that RhoA is associated with caveolae-enriched membrane domains, possibly through physical interaction with Caveolin-1 [26]. Although caveolae regulate cell survival processes [37], we show that overexpression of Caveolin-1 induced either by IR or cDNA cell transfection did not influence cell proliferation but may potentially have

regulated RhoA's functions or localization. The interaction between RhoA and Caveolin-1 in the context of IR is currently under investigation.

One of the major features characterizing high-grade radioresistant gliomas is their infiltrating character. Recently, we showed that this phenotype might be linked to RhoA/Rok-mediated CD44 cell surface shedding which could be targeted and inhibited by EGCg, a green tea-derived polyphenol with anticancerous properties [5]. In light of this evidence and of recent studies which have shown that EGCg could serve as an IR enhancer on cancer cell lines [8], we investigated whether EGCg pretreatment prior to IR could reverse the cytoprotective effect of Survivin. We observed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments. Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to downregulate RhoA expression and reverses the cytoprotective effect that Survivin exhibited towards IR. Although the exact molecular mechanism of EGCg is not completely understood, recent studies have shown that the inhibitory effect of EGCg on tumor cell proliferation might be transduced through its binding to the 67-kDa laminin receptor (67LR) [50], a protein whose expression is strongly correlated with tumor aggressiveness [11]. Interestingly, studies have shown that through its binding to the 67LR, EGCg may also reduce the phosphorylation of myosin II regulatory light chain, resulting in an increase in cells in the G2/M phase of cell cycle and ultimately in an inhibition of cell growth [56] possibly through increased cell radiosensitivity [10].

In conclusion, we demonstrate that the growth inhibitory effect of EGCg pretreatment efficiently antagonized both the IR-induced expression of Survivin, as well as that of RhoA induced by overexpression of recombinant Survivin. A potential therapeutic impact of EGCg in targeting pro-survival and RhoA-mediated

intracellular pathways in cancer cells is suggested to act synergistically with IR. We propose that EGCg might potentiate IR's inhibitory effect on tumor cell proliferation by increasing the proportion of cells in a radiosensitive state. This natural phytochemical might thus be an important molecule to consider in combined adjuvant chemotherapy and radiotherapy.

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Fig.1

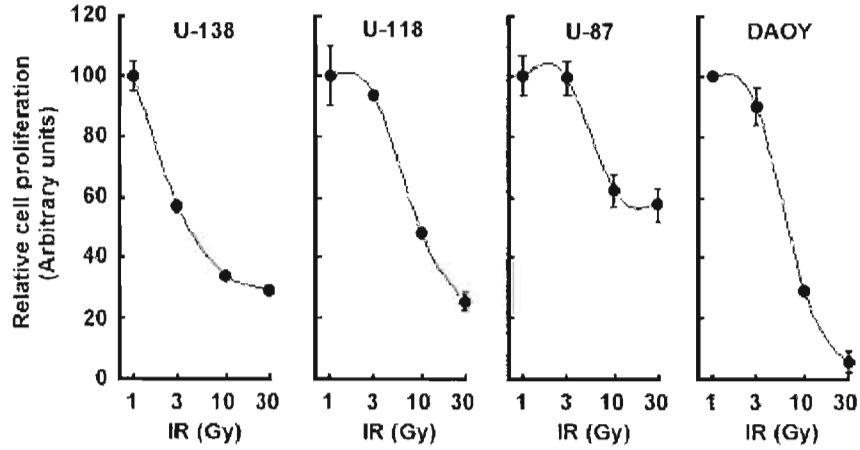

Fig.1: Effects of ionizing radiation on the proliferation rates of malignant glioma and medulloblastoma cell lines. Single dose ionizing radiation (IR) was applied to subconfluent cells and cell proliferation rate was assessed 18 hours after IR using an automatic nuclear counter and Trypan blue staining. Cell proliferation is expressed as a percent of the non-irradiated (control) cell proliferation.

Fig.2

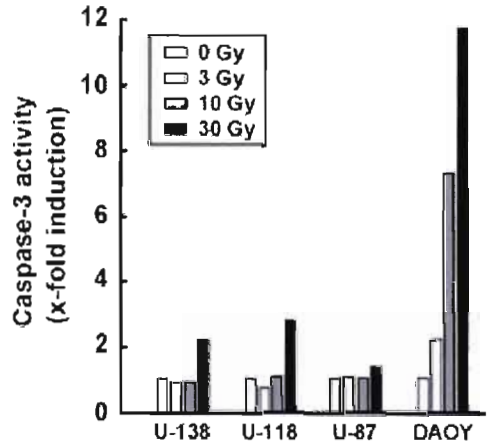

Fig.2: Ionizing radiation effects on caspase-3 activity in malignant glioma and medulloblastoma cell lines. Cells were grown to 60% confluence and were exposed to a single dose of IR. A representative caspase-3 activity profile for each cell line tested is presented and was assessed as described in the Methods section.

Fig.3

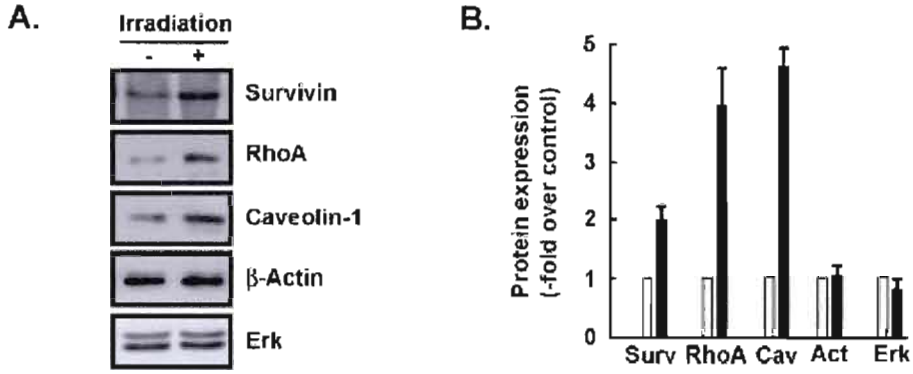

Fig.3: Expression of prosurvival proteins in malignant glioma cells exposed to ionizing radiation. U-87 cells were cultured and exposed to 10 Gy IR. Cell lysates isolated from control and irradiated conditions were electrophoresed on SDS-gels, and transferred to PVDF membranes. (A) Immunodetection of the specific proteins was carried out as described in the Methods section. (B) Quantification of protein expression was performed by scanning densitometry of control, non-irradiated U-87 cells (white bars) and of irradiated cells (black bars).

Fig.4

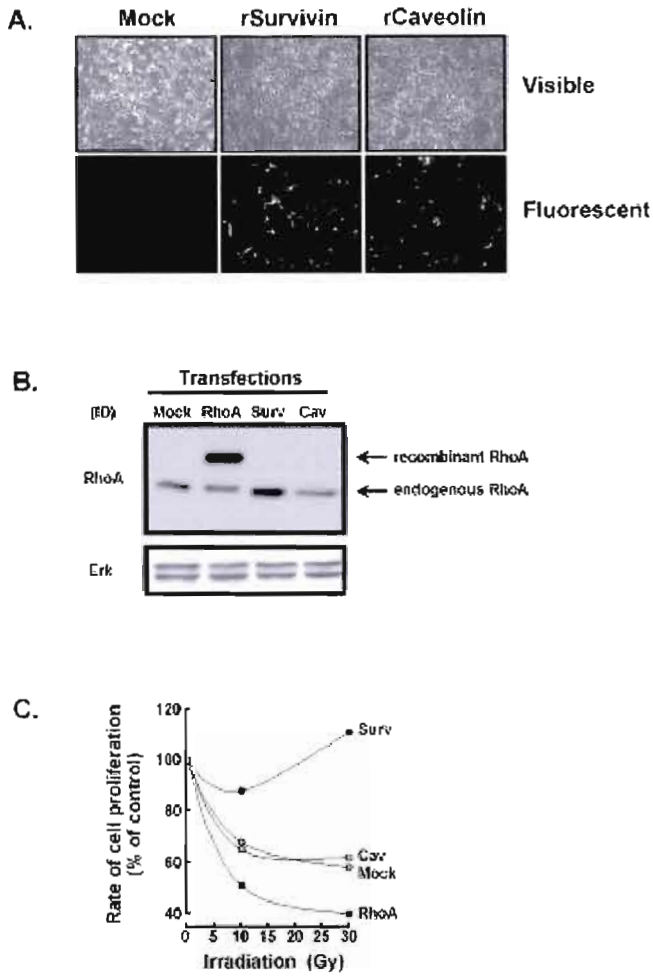

Fig.4: Effect of IR exposure on the proliferation rates of malignant glioma cells transfected with proteins involved in radioresistance. U-87 cells were transfected with cDNA constructs encoding for recombinant Survivin, Caveolin-1 or RhoA. (A) Transfection efficiency was confirmed by fluorescent microscopy in cells transfected with Survivin and Caveolin-1 and (B) by Western blotting in cells transfected with RhoA. (C) A dose-dependent decrease in the mock U-87 (open circles) proliferation rate is observed as cells are irradiated with increasing sublethal doses. The proliferation rate of U-87 cells transfected with Survivin (closed circles), RhoA (closed squares), and Caveolin-1 (open squares) is also shown.

Fig.5

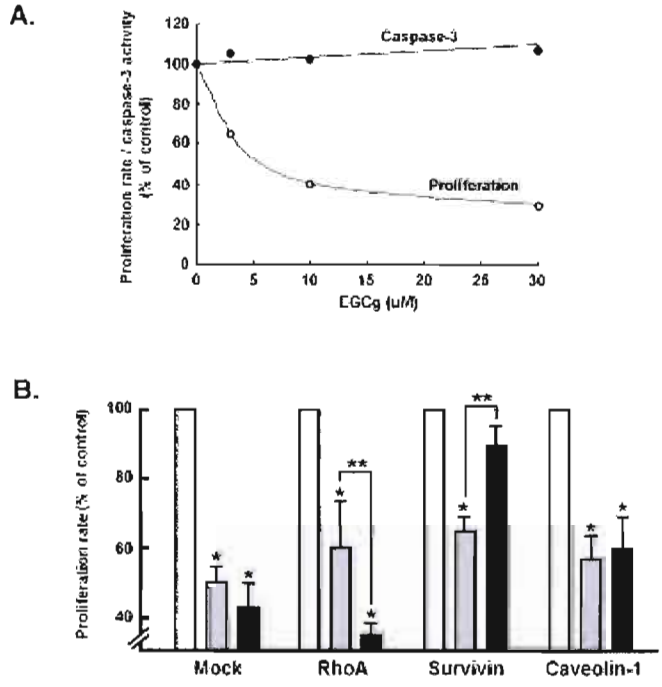

Fig.5: Survivin overexpression does not antagonize EGCg's antiproliferative effect. Subconfluent cells were exposed for 6 hours to increasing concentrations of EGCg (0-30 □M) and cell proliferation rate (white circle) and caspase-3 activity (black circle) were assessed following EGCg treatment as described in the Methods section. Results are expressed as a percent of the control untreated Mock cells (A). The proliferation rate was also assessed in U-87 cells transfected with Survivin, RhoA or Caveolin-1 cDNAs and further exposed to IR (black bars) or EGCg treatment (grey bars). Cell proliferation rate is expressed as a ratio of control untreated Mock cells (white bars).

Fig.6

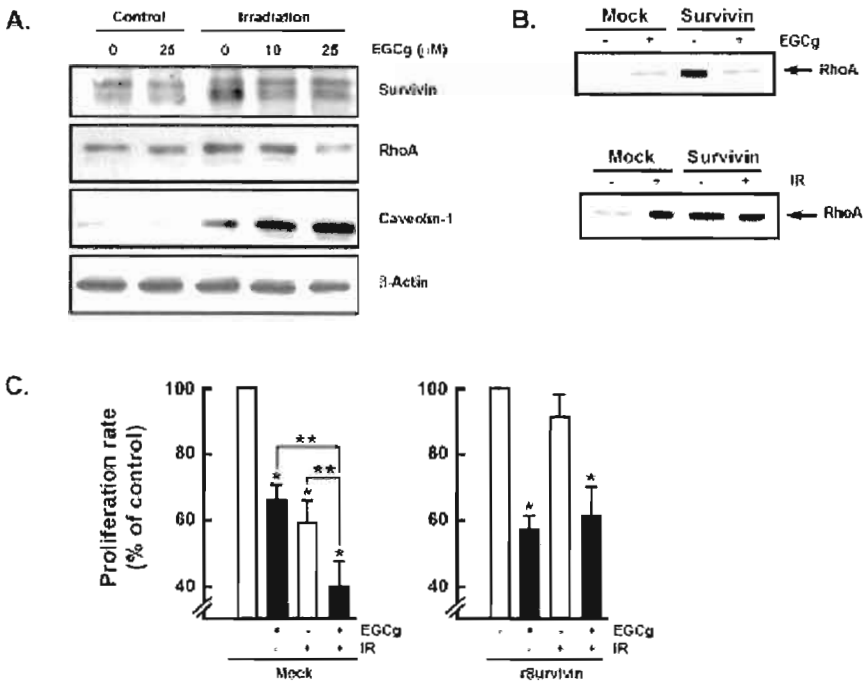

Fig.6: Effect of combined EGCg and low dose IR on U-87 glioma cell proliferation. U-87 cells were cultured and treated with EGCg (0-25 □M) for 6 hours before IR (10 Gy) and left to recuperate overnight at 37°C. (A) Cell lysates of each condition were electrophoresed on SDS-gels and immunodetection was carried out as described in the Methods section. (B) Mock or Survivin-transfected U-87 cells were also treated with either EGCg (upper panel) or IR (lower panel) and RhoA immunodetection performed in cell lysates. (C) Cell proliferation was performed in Mock and Survivin-transfected cells that were either treated with EGCg, irradiated (IR), or which were submitted to combined EGCg/IR treatment.