UNIVERSITÉ DU QUÉBEC À MONTRÉAL

IMPLICATION D'UN AXE DE SIGNALISATION MT1-MMP/G6PT DANS LA MIGRATION ET LA SURVIE DES CELLULES SOUCHES MÉSENCHYMATEUSES

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RÉSUMÉ

La contribution des cellules souches au développement tumoral est une percée conceptuelle récente dans notre compréhension des mécanismes moléculaires et cellulaires impliqués dans la carcinogenèse. En ce sens, il est reconnu depuis quelques années qu'une sous-population de cellules souches mésenchymateuses (MSC) mobilisables en réponse à des facteurs de croissance tumoraux pourrait contribuer au développement tumoral. Les recherches rapportées dans ce mémoire nous ont permis d'étudier certains partenaires clé dans la régulation de la migration et de la survie cellulaire des MSC. L'observation préalable d'une modulation conjointe de l'expression d'une métalloprotéase matricielle de type membranaire (MT1-MMP) et du transporteur microsomal de glucose-6-phosphate (G6PT) nous a permis d'évaluer la contribution respective de ces joueurs dans la signalisation affectant la chimiotaxie des cellules souches ainsi que des cellules tumorales cérébrales. De plus, nous avons évalué l'impact de certains « mannosides » synthétisés en vue de cibler spécifiquement les fonctions de surfaces de MT1-MMP et qui pourraient être à l'origine de nouvelles approches thérapeutiques anticancéreuses affectant le recrutement des cellules souches au foyer tumoral. Finalement, l'importance de l'axe de signalisation MT1-MMP/G6PT dans la mobilisation du calcium intracellulaire en réponse à la sphingosine-1-phosphate, un lipide bioactif synthétisé par des niveaux d'expression élevés de sphingosine-kinase retrouvé au niveau tumoral, permet également de concevoir le ciblage effectif de l'un ou l'autre de ces partenaires dans la progression tumorale. L'ensemble de nos résultats permettra de mieux comprendre les phénomènes régulant la survie et le recrutement des MSC aux sites de foyers tumoraux, en plus de fournir de précieux renseignements sur un nouvel axe original de signalisation liant les fonctions de MT1-MMP à celles, inattendues, de G6PT.

Mots clés : Cellules souches, cancer, MT1-MMP, G6PT, sphingosine-1-phosphate

CHAPITRE I

Introduction

1.1 Les cellules souches

1.1.1 Éthique et problématique de travail

Il existe deux critères majeurs permettant de classifier une cellule comme étant une cellule souche. Elle doit premièrement être capable d'auto-renouvellement en plus de posséder la capacité à se différencier en différents types cellulaires plus spécialisés (Bongso et Lee, 2005). Il est cependant nécessaire d'effectuer les tests adéquats de différenciation et d'identification de certains marqueurs de surface avant de statuer qu'une cellule est dite « souche ». L'intérêt pour les cellules souches provient du fait qu'elles peuvent être maintenues en culture et divisées un nombre important de fois, voire même indéfiniment dans certaines conditions, tout en restant non-différenciées et en conservant leur capacité à produire plusieurs types cellulaires spécialisés (Keller, 2005), d'où l'espoir d'utiliser de façon adéquate ces cellules comme nouvelle approche thérapeutique. Cet intérêt de la médecine pour les cellules souches est tout de même récent. Il faut en effet remonter au début des années 1980 pour voir apparaître de plus en plus d'études sur les populations de cellules souches, la classe la plus étudiée à l'époque étant celle des cellules souches embryonnaires. Ces dernières ont été pour la première fois isolées suite à un prélèvement de la masse cellulaire interne de l'embryon à son stade de développement de blastocyte (Evans et Kaufman, 1981). La recherche sur les cellules souches embryonnaires soulevant des questions d'ordre éthique, puisqu'elle implique de stopper le processus de développement d'un être vivant en devenir, on dénote dans le domaine deux axes de recherches principaux : les chercheurs travaillant à partir de cellules souches embryonnaires/fétales et ceux travaillant sur les cellules souches adultes.

1.1.2 Classification des différentes populations de cellules souches

La classification des populations de cellules souches est relativement complexe. Le domaine étant tout de même récent et en constant développement, les définitions que l'on y retrouve peuvent parfois sembler contradictoires. Toutefois, on s'entend pour dire qu'il existe quatre grandes familles de cellules souches classées selon leur origine : les cellules souches embryonnaires, les cellules souches fœtales, les cellules souches du cordon ombilical et les cellules souches adultes (Bongso et Lee, 2005). La figure 1 expose la hiérarchie du développement de ces cellules. En haut de la pyramide, on remarque les cellules dites totipotentes obtenues à partir d'un blastocyte de 1 à 4 jours et qui sont aptes à donner naissances à tous les types de cellules du corps humain. Elles sont les seules à pouvoir donner naissance à un organisme viable, les expériences démontrant qu'il est possible de générer des jumeaux identiques en divisant *in vitro* le blastocyte (Bongso et Richards, 2004). Toutefois, puisque ces cellules ne possèdent pas la capacité à s'auto-renouveler indéfiniment, elles ne peuvent être officiellement considérées comme étant « souches ». Par contre, à un stade de division plus avancé, on retrouve les cellules dites pluripotentes, c'est-à-dire qu'elles ont la capacité de produire pratiquement tous les types cellulaires possibles, mais qui sont plutôt prélevées sur un jeune embryon, soit entre les jours 5 à 7 du développement. On les appelle communément, de par leur origine, les cellules souches embryonnaires (Lanza et al., 2004).

En ce qui a trait aux cellules souches issues du cordon ombilical, on y retrouve la sous-catégorie de cellules souches hématopoïétiques. Ces cellules multipotentes possèdent des caractéristiques différentes des cellules souches hématopoïétiques issues de la moëlle osseuse, en particulier par rapport aux facteurs de croissance nécessaires à leur développement *in vitro* ainsi que par un risque de rejet « donneur versus receveur » beaucoup moins élevé lors de transplantations que dans le cas des cellules de la moëlle osseuse (Rogers et Casper, 2004). Finalement, chez les cellules souches adultes, on retrouve encore une fois plusieurs sous-catégories, les plus importantes étant les cellules souches hématopoïétiques et mésenchymateuses (figure 2). Les cellules souches hématopoïétiques issues de la moëlle osseuse sont multipotentes et peuvent donner naissance aux différentes cellules sanguines requises dans la circulation périphérique. Le lieu de prélèvement des cellules souches hématopoïétiques et mésenchymateuses étant le même, la distinction entre les deux

populations repose entre autre sur leur capacité d'adhésion *in vitro,* sur la présence de certains marqueurs de surface et sur leur potentiel de différentiation. En effet, les cellules souches mésenchymateuses en culture sont adhérentes, tandis que les précurseurs hématopoïétiques restent normalement en suspension (Lanza et al., 2004). Les cellules souches mésenchymateuses possèdent aussi la capacité à former du cartilage, les os, les muscles, les tendons, les ligaments et les cellules adipeuses (Bongso et Lee, 2005). De plus, les MSC peuvent être identifiées par certaines glycoprotéines membranaires. Une population de MSC doit être négative pour les marqueurs CD34, CD38 et CD45, alors qu'elle doit être positive pour CD105, CD73 et CD90 (Dvorakova et al., 2008).

Les cellules souches sont connues pour être impliquées dans diverses pathologies. L'origine des cas de leucémies aigües lymphoblastiques se trouve chez les cellules lymphoïdes progénitrices, causant chez les personnes souffrant de cette maladie d'importants problèmes au niveau du système immunitaire (Hoelzer et al., 2002). Un autre cas bien étudié de pathologie au niveau des cellules souches est celui de l'ostéogénèse imparfaite (OI), un désordre génétique affectant la synthèse adéquate du collagène, la protéine constituant majoritairement la structure de la matrice extracellulaire (MEC) entourant les os (Bobis et al., 2006). On note chez les patients souffrant de OI des problèmes au niveau de la croissance osseuse, des déformations au niveau de la colonne cervicale ainsi que des fractures fréquentes et multiples. Plus récemment, l'implication des populations de cellules souches dans le développement tumoral a été relevée (Annabi et al., 2004).

1.1.3 Les cellules souches cancéreuses

Il y a une quarantaine d'années, une équipe de chercheurs a fait le postulat que les cancers proviendraient d'un arrêt dans la maturation des cellules souches (Pierce, 1967), et ce environ dix ans après que les premiers chercheurs aient émis l'hypothèse que des cellules souches spécifiques à un tissu pourraient être à la base de la formation du cancer (Till et McCulloch, 1961). On savait déjà à cette époque qu'une très faible proportion de cellules leucémiques (1-4%) étaient capables de former une masse de cellules lors d'implantation *in vivo*, laissant place à deux interprétations : soit toutes les cellules possédaient une très faible capacité de prolifération, soit seulement quelques unes d'entre elles étaient hautement prolifératives (Bruce et Van Der Gaag, 1963). L'existence d'une sous-population cellulaire

qui possédait des caractéristiques propres aux cellules souches, dans ce cas une prolifération accrue et la capacité à générer de nouvelles tumeurs, dans l'environnement tumoral a été démontrée récemment (Bonnet et Dick, 1997; Reya et al., 2001). Toutefois, les études de l'époque n'étaient pas acceptées par toute la communauté scientifique, dû en partie au fait que l'on mesurait *in vitro* des paramètres s'apparentant à l'auto-renouvellement des cellules souches, mais qui en vérité étaient plutôt des tests de prolifération. Cette théorie a cependant refait surface aux cours des dernières années, l'avancement scientifique dans le domaine des cellules souches ayant permis la mise au point des tests plus fiables d'auto-renouvellement. Plusieurs raisons permettent de croire en ce concept récent. Tout d'abord, de par leur capacité à vivre très longtemps, les cellules souches sont assujetties à un risque plus élevé d'accumuler des mutations (Wicha et al., 2006). Par la suite, les cellules souches et les cellules tumorales possèdent plusieurs caractéristiques en commun : la capacité à s'auto-renouveler, la capacité à se différencier, l'expression active de la télomérase, l'activation de voies anti-apoptotiques, une augmentation des activités de transport membranaire ainsi qu'une capacité à migrer et à former des métastases (Dontu et al., 2003; Reynolds et Weiss, 1996; Weiss et al., 1996). Un des premiers processus à être affecté lors de la transformation des cellules souches en leur forme cancéreuse est possiblement celui d'auto-renouvellement, qui est normalement hautement régulé. Normalement, la division des cellules souches est asymétrique, c'est-à-dire que la cellule mère se divise en donnant naissance à une copie identique d'elle-même et d'une cellule « fille », qui se différenciera par la suite pour produire une cellule spécialisée. Dans le cas d'un développement tumoral, on pense qu'il pourrait s'agir d'un dérèglement produisant une division symétrique de la cellule mère en deux copies d'elle-même, produisant ainsi une accumulation et un arrêt de la production de cellules spécialisées (Liu et al., 2005).

Récement, les principales voies de signalisation régissant l'auto-renouvellement des cellules souches ont été identifées, soit celles de Wnt, Notch et Hedgehog (Dontu et al., 2004; Liu et al., 2005). Le dérèglement de la voie de Hedgehog peut, par ailleurs, mener au développement de carcinomes de la peau, gastriques, pancréatiques, de la prostate et du sein (Karhadkar et al., 2004; Olsen et al., 2004). Des altérations dans la voie de signalisation Notch peuvent mener au développement de cancers cervicaux, de cancer du sein et de leucémies lymphoblastiques aigues (Benson et al., 2004; Dievart et al., 1999; Nam et al.,

2002; Nickoloff et al., 2003; Weijzen et al., 2002). L'avènement du modèle des cellules souches tumorales fait émerger des implications importantes par rapport au « modèle stochastique » classique. Cette conception de la carcinogénèse impliquait que n'importe quelle cellule pouvait aléatoirement acquérir une ou plusieurs mutations la rendant cancéreuse. Par opposition à ce modèle, celui des cellules souches cancéreuses propose que le développement de tumeurs serait plutôt imputable à des dérèglements affectant les populations de cellules souches spécifiques au tissu impliqué (figure 3). Cette différence de conception pourrait avoir des conséquences majeures sur la façon de diagnostiquer, traiter et prévenir l'avancée tumorale (Wicha et al., 2006). Par exemple, il a été démontré récemment par Guzman et al. que les cellules souches leucémiques étaient plus résistantes aux traitements que leurs « filles » plus différenciées (Guzman et al., 2002). C'est dans cette optique qu'il est important d'établir des marqueurs spécifiques aux cellules souches tumorales permettant de mieux les étudier et de les cibler dans d'éventuels traitements. En ce moment, un des marqueurs prévalant dans le milieu scientifique est CD133, une glycoprotéine membranaire qui a longtemps été un marqueur des cellules souches hématopoïétiques, mais qui a été identifiée récemment comme une protéine préférentiellement exprimée chez les cellules tumorales capables d'auto-renouvellement (Mizrak et al., 2008).

1.1.4 Contribution des MSC au développement tumoral

De récentes découvertes ont fait état de l'implication des MSC au niveau du développement tumoral cérébral. En effet, il a été démontré que les MSC étaient avidement recrutées par les cellules endothéliales différenciées participant à la néovascularisation des tumeurs de même que par les cellules tumorales elles-mêmes en réponse au facteur de croissance de fibroblastes (bFGF) (Annabi et al., 2004). De plus, une équipe de Houston a démontré en 2005 que des MSC implantées de façon intravasculaire étaient avidement recrutées au niveau des glioblastomes, mettant en évidence la possibilité que ces cellules puissent participer à la croissance de la tumeur (Nakamizo et al., 2005).Ils ont d'ailleurs pu isoler des MSC à partir de tumeurs prélevées chez des patients. La quantité de MSC pouvant être isolées de ces tumeurs était proportionnelle au grade de cette dernière, relevant ainsi l'importance de la contribution des MSC à la croissance tumorale. Une équipe coréenne a

d'ailleurs récemment démontré qu'une classe de cellules de glioblastomes isolés de patients montrait un profil d'expression génique comparable aux cellules mésenchymateuses, et que dans ces cas les patients avaient de très faibles chances de survie (Joo et al., 2008).

1.2 Cancer et invasion

1.2.1 Angiogénèse tumorale

On entend par angionénèse la formation de nouveaux vaisseaux sanguins à partir de ceux déjà existants (Hanahan et Folkman, 1996). Ce processus requiert donc la participation de cellules endothéliales qui, par divisions successives, vont permettre la mise en place des vaisseaux sanguins. On peut cependant distinguer deux contextes angiogéniques; l'angiogénèse normale, impliquée, entre autre, dans l'implantation et le développement embryonnaire, l'organogénèse et la cicatrisation; et l'angiogénèse pathologique, impliquée dans des processus néfastes pour l'organisme comme la croissance et l'invasion tumorale, les rétinopathies, les hémangiomes infantiles, l'arthrite rhumatoïde, le psoriasis, les ulcères duodénaux et la fibrose (Hanahan et al., 1996). Le processus d'angiogénèse tumorale est déclenché lorsque le foyer tumoral atteint un volume d'environ $1 mm^3$ (Folkman, 1996). À ce moment, l'apport en nutriments essentiels et en oxygène n'est plus suffisant pour maintenir les cellules cancéreuses en vie. L'hypoxie joue un rôle de premier plan dans le processus angiogénique. En effet, la réponse cellulaire à l'hypoxie se traduit par un changement allant de la phosphorylation oxydative à la glycolyse anaérobique, par une augmentation de l'entrée de glucose dans les cellules et par une augmentation importante de gènes codant pour différentes molécules de stress (Ingber et Folkman, 1989; Zhang et al., 2007). Un acteur important dans ces réponses est le facteur de transcription HIF-1 (Hypoxia Inducible Factor-1) qui, à lui seul, est responsable de l'activation de plus de 70 gènes en réponse à l'hypoxie (Semenza, 2004). Un de ces gènes hautement impliqué dans l'angiogénèse tumorale est le facteur de croissance de l'endothélium vasculaire (VEGF) (Annabi et al., 2003; Hanahan et al., 1996). L'action de ce facteur de croissance, en concert avec l'augmentation de l'expression des métalloprotéases matricielles (MMP) permettant la dégradation de la MEC, résulte en une migration des cellules endothéliales pour finalement permettre la formation de nouveaux capillaires (Annabi et al., 2003; Ingber et Folkman, 1989; Lelievre et al., 1996).

1.2.2 Matrice extracellulaire (MEC)

Le processus d'angiogénèse requiert d'importantes modifications de l'espace environnant requis pour l'organisation des vaisseaux. La MEC régule d'une façon importante les interactions entre les épithéliums et leurs stromas. La matrice occupe un rôle de premier plan dans la survie cellulaire, en maintenant un fin équilibre entre la synthèse et la dégradation de ses diverses composantes. Dans l'environnement cérébral, la glie est le nom porté par la MEC entourant les cellules localisées au cerveau. Composée principalement de collagènes (types I, II et III), la glie est formée d'autres constituants secondaires, comme la laminine, la fibronectine et différentes protéoglycanes. Les variations de la quantité de ces composantes est la résultante d'un accroissement de la synthèse et/ou d'une réduction de la dégradation de chacune d'elles. La dégradation proprement dite est le résultat d'une cascade enzymatique importante impliquant diverses protéases, notamment les protéases à cystéine (cathepsines B et L), les protéases à aspartate (cathepsine D), les protéases à sérine et finalement les MMP (Cuvelier et al., 1997).

1.2.3 Métalloprotéases

Les MMP jouent un rôle crucial dans le phénomène d'invasion tumorale. On dénote deux types de MMP. Les MMPs solubles, sécrétées dans le milieu extra-cellulaire, et celles du type membranaire (MT-MMP) qui sont ancrées à la membrane plasmique (Malemud, 2006). Des 28 membres de la grande famille des MMP identifiés à ce jour, 22 sont présents chez l'humain, les principales étant énumérées dans le tableau I. Dans l'organisme, les MMP sont régulées en majeur partie par leurs inhibiteurs naturels, les TIMP (tissue inhibitors of metalloproteases) (Hamacher et al., 2004).

De toutes les métalloprotéases, l'expression de la MMP-2 (gélatinase A) ainsi que celle de la MMP-14 (membrane type-1 matrix metalloprotease, MT1-MMP) a été reconnue pour être plus élevée dans plusieurs cas de tumeurs, entre autre celles des poumons (Tokuraku et al., 1995), du foie (Harada et al., 1998), du sein (Ishigaki et al., 1999) et du cerveau (Forsyth et al., 1999). L'expression anormalement élevée de ces deux MMP corrèle souvent avec un faible taux de survie des patients atteints de ces types de cancers (Seiki, 2003).

La MMP-2 est une métalloprotéase soluble de 72 kDa sécrétée sous forme de zymogène (proMMP-2), possédant un peptide signal, un pro-domaine, un domaine de liaison du zinc contenant le site catalytique et un domaine hémopexine situé à l'extrémité C-terminale (Murphy et al., 1999). La MMP-2 activée hydrolyse de façon préférentielle la gélatine et le collagène de type IV, mais possède tout de même la capacité de cliver d'autres types de collagènes, l'élastine, la décorine, la fibronectine et les protéoglycanes du cartilage (Lehti et al., 2002).

L'activation de la proMMP-2 (figure 4) nécessite une cascade d'évènements impliquant deux autres joueurs, MT1-MMP et TIMP-2. La proMMP-2 se lie tout d'abord à un complexe MT1-MMP/TIMP-2 à la surface cellulaire (Polette et Birembaut, 1998). La liaison de TIMP-2 via son domaine N-terminal au domaine catalytique de MT1-MMP inhibe l'activité de cette dernière. Par la suite, la liaison de la proMMP-2 via son domaine hémopexine au domaine C-terminal de TIMP-2 permet la formation d'un complexe tri-moléculaire. Une MT1-MMP libre située dans les environs du complexe clivera tout d'abord la proMMP-2 entre l'Asn37 et la Leu38, générant ainsi la forme intermédiaire de la MMP-2 à 64 kDa. S'ensuivra ensuite l'autoprotéolyse de la MMP-2 déstabilisée entre l'Asn90 et la Tyr91, générant enfin la forme active de la MMP-2 (Strongin et al., 1995).

1.2.4 Fonctions de MT1-MMP

MT1-MMP est une protéine de 60 kDa constituée d'un segment transmembranaire, d'une portion extracellulaire comprenant le site actif et d'une portion intracellulaire servant à la transduction des signaux (Gingras et al., 2001). La MT1-MMP peut, en effet, sous l'influence de son domaine cytosolique, induire une cascade de signalisation permettant d'augmenter la migration cellulaire (Annabi et al., 2002; Gingras et al., 2001). Son expression génique est régulée par le facteur de transcription EGR-1 (Haas et al., 1999). Il a été démontré que la MT1-MMP peut se retrouver préférentiellement dans les cavéoles, des invaginations membranaires riches en cholestérol et dont le marqueur est la cavéoline, permettant ainsi de réunir dans un même espace plusieurs molécules nécessaires à la signalisation intracellulaire (Annabi et al., 2001). Lorsque surexprimée, il a été montré que la MT1-MMP pouvait augmenter l'invasion et la formation de structures capillaires de cellules endothéliales sur matrigel (tubulogénèse) (Hiraoka et al., 1998; Hotary et al., 2000). De plus,

l'expression de MT1-MMP est augmentée chez les cellules endothéliales stimulées avec les facteurs pro-angiogéniques VEGF, FGF-2 et TNF-α, expliquant ainsi la hausse de la tubulogénèse en réponse à ces facteurs (Lafleur et al., 2002). Un autre rôle important de MT1-MMP est son implication dans le processus de migration cellulaire des glioblastomes (Annabi et al., 2002; Belien et al., 1999). Une étude récente a montré que l'augmentation de la migration suite à une stimulation par un biolipide actif sécrété au niveau tumoral, la sphingosine-1-phosphate (S1P), était le résultat d'une augmentation de la transactivation du récepteur au facteur de croissance épidermal (EGFR). Cette transactivation se produit via la portion cytoplasmique de MT1-MMP, et implique la voie de signalisation de ERK (Langlois et al., 2007).

De plus, le domaine catalytique de MT1-MMP peut activer des MMP solubles sécrétées sous forme de zymogène. Par exemple, il est connu qu'un substrat préférentiel de MT1-MMP est la proMMP-2, qui une fois clivée en sa forme active peut alors dégrader plusieurs protéines de la MEC. Un rôle récent et plutôt inédit de MT1-MMP implique l'augmentation des mécanismes régulant la mort cellulaire lors d'une surexpression de la forme native de cette protéine (Belkaid et al., 2007). De plus, l'introduction dans les cellules d'un mutant de MT1-MMP tronqué de sa partie cytoplasmique ne parvient pas à reproduire cette induction de la mort cellulaire, confirmant ainsi l'importance de la portion intracellulaire de MT1-MMP dans le processus de signalisation.

1.2.5 Cibler les fonctions de MT1-MMP

Étant donné son important rôle dans le développement de tumeurs cancéreuses, MT1-MMP est une cible de choix pour l'élaboration de nouvelles thérapies. Bien évidemment, on peut immédiatement penser à des inhibiteurs synthétiques des MMP, qui viennent mimer l'action de molécules endogènes remplissant ce rôle, comme les TIMP, l'arrestine et la canstatine (Kamphaus et al., 2000). À ce titre, certaines molécules pharmacologiques, par exemple le marimastat, le neovastat, le prinomastat (AG3340) et COL-3, sont déjà disponibles ou en phase d'essais cliniques dans divers types de cancer comme ceux des poumons, du pancréas, du cerveau et de la prostate (Coussens et al., 2002; Drummond et al., 1999; Lafleur et al., 2003).

Les conclusions tirées des essais cliniques sont, à ce jour, décevantes. Tout d'abord, le passage du modèle animal à l'humain amène parfois à des résultats différents, justifiant ainsi un arrêt prématuré des essais à grande échelle. Sur le modèle murin, les tests se font normalement à des stades précoces du développement tumoral, et les traitements se poursuivent tout au long de la progression, tandis que chez l'humain les études portent plutôt sur des patients en phase avancée de développement tumoral sur lesquels on ne peut qu'évaluer le taux de survie relatif et la vitesse de progression.

De plus, puisque pour la plupart de ces médicaments il s'agit de statines, leur effet est cytostatique plutôt que cytotoxique. Cela complique les tests cliniques puisque comparativement à un produit toxique pour les cellules qui induira la mort cellulaire et que l'on pourra facilement quantifier par diminution de la taille tumorale, les statines provoquent plutôt un arrêt de la croissance cellulaire en gardant les cellules viables (Coussens et al., 2002). Les tests classiques n'étant plus applicables, il faut alors, comme dans le cas du marimastat, calculer la diminution du taux de croissance du cancer via des marqueurs tumoraux retrouvés dans le sérum (Coussens et al., 2002). Cette méthode est controversée puisqu'il a été récemment démontré que les marqueurs retrouvés dans le sérum, par exemple la protéine CA125 servant au diagnostique du cancer des ovaires, ne réflètaient pas une régression de la tumeur (Gore et al., 1996). De plus, étant donné que les MMP sont impliquées dans plusieurs processus non-pathologiques, l'inhibition de certaines d'entre elles provoque parfois des effets secondaires indésirables, comme de l'inflammation et de la douleur musculo-squelettique (Coussens et al., 2002).

D'autres approches existent afin de s'attaquer aux MMP. Par exemple, au lieu de les cibler directement, l'utilisation d'anticorps dirigés contre VEGF ou son récepteur, VEGFR, permet d'inhiber partiellement l'action du VEGF sur la migration et l'invasion cellulaire lorsque l'expression de MT1-MMP est augmentée (Lafleur et al., 2003). D'autres techniques utilisant les anticorps permettent de cibler spécifiquement les fonctions de MT1-MMP, par exemple l'emploi d'anticorps dirigés contre l'intégrine $\alpha v\beta 3$ ou encore la glycoprotéine CD44, une molécule importante dans l'adhésion cellulaire et qui est normalement clivée par MT1-MMP lors du processus de migration cellulaire (Annabi et al., 2004; Lafleur et al., 2003; Seiki, 2003).

Une autre approche thérapeutique récente est l'utilisation de petits ARN (acides ribonucléiques) interférants (siRNA). L'emploi de ces petites molécules d'oligonucléotides s'appariant à l'ARN messager homologue conduit à la dégradation de ce dernier par le complexe RISC (RNA-induced silencing complex), et donc à une régulation à la baisse du gène et ultimement de la protéine d'intérêt. Cette technique a permis de mettre à jour plusieurs voies de signalisation en inhibant l'expression des différentes protéines impliquées dans ces processus. De plus, l'émergence récente de véhicules de livraison des siRNA n'affectant pas la viabilité cellulaire *in vitro* a permis l'utilisation directe des siRNA comme agents thérapeutiques. À ce jour, des essais cliniques impliquant l'utilisation de siRNA dirigés contre MT1-MMP ou certains de ses partenaires potentiels, comme VEGF, MMP-2 et MMP-9, ainsi que d'autres joueurs clés dans le processus de développement tumoral, sont en cours (Lu et al., 2005).

1.2.6 Glioblastomes

L'Organisation Mondiale de la Santé (OMS) classifie les cancers cérébraux relatifs à la glie (gliomes) en quatre grades relativement aux propriétés intrinsèques de ces derniers. Dans la catégorie des astrocytomes, le type de gliome le plus fréquent, les glioblastomes multiformes sont classés catégorie IV. L'espérance de vie moyenne d'une personne diagnostiquée pour un glioblastome multiforme est de 9 à 12 mois (Scott et al., 1998). Les méthodes de traitements actuelles utilisées par la médecine sont l'ablation, la chimiothérapie et la radiothérapie. Les traitements classiques de chimiothérapie disponibles à ce jour ne permettent un arrêt de la croissance des glioblastomes que dans moins de 15% des cas, tandis que, dans le cas d'une réapparition du cancer après une première ablation, le taux de survie moyen n'est plus que d'à peine 25 semaines (Sathornsumetee et Rich, 2007).

Les glioblastomes sont classés en deux catégories : les glioblastomes primaires, qui apparaissent *de novo* en tumeur de grade IV selon l'OMS, et les glioblastomes secondaires, qui eux résultent de tumeurs de grades inférieurs qui se développent en grade IV (Aldape et al., 2003). Même si au point de vue génétique et pathologique les cellules de glioblastomes sont très hétérogènes, et ce au sein d'une même tumeur, il existe certaine altérations communes à ce type de cancer. En effet, on remarque dans plusieurs cas une inactivation du gène du suppresseur de tumeur p53, de même que celui de la phosphatase suppresseur de

tumeur PTEN. On retrouve dans plusieurs cas de glioblastomes une surexpression du facteur de croissance dérivé des plaquettes (PDGF) et du facteur de croissance épidermal (EGF), de même que de leurs récepteurs respectifs PDGFR et EGFR (Kleihues et Ohgaki, 1999). Au niveau métabolique, les glioblastomes présentent une activité de glycolyse élevée afin de suffire à leur forte demande d'ATP (Oudard et al., 1997; Oudard et al., 1998).

1.3 Le système glucose-6-phosphatase (G6Pase)

L'apport en glucose dans l'organisme est essentiel à la production d'énergie permettant son bon fonctionnement. Dans un corps sain, le niveau de glucose sanguin est maintenu à une concentration d'environ 5 mM. La capture du glucose est facilitée par des transporteurs spécifiques à certains tissus (tableau II) (Takata, 1996). La phosphorylation du glucose par une glucokinase dans les hépatocytes, et par une hexokinase dans les autres tissus, permet la formation immédiate du glucose-6-phosphate (G6P), première étape d'un processus qui mènera le G6P à être métabolisé en empruntant la voie glycolytique, se terminant ultimement par la production d'ATP et de pyruvate via la voie aérobie (figure 5). En cas d'excès dans la circulation, le glucose peut aussi suivre la voie glycogénique pour former le glycogène, structure permettant son stockage. Dans le cas contraire, c'est-à-dire lors d'une diminution de la concentration de glucose dans le sang, le processus inverse est enclenché et le glycogène est alors dégradé en G6P. On remarque donc qu'à chacune des étapes du processus de régulation du glucose, le G6P est présent et qu'il remplit donc un rôle important dans la régulation de l'homéostasie glucidique.

1.3.1 Localisation et fonctionnement du système G6Pase

En 1975, Arion et al. ont postulé que, contrairement au modèle conformationnel suggérant que la reconnaissance du G6P de même que son hydrolyse se produisaient sur la membrane microsomale externe avant translocation à l'intérieur de la lumière microsomal, le G6P suivait plutôt le modèle de transport du substrat (Arion et al., 1975). Dans ce modèle, ils proposent que la G6Pase est l'unité catalytique permettant l'hydrolyse du G6P en glucose et qu'elle est orientée vers l'intérieur de la lumière microsomale. Ce postulat implique donc la présence d'un transporteur capable d'importer le G6P dans la lumière microsomale (G6PT ou T1), de même que deux transporteurs capables d'exporter vers la circulation sanguine les

produits de la dégradation du G6P, soit le glucose (T3) et le phosphate inorganique (T2) (figure 6). Plusieurs études sont par la suite venues confirmer le postulat d'Arion, soient :

1. L'identification et le clonage du gène codant pour le transporteur de G6P (T1) indépendant de la G6Pase.

2. L'identification d'inhibiteurs spécifiques à T1.

3. L'existence de maladie due à une déficience de T1.

En plus d'appuyer cette théorie, des études plus récentes ont démontré que le transport du G6P par G6PT était l'étape limitant la conversion du G6P en glucose, soulignant ainsi l'importance de cette protéine dans la régulation du niveau de glucose sanguin (Foster et al., 1997; Gerin et al., 2001; van de Werve et al., 2000; van Schaftingen et Gerin, 2002).

La présence des différentes composantes du système G6Pase a récemment été relevée dans plusieurs types cellulaires. Des niveaux élevés d'expression du système ont été retrouvés dans le foie, les reins et l'intestin, en raison de leur forte activité glyconéogénique. Toutefois, on retrouve aussi des composantes du système dans les tissus non-producteurs de glucose, laissant présager un rôle secondaire autre que celui impliquant uniquement le transport du G6P dans un but d'hydrolyse (Ghosh et al., 2005).

1.3.2 Caractéristiques des composantes du système G6Pase

Le système G6Pase, tel que mentionné plus haut, est composé de 4 protéines majeures : un transporteur de G6P (G6PT, T1), une phosphohydrolase (G6Pase) responsable de l'activité catalytique et deux translocases T2 et T3, responsables respectivement de l'exportation du phosphate inorganique et du glucose vers la circulation sanguine. Tout récemment, il a été postulé et démontré que G6PT était en vérité responsable non seulement de l'activité d'importation du G6P dans la lumière microsomale, mais servirait également d'antiport pour l'exportation du phosphate inorganique (Chen et al., 2008).

Ces protéines sont toutes localisées à la membrane du réticulum endoplasmique. La théorie d'Arion repose en grande partie sur les fonctions de transport du G6P par G6PT. La

protéine G6PT est codée par un seul gène de 5.3 Kb contenant 9 exons localisés sur le chromosome 11q23 (Annabi et al., 1998; Lin et al., 1998). Diverses techniques ont été utilisées pour caractériser G6PT. Les premières méthodes de caractérisation étaient plutôt chimiques, comme celle utilisée par Arion et son équipe, et comparaient les concentrations de G6P et de mannose-6-phosphate intramicrosomal après une incubation de microsomes hépatiques avec ces deux produits. Cette méthode étant la première utilisée pour expliquer le transport du G6P dans la lumière microsomale, elle n'était pas parfaite. Des études suivantes ont prouvé, à l'aide d'inhibiteurs plus spécifiques, l'existence de G6PT de même que sa masse de 46 kDa, migrant à un poids apparent de 37 kDa sur un gel SDS/Page (Herling et al., 1999; Kramer et al., 1999). Le cDNA de G6PT a été cloné et séquencé en 1998 (Lin et al., 1998). Les résultats montrent qu'il s'agit d'une protéine de 429 acides aminés (figure 7), possédant théoriquement entre 9 et 10 domaines transmembranaires.

1.3.3 Les déficits associés aux fonctions de G6PT

Les maladies reliées au métabolisme du glucogène sont appelées glycogénoses (Glycogen Storage Disease, GSD) et sont des maladies héréditaires. Jusqu'à récemment, on comptait sept types distincts de GSD, retrouvés principalement dans le foie. Toutefois, il est maintenant accepté qu'on peut retrouver environ 12 formes de GSD, classées selon l'enzyme et le tissu affectés, et que certaines d'entre elles se retrouvent au niveau musculaire (Shin, 2006). Toutes les formes ont en commun d'affecter les voies métaboliques de mise en réserve du glucose sous forme de glycogène, ou encore l'utilisation de ce dernier dans le but de maintenir la glycémie sanguine. L'incidence totale des GSD toutes confondues est estimée à 1 sur 20 000-43 000 (Ozen, 2007). Dans le cas présent, on s'intéressera principalement à la GSD de type I, puisqu'elle implique les différentes composantes du système G6Pase.

La GSD-I, ou maladie de von Gierke, est la forme la plus grave de GSD. La transmission de la maladie survient de façon autosomale récessive et son incidence dans la population est d'environ 1 sur 400 000 naissances (Ozen, 2007). On dénombre quatre formes principales de GSD-I, soit les types a, b, c et d. Le type Ib a été relevé pour la première fois en 1968, quand un groupe de chercheurs a constaté que l'activité de la G6Pase était normale *in vitro*, mais que l'hydrolyse du G6P en glucose était inexistante *in vivo* (Senior et Loridan, 1968). Il a alors été démontré qu'il s'agissait en fait d'un problème au niveau du transporteur

G6PT. En plus des symptômes servant au diagnostic de la GSD-1a, des infections récurrentes, une neutropénie et une dysfonction des neutrophiles sont des signes caractéristiques de la GSD-1b. Soixante-neuf mutations mutations ont été relevées dans divers cas de GSD-1b, 30 de celles-ci se retrouvant dans des régions codantes du gène de G6PT. 20 d'entre elles inhibent totalement le transport de G6P vers la lumière microsomale, alors que les 10 autres n'inhibaient que partiellement l'activité du transporteur (Chen et al., 2002).

1.3.4 Implication de G6PT dans la survie cellulaire et le flux calcique

Il a récemment été relevé que certaines tumeurs montraient des déplétions en glucose (Linder et Shoshan, 2005), suggérant ainsi une possible implication du système G6Pase. L'implication de la protéine G6PT dans d'autres types de pathologies que la GSD-Ib a récemment été soulignée, notamment son rôle dans le chemotaxisme et la survie des glioblastomes et potentiellement dans l'hydrolyse de la MEC, puisque l'utilisation d'un inhibiteur fonctionnel de G6PT, l'acide chlorogénique, diminuait la sécrétion de MMP dans le milieu extracellulaire (Belkaid et al., 2006; Belkaid et al., 2006). Une délétion du gène de G6PT causait chez des souris des problèmes importants au niveau de la chimiotaxie, du flux calcique ainsi que de la réponse des neutrophiles à un stimulus. « L'emballement respiratoire » (respiratory burst) survient au contact d'une cellule sécrétrice (neutrophile, macrophage) et d'un pathogène. La cellule réagit en relâchant rapidement une quantité importante de molécules d'oxygène réactives (superoxide et peroxyde d'hydrogène) comme réponse immunitaire de défense. Ce processus est un mécanisme d'élimination de pathogène très important dans l'organisme. Or, lorsque G6PT est inhibé, on dénote des problèmes au niveau de l'emballement respiratoire, suggérant ainsi un rôle pour G6PT dans le processus de défense immunitaire (Chen et al., 2003). En plus de son impact sur les neutrophiles, l'inhibition de G6PT par une molécule dérivée de l'acide chlorogénique provoquait la mort cellulaire par apoptose des cellules de leucémie humaine (HL-60), relevant ainsi l'implication de ce transporteur dans la survie cellulaire (Leuzzi et al., 2003). D'une autre part, G6PT régule la séquestration du Ca^{2+} à l'intérieur du RE, un processus ATP-dépendant perturbé lorsque le gène de G6PT est inhibé (Chen et al., 2003). L'homéostasie calcique est aussi assurée à l'intérieur de la cellule par la S1P via son récepteur (S1PR) (Rapizzi et al., 2007),

qui provoque une rapide activation de la voie de ERK, contribuant ultimement en une croissance accrue et une augmentation du potentiel invasif de cellules tumorales (Van Brocklyn et al., 2003).

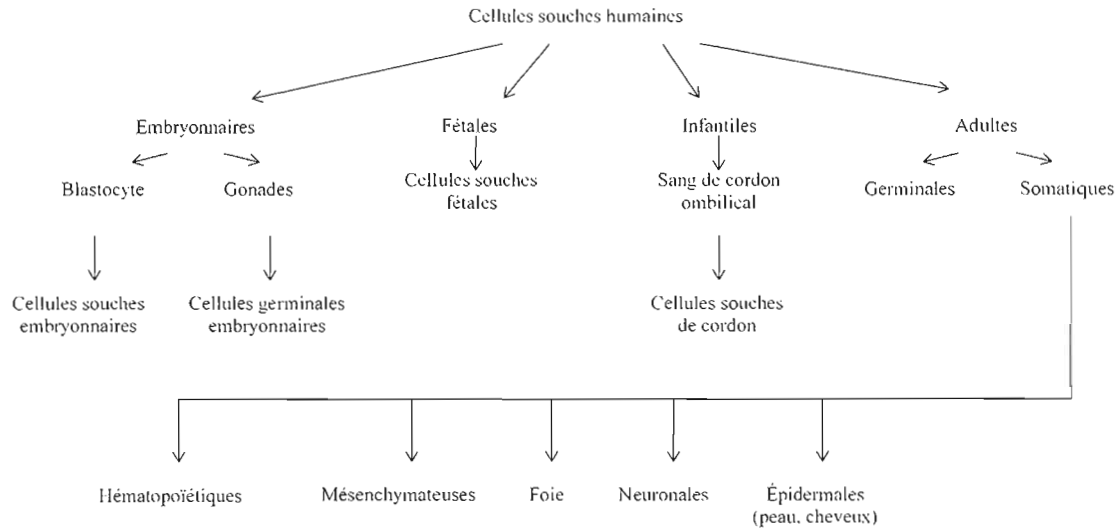

Figure 1 : *Hiérarchie des cellules souches humaines*. La nomenclature des cellules souches dépend du stade de développement duquel elles ont été prélevées. Ces classifications sont celles le plus couramment employées dans le domaine scientifique. (Adaptée de Bongso et Lee, 2005)

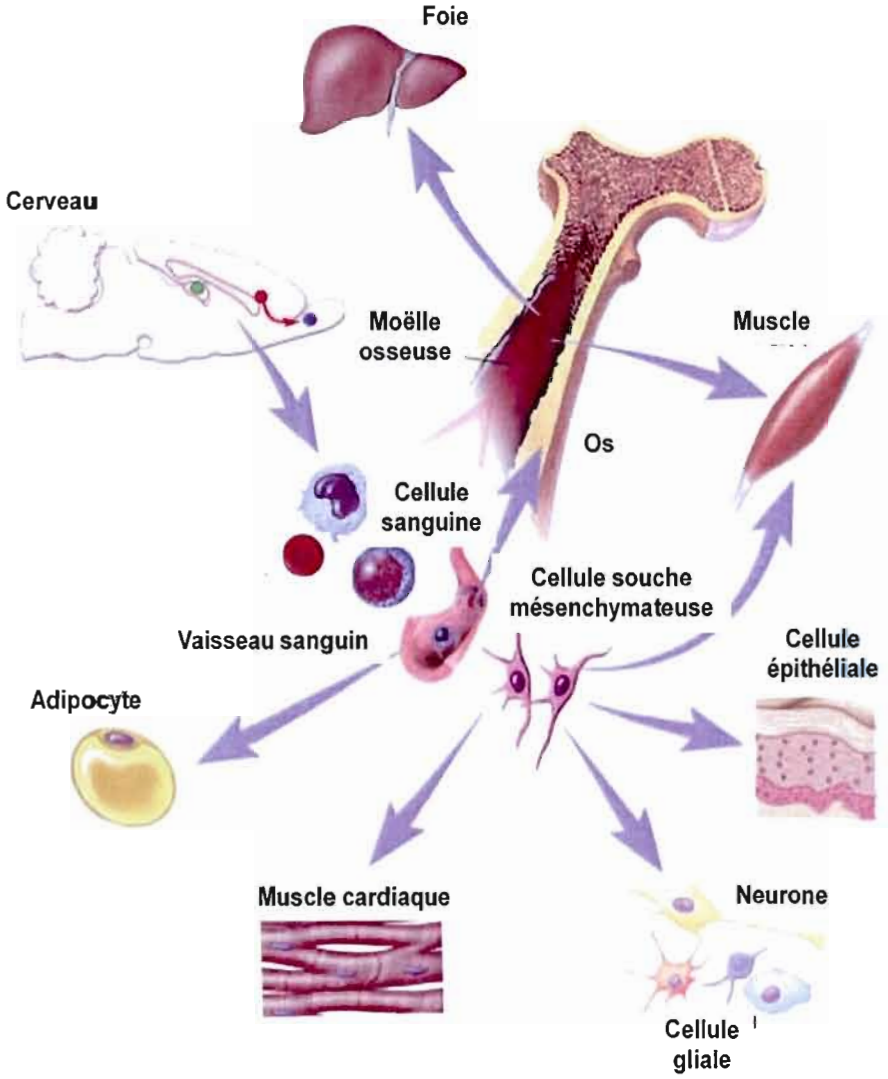

Figure 2 : *Développement des cellules souches issues de la moelle osseuse.* Les cellules souches issues de la moelle osseuse peuvent contribuer au développement des os, des adipocytes et des muscles (cellules souches mésenchymateuses) ou encore au processus d'hématopoïèse (cellules souches hématopoïétiques).

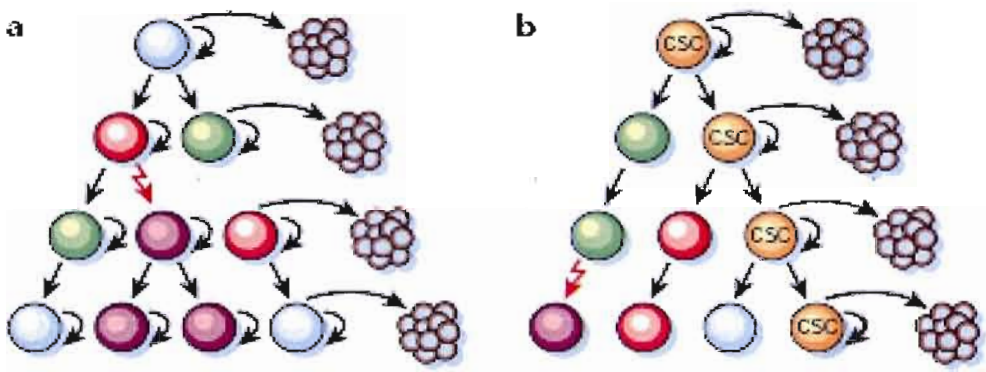

Figure 3: *Modèles du développement tumoral.* A. Modèle aléatoire. Chaque cellule a un faible potentiel prolifératif et n'importe laquelle peut subir des mutations qui la rendra cancéreuse. B. Modèle « Cellules Souches Cancéreuses » (CSC). Les cellules souches cancéreuses ont un potentiel prolifératif plus élevé que les cellules différenciées. Lorsqu'elles subissent des mutations, elles peuvent donner naissance à un foyer tumoral et produire des « filles » cancéreuses. (Adaptée de Reya et al., 2001)

Tableau I : *Liste des métalloprotéases*

Groupe	Membres	Nomenclature	Principaux substrats
Collagénases	Collagénase interstitielle	MMP-1	Collagène fibrillaire
	Collagénase des neutrophiles	MMP-8	Collagène fibrillaire
	Collagénase-3	MMP-13	Collagène fibrillaire
	Collagénase-4	MMP-21	Collagène fibrillaire
Gélatinases	Gélatinase-A	MMP-2	Gélatine, fibronectine, collagène IV, V
	Gélatinase-B	MMP-9	Gélatine, fibronectine, collagène IV, V
MT-MMP	MT1-MMP	MMP-14	ProMMP-2, collagène, gélatine
	MT2-MMP	MMP-15	ProMMP-2, collagène, gélatine
	MT3-MMP	MMP-16	ProMMP-2, collagène, gélatine
	MT4-MMP	MMP-17	ProMMP-2, collagène, gélatine
	MT5-MMP	MMP-24	ProMMP-2, collagène, gélatine
	MT6-MMP	MMP-25	ProMMP-2, gélatine, collagène
Autres	Métalloélastase	MMP-12	Élastine
	Énamélysine	MMP-20	Inconnu
	MMP-xénopus	MMP-18	Inconnu
	Inconnu	MMP-19	Aggrecan
	Inconnu	MMP-23	Inconnu

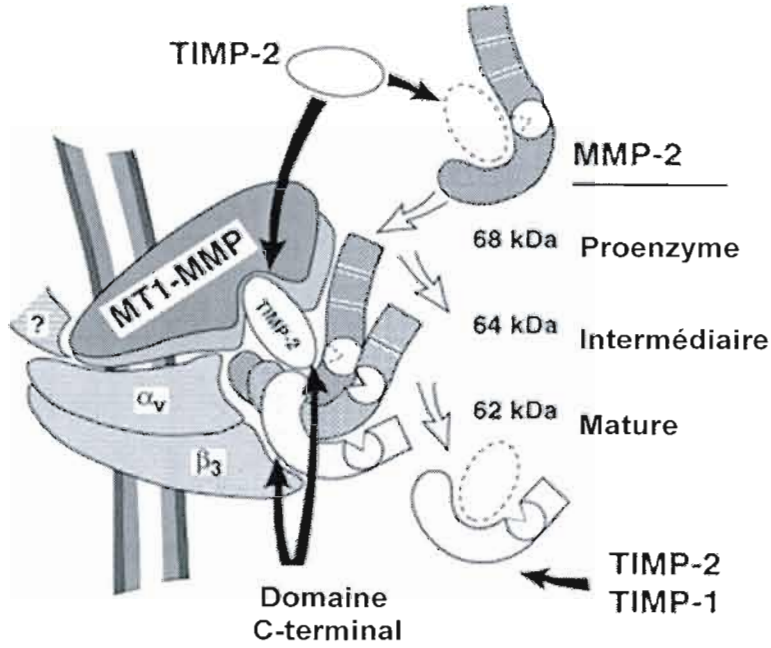

Figure 4 : *Processus d'activation de la proMMP-2*. La proMMP-2 est sécrétée sous forme de zymogène. Un complexe est formé entre MT1-MMP, TIMP-2, l'intégrine αvβ3 et la proMMP-2. Une autre MT1-MMP libre viendra cliver le propeptide produisant ainsi la forme intermédiaire de la MMP-2, qui sera ensuite clivée une seconde fois et devenir active. (Adaptée de Strongin et al., 1995)

Tableau II: *Transporteurs de glucose du type diffusion facilitée*

Famille GLUT	Sites principaux d'expression
GLUT1	Érythrocytes, reins
GLUT2	Foie, intestin grêle, pancréas
GLUT3	Neurones, placenta
GLUT4	Tissu adipeux, muscles
GLUT5	Intestin grêle
GLUT6	Pseudogène

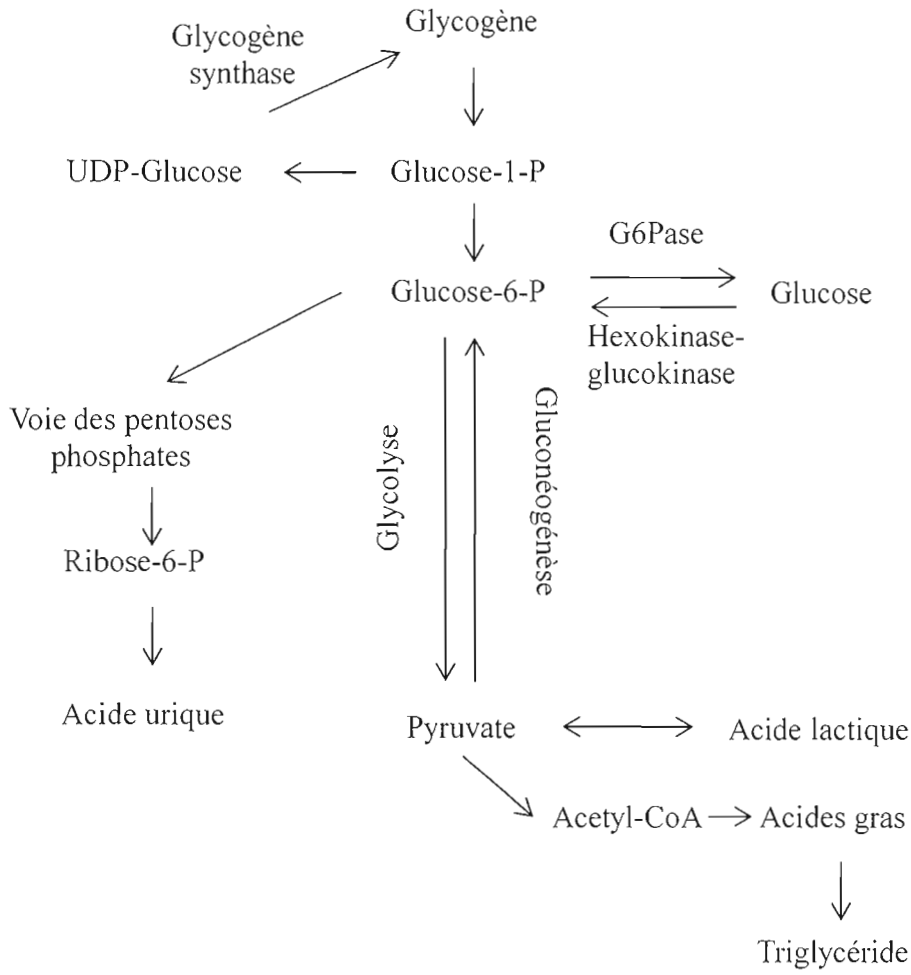

Figure 5 : *Métabolisme du glucose*. Le glucose peut être phosphorylé par une glucokinase (hexokinase) pour produire le glucose-6-phosphate. Par la suite, ce composé peut être stocké sous forme de glycogène ou encore, via la glycolyse, être transformé en pyruvate afin de produire de l'énergie sous forme d'ATP.

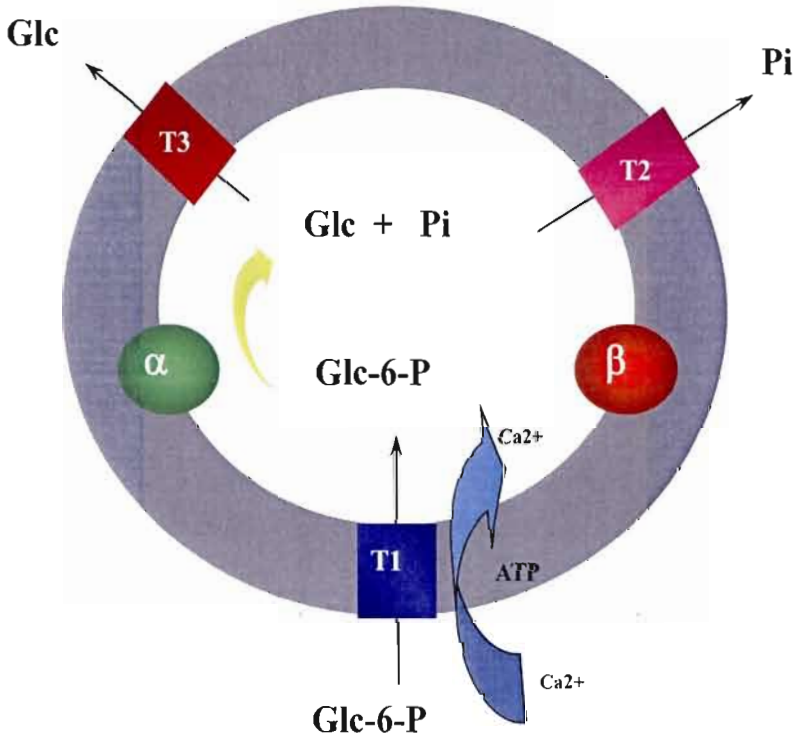

Figure 6 : *Le système glucose-6-phosphatase.* Le glucose-6-phosphate (G6P) est transporté dans la lumière du réticulum endoplasmique via un transporteur (G6PT, T1), avant d'être hydrolysé par la forme α ou β de la glucose-6-phosphatase en phosphate inorganique (Pi) et en glucose (Glc). Ces composés seront par la suite acheminés vers la circulation sanguine via leur transporteur respectif, soient T2 et T3.

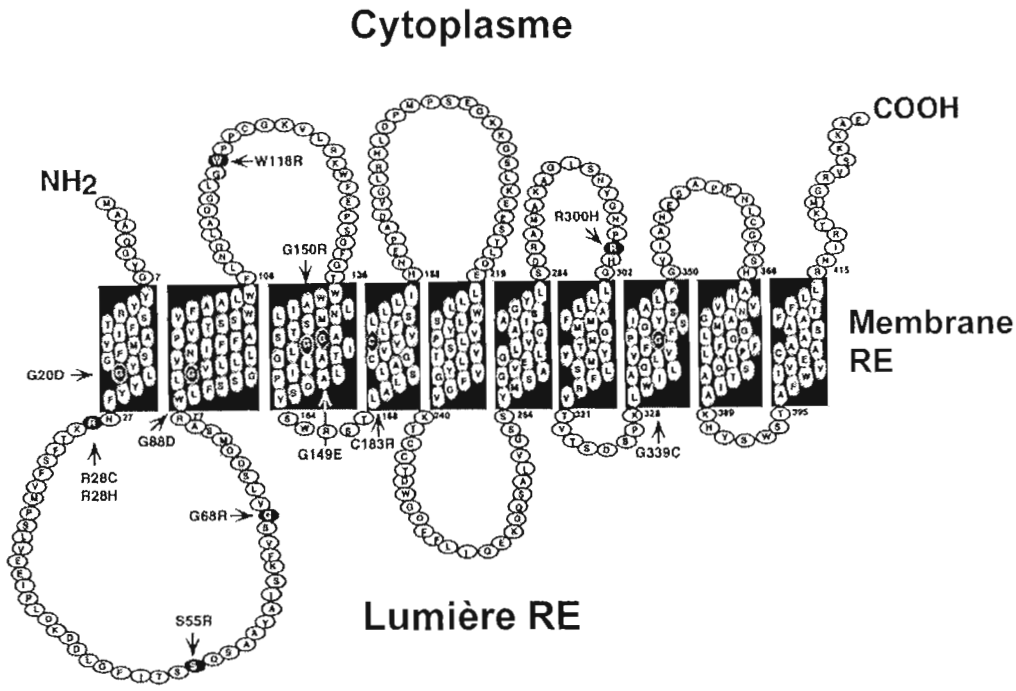

Figure 7 : *Structure moléculaire de G6PT.* Représentation schématique du G6PT, comprenant 9 boucles extra-membranaires et 10 domaines transmembranaires. À noter que les extrémités N- et C-terminales se trouvent à l'extérieur du réticulum endoplasmique. Les flèches pointent différentes mutations répertoriées pour inactiver les fonctions de G6PT. (Adapté de Pan et al., 1999)

CHAPITRE II

Projet de recherche

2.1 Hypothèse de travail

La présence ubiquitaire de G6PT dans des tissus non néoglucogéniques est reconnue, quoique son rôle le soit moins. Nous avons déjà montré que cette protéine avait un rôle dans la chimiotaxie et la survie cellulaire (Belkaid et al., 2006). De plus, nous avons remarqué que la modulation de l'expression de MT1-MMP provoquait des variations sur celle de G6PT (Belkaid et al., 2007). Nous avons donc tenté de mettre à jour les événements de signalisation ayant cours lors de processus impliquant ces deux protéines, en répondant principalement à ces trois questions :

1. Y a-t-il un lien moléculaire entre l'expression de la métalloprotéase MT1-MMP et celle de G6PT, une protéine impliquée dans le transport intra-microsomal du glucose-6-phosphate, dans la chimiotaxie et la survie des cellules souches mésenchymateuses?

2. Peut-on mettre au point des molécules capables d'inhiber les fonctions de signalisation de MT1-MMP chez les cellules souches?

3. L'axe MT1-MMP/G6PT est-il nécessaire à la régulation normale de l'homéostasie calcique chez les cellules de glioblastomes?

2.2 Un axe de signalisation MT1-MMP/G6PT régule la chimiotaxie et la survie des cellules souches mésenchymateuses

La première partie de notre étude avait pour but d'évaluer de la contribution possible de G6PT et MT1-MMP dans le processus de migration cellulaire, en plus de documenter l'importance de la portion cytoplasmique de MT1-MMP dans le processus de signalisation régulant la mort cellulaire.

La chimiotaxie des cellules souches mésenchymateuse est d'une importance capitale puisqu'on sait maintenant que ces cellules pourraient être recrutées et contribuer au développement tumoral. De plus, il a été relevé que la présence de G6PT dans des cellules non-productrices de glucose pourrait avoir un rôle à jouer dans le processus de migration de même que la survie de la population cellulaire. Cette étude permettrait donc de jeter les bases moléculaires liant possiblement la métalloprotéase MT1-MMP, déjà connue comme étant fortement impliquée dans la dégradation de la MEC, et G6PT. Nos résultats montrent clairement que la présence de G6PT influe sur la migration des cellules souches mésenchymateuses en réponse à la sphingosine-1-phosphate (S1P), un biolipide actif retrouvé au niveau tumoral. De plus, nous avons démontré que le niveau d'expression de G6PT dépendait de celui de MT1-MMP. Les résultats obtenus sont présentés sous forme d'article publié dans *Journal of Biological Chemistry* (section expérimentale)

2.3 Construction de mannosides permettant d'inhiber les fonctions de MT1-MMP

La Concanavaline-A (ConA) est une lectine capable de lier des glycoprotéines exprimées à la surface cellulaire. Les lectines sont elles-mêmes des glycoprotéines qui ont une affinité pour les groupements « oses » d'autres protéines glycosylées. Les voies de signalisations activées en présence de la ConA sont peu connues, mais on sait que cette dernière induit l'expression de MT1-MMP (Gervasi et al., 1996). Nous avons démontré que cette métalloprotéase jouait un rôle important, conjointement avec G6PT, dans le processus de migration et de survie cellulaire. Le « design » de glycomimétiques pouvant lier la ConA et en réduire ses effets sur l'expression de MT1-MMP pourrait permettre ultimement la mise au point de nouvelles thérapies. Les résultats de ces études ont permis d'évaluer les meilleurs partenaires d'interactions avec la ConA, en plus de déterminer leurs effets sur les fonctions de signalisation impliquant MT1-MMP. Ces recherches sont présentées sous forme d'article publié dans *Glycobiology* (section expérimentale).

2.4 Rôle de l'axe moléculaire MT1-MMP/G6PT dans la mobilisation du calcium intracellulaire

Il a déjà été relevé que G6PT régulait aussi la séquestration du calcium à l'intérieur du réticulum endoplasmique. Sachant que la sphingosine-1-phosphate induit le flux calcique via la fixation à son récepteur S1PR, et que ce dernier est un puissant chemo-attractant régulant la migration cellulaire des cellules tumorales cérébrales gliales (glioblastomes U-87), nous avons voulu évaluer l'impact de modulations à l'axe MT1-MMP/G6PT sur l'homéostasie calcique. Ces travaux ont permis de démontrer que des modulations aux niveaux d'expressions de MT1-MMP ou G6PT modulaient la réponse calcique à la S1P, en plus de démontrer une fonction potentielle des acteurs de cet axe de signalisation. Les résultats obtenus sont présentés sous forme d'article publié dans *FEBS Letters* (section expérimentale).

CHAPITRE III

Articles

3.1

MT1-MMP down-regulates the glucose 6-phosphate transporter expression in marrow stromal cells: a molecular link between pro-MMP-2 activation, chemotaxis, and cell survival

From

Currie, J. C., Fortier, S., Sina, A., Galipeau, J., Cao, J. and Annabi, B. (2007). MT1-MMP down-regulates the glucose 6-phosphate transporter expression in marrow stromal cells: a molecular link between pro-MMP-2 activation, chemotaxis, and cell survival. Journal of Biological Chemistry 282(11): 8142-9

MT1-MMP down-regulates the glucose 6-phosphate transporter expression in marrow stromal cells: a molecular link between pro-MMP-2 activation, chemotaxis, and cell survival

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Running title : MT1-MMP/G6PT signalling axis in marrow stromal cells

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Simon Fortier a participé aux expériementations (figures 3, 5 et 6), à la rédaction et à l'interprétation des résultats. Jean-Christophe Currie a réalisé et organisé la majorité des expérimentations, rédigé l'article et analysé les résultats. Asmaa Sina a participé à certaines expériementations. Jacques Galipeau a fourni les MSC. Jian Cao a fourni les cellules EGR-1 mutantes, et Borhane Annabi est le directeur du laboratoire.

ABSTRACT

Bone marrow-derived stromal cells (BMSC) are avidly recruited by experimental vascularizing tumors, which implies that they must respond to tumor-derived growth factor cues. In fact, BMSC chemotaxis and cell survival are regulated, in part, by the membrane type-1 matrix metalloproteinase (MT1-MMP), a MMP also involved in proMMP-2 activation and in the degradation of the extracellular matrix (ECM). Given that impaired chemotaxis was recently observed in bone marrow cells isolated from a glucose-6-phosphate transporter deficient ($G6PT^{-/-}$) mouse model, we sought to investigate the potential MT1-MMP/G6PT signaling axis in BMSC. We show that MT1-MMP-mediated activation of proMMP-2 by concanavalin-A (ConA) correlated with an increase in subG1 cell cycle phase as well as of cell necrosis indicative of a decrease in BMSC survival. MT1-MMP gene silencing with small interfering RNA (siMT1-MMP) antagonized both the ConA-mediated activation of proMMP-2 and the induction of cell necrosis. Overexpression of the recombinant full-length MT1-MMP also triggered necrosis, and this was signaled through MT1-MMP's cytoplasmic domain. ConA inhibited both the gene and protein expression of G6PT, while overexpression of the recombinant G6PT inhibited MT1-MMP-mediated proMMP-2 activation but could not rescue BMSC from ConA-induced cell necrosis. Cell chemotaxis in response to the tumorigenic growth factor sphingosine-1-phosphate was significantly abrogated in siMT1-MMP and in siG6PT BMSC. Altogether, we provide evidence for an MT1-MMP/G6PT signaling axis that regulates BMSC survival and ECM degradation. This may lead to optimized control of cell mobilization in clinical applications that use BMSC as a platform for the systemic delivery of therapeutic recombinant proteins *in vivo*.

INTRODUCTION

Our recent increased comprehension of stem cells mobilization, cell-matrix interaction, and biodistribution has enabled the development of new therapeutic strategies (1, 2). While locally transplanted bone marrow-derived stromal cells (BMSC) have already been used clinically to treat osteogenesis imperfecta (3), neurological diseases (4), and for cellular cardiomyoplasty (5), less invasive routes of BMSC transplantation have been the focus of recent attention (6-8). In fact, several clinical applications now use intravenous administration of genetically-engineered BMSC either as an ideal vehicle for gene transfer (6), or as a platform for the systemic delivery of therapeutic recombinant proteins *in vivo* (9, 10). This implies that these circulating systemically infused cells have to respond to serum-derived cues that will direct their ultimate biodistribution. The molecular players regulating the cellular mobilization and chemotaxis of BMSC in response to physiological hematopoietic or to pathological tumor-derived growth factors have, however, received little attention.

Among the mediators known to exert potent cellular chemotactic effects, sphingosine-1-phosphate (S1P) is one of the most important bioactive lysophospholipid secreted in blood plasma either upon platelet activation (11) or from brain tumor-derived glioma cells (12). In fact, we have demonstrated that BMSC chemotaxis was very strong in response to S1P (13) and required reorganization of the actin cytoskeleton and remodelling of the extracellular matrix (ECM) through a complex cooperative signal transduction network involving cell surface matrix metalloproteinase (MMP) activity (14). So far, the molecular characterization and the nature of that MMP, regulating both BMSC chemotaxis and interaction with the ECM protein microenvironment, remain poorly understood. Recently, we highlighted a functional cross-talk between the membrane type-1 MMP (MT1-MMP) and the S1P receptor EDG-1-mediated signaling in BMSC chemotaxis (13). Interestingly, besides its classical role in ECM proteolysis, MT1-MMP is also involved in transducing crucial intracellular signalling that may control several processes related to BMSC mobilisation and cell survival (13-16).

Given that impaired chemotaxis was recently observed in bone marrow cells isolated from a microsomal glucose-6-phosphate transporter deficient ($G6PT^{-/-}$) mouse model (17, 18), and that G6PT was demonstrated to regulate cell migration (19), we hypothesized that MT1-MMP and G6PT may share some common molecular and cellular impact on the regulation of BMSC chemotaxis and ECM degradation. Since its discovery, G6PT has been shown to integrate and regulate many metabolic functions such as glycemia, lipidemia, uricemia, and lactic acidemia (20). More importantly, its activity cannot be substituted as G6PT deficiencies lead to glycogen storage disease (GSD) type Ib characterized by, not only disturbed glucose homeostatsis, but with severe myeloid dysfunctions (20). Lack of G6PT functions alters, for instance, neutrophil chemotaxis and calcium flux (21), and its functional or expression inhibition decreases cell survival (19, 22, 23).

Recent evidence has divulgated new functions for MT1-MMP in modulating cell survival through yet unidentified intracellular processes (13, 24). Since we have shown that cell surface MMP signalling was essential in S1P-induced chemotaxis of BMSC (14), that G6PT overexpression triggered cell migration, and that its functional inhibition with chlorogenic acid antagonized S1P-induced ERK (Extracellular-signal-Regulated protein Kinase) phosphorylation and chemotactic response (19), we decided to investigate the potential molecular and functional link that may exist between MT1-MMP and G6PT functions.

EXPERIMENTAL PROCEDURES

Materials. Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibody against MT1-MMP was from Chemicon

(Temecula, CA), while the polyclonal antibody against G6PT was generated against the 5-GYGYYRTVIFSAMFGGY-21 peptide derived from the human G6PT primary sequence (accession # AAD19898) (25) at the Biotechnology Research Institute (Montreal, QC). All other reagents were from Sigma-Aldrich Canada.

Cell culture : Bone marrow-derived stromal cells (BMSC) were isolated from mouse whole femur and tibia bone marrow, cultured and characterized as previously described (14). Analysis by flow cytometry performed at passage 14 revealed that BMSC expressed CD44, yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3), and Tie2 (angiopoietin receptor) (data not shown).

Chemotactic cell migration assay : Mock or BMSC transfected with the siMT1-MMP or siG6PT were trypsinized and seeded at 10^5 cells on 0.15% gelatin/PBS precoated Transwells (Corning/Costar; Acton, MA; 8-μm pore size) assembled in 24-well Boyden chambers which were filled with 600 μl of DMEM supplemented or not with 1 μM S1P, or of a growth factors-enriched media isolated from serum-starved U87 glioblastoma cells in the lower compartment. Cell migration was allowed to proceed for 6 hours at 37°C in 5% CO_2. Non-migrating cells that remained on the upper side of the Transwell filter were carefully removed with cotton swabs. Cells that had migrated to the lower side of the filters were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet/20% MeOH and counted. The migration was quantified by analyzing at least ten random fields per filter for each independent experiment using computer-assisted imaging software Northern Eclipse 6.0 (Empix Imaging Inc., Mississauga, ON).

RNA interference : RNA interference experiments were performed using Lipofectamine 2000. A small interfering RNA against MT1-MMP (siMT1-MMP) and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield, IN), and annealed to form duplexes. The sequence of the siMT1-MMP used in this study is as follows : 5'-CCAGAAGCUGAAGGUAGAAdTdT-3' (sense) and 5'-UUCUACCUUCAGCUUCUGGdTdT-3' (antisense) (26). Knockdown of MT1-MMP expression, as assessed by RT-PCR, ranged routinely from 65-90%.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis : Total RNA was extracted from cultured BMSC 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' sequences for MT1-MMP, G6PT, G6Pase-α, and GAPDH have been validated and published (19). PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification and the products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Gelatin zymography : Gelatin zymography was used to assess the extent of proMMP-2 activation in the culture medium as previously described (19). Gelatinolytic activity was detected as unstained bands on a blue background.

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 for 1 hr at room temperature 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 (1/2,500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

Analysis of cell cycle by flow cytometry: Distribution of BMSC throughout the cell cycle was assessed by flow cytometry (27). Serum-fasting preparation was performed prior to analysis and therefore the cell populations were synchronous. Cells were harvested by gentle scraping, pelleted by centrifugation, washed with ice-cold PBS/EDTA (5 mM), then resuspended in 1 volume PBS/EDTA and fixed with 100% ethanol overnight. Three volumes of staining solution, containing propidium iodide (PI, 50 μg/ml) (Sigma) and DNAse-free RNAse (20 μg/ml), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a Becton Dickinson FACS Calibur

flow cytometer equipped with CellQuest Pro software. In particular, the characteristics of cell distribution in the subG1 region were studied on the DNA histogram.

Analysis of apoptosis by flow cytometry: Cell death was assessed as follows. Cells floating in the supernatant and adherent cells harvested by trypsin solution were gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with PBS. Then, $2x10^5$ cells were pelleted and suspended in 200 µL of buffer solution and stained with annexin V-fluorescein isothiocyanate (FITC) and PI as described by the manufacturer (BD Bioscience). The cells were diluted by adding 300 µL of buffer solution and processed for data acquisition and analysis on a Becton Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The X- and Y-axes indicated the fluorescence of annexin-V and PI respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is translocated to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the die is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V^+/PI^-). Cells in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+).

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 Ilomastat 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

MT1-MMP-mediated activation of latent proMMP-2 by concanavalin-A correlates with a decrease in G0/G1 and an increase in subG1 cell cycle phases. Concanavalin-A (ConA) is thought to promote MT1-MMP trafficking from a storage pool to the cell surface (28) and is, thus, routinely used to assess the MT1-MMP-mediated processes involved in proMMP-2 activation (13, 16). A dose-response to ConA was performed in BMSC and proMMP-2 activation assessed by zymography. We show that latent proMMP-2 was increasingly processed to its active MMP-2 form (Fig.1A), and that, concomitantly, MT1-MMP proteolytic processing to its inactive 43 kDa form was also induced (Fig.1B). Because proMMP-2 activation is thought to potentially interfere with cell survival and proliferation (29, 30), we also sought to investigate the effect of ConA on BMSC cell cycle division processes. Cells were treated with increasing doses of ConA, staining performed with propidium iodide (PI), and flow cytometry was used to assess the percent of cells found in G0/G1, S, G2/M, and subG1 cell cycle phases (Fig.1C). We show that ConA induced a dose-dependent decrease in G0/G1 that was concomitantly observed with and increase in subG1 (Fig.1D). While S and G2/M cell cycle phases remained unaffected, these observations suggest that ConA triggers some cell death process.

Ilomastat inhibits concanavalin-A-induced proMMP-2 activation and cell necrosis. In light of our previous observation that ConA potentially decreased BMSC survival, we next assessed whether cell apoptosis or necrosis was involved. First, cells were treated or not with ConA and in the presence or not of Ilomastat, a broad range MMP inhibitor. Gelatin zymography shows that Ilomastat was efficiently able to inhibit both proMMP-2 activation and MT1-MMP proteolytic activation by ConA (Fig.2A). When cell death was assessed through Annexin V/PI staining upon increasing concentration of ConA treatment, we observed that only cell necrosis (Fig.2C, open circles) was induced while cell apoptosis was unaffected (Fig.2C, open triangles). Interestingly, only partial inhibition of approximately 65% by Ilomastat was observed when cells were treated with both conA and Ilomastat (Fig.2C, closed circles) while it did not affect cell apoptosis (Fig.2C, closed triangles). This differential inhibitory efficacy of Ilomastat on proMMP-2 activation and against cell necrosis

suggests that inhibiting the catalytic function of cell surface MT1-MMP is not sufficient to completely inhibit ConA-induced cell necrosis.

Gene silencing of MT1-MMP diminishes concanavalin-A ability to induce cell necrosis. To further investigate the role of MT1-MMP in ConA-mediated activation of proMMP-2 and cell necrosis, we downregulated MT1-MMP gene expression using small interfering RNA (siRNA). When Mock or siMT1-MMP transfected BMSC were treated with ConA, we observed a significant reduction in proMMP-2 activation (Fig.3A). Moreover, when these same cells were assessed for ConA-induced cell death, ConA was unable to trigger cell necrosis in siMT1-MMP transfected cells (Fig.3B). Next, total RNA was isolated from the same conditions and assessed for the glucose-6-phosphate transporter (G6PT) and MT1-MMP gene expression. We observed that ConA, as expected, induced MT1-MMP gene expression in Mock and still was able to induce the remaining low endogenous MT1-MMP gene expression in siMT1-MMP-transfected cells (Fig.3C). However, while ConA downregulated G6PT gene expression, it was found ineffective in those BMSC transfected with siMT1-MMP (Fig.3D). These results suggest that specific domains, independent from the catalytic domain of MT1-MMP, are required to signal cell necrosis in BMSC, and that MT1-MMP is required in the regulation of the G6PT gene expression.

Induction of cell death by MT1-MMP requires an intact intracellular domain. In order to test the hypothesis that alternate domains in MT1-MMP were involved in potentially signalling cell death in BMSC, we transiently transfected cells with the cDNAs encoding the recombinant forms of a GFP-fused full-length wild-type (Wt) MT1-MMP as well as of a GFP-fused cytoplasmic-truncated MT1-MMP (Δ-Cyto) (31). Overexpression of both of the recombinant forms of MT1-MMP was confirmed through fluorescent visualization (Fig.4A). Interestingly, when annexin-V/propidium iodide (PI) staining of the cells was performed, a very significant (~8.5-fold increase) proportion of the cells overexpressing the Wt MT1-MMP shifted into necrosis, while overexpression of the Δ-Cyto MT1-MMP only partially (~3-fold increase) triggered cells into necrosis (Fig.4B). Functionality of the membrane bound recombinant MT1-MMP forms was also confirmed by their capacity to activate latent proMMP-2 into MMP-2 by gelatine zymography, and such catalytic function was inhibited by Ilomastat (Fig.4C). Noteworthy, when cell necrosis was assessed in the MT1-MMP

transfected cells, Ilomastat, as it did in ConA-treated cells, inhibited partially the Wt MT1-MMP-induced cell death and completely antagonized the slight increase in cell necrosis that was due to the overexpression of the Δ-Cyto MT1-MMP. Collectively, we show that the catalytic function of MT1-MMP is partially responsible for the induction of cell death, but that the intracellular domain of MT1-MMP is the most crucial player in signalling intracellular events that diminish cell survival.

Concanavalin-A inhibits G6PT gene and protein expression. In light of the documented prosurvival functions of G6PT (22, 23), and because of the potential common roles that G6PT and MT1-MMP possess in regulating cell chemotaxis, we assessed whether MT1-MMP mediated events also affected G6PT expression. BMSC were treated with concentrations of ConA known to trigger cell necrosis and G6PT protein expression was immunodetected in cell lysates. We show that ConA at 10-30 μg/ml significantly inhibited G6PT expression, while Ilomastat prevented that decrease (Fig.5A) suggesting that G6PT potentially regulated some ConA-induced MT1-MMP-mediated events. In order to further assess the impact of G6PT, conditioned media was harvested from Mock or G6PT-transfected cells that were treated or not with ConA. We observed that ConA induced an MT1-MMP-mediated activation of proMMP-2, while cells overexpressing G6PT completely inhibited proMMP-2 activation (Fig.5B).

MT1-MMP and G6PT gene silencing abrogates BMSC chemotaxis in response to sphingosine-1-phosphate. In order to assess the impact of MT1-MMP and G6PT in BMSC chemotaxis, we specifically downregulated either MT1-MMP or G6PT gene expression and evaluated BMSC response to either S1P or to tumor growth factors-enriched media (U-87 CM). We found that BMSC efficiently responded to S1P or to U-87 CM chemoattraction as demonstrated by their increased migration (Fig.6). However, MT1-MMP and G6PT gene silencing abrogated BMSC response to S1P, while only G6PT but not MT1-MMP silencing abrogated cell migration in response to U-87 CM. Collectively, this shows that MT1-MMP and G6PT are essential for BMSC chemotaxis. The lack of G6PT expression seems to further impact on the effects of the multiple growth factors secreted by brain tumor cells.

DISCUSSION

Understanding of the molecular players involved in BMSC mobilization has led to improved usage of these cells in therapeutic oncology modalities (32, 33). Accordingly, BMSC migration capacity and chemotactic response to circulating tumor-derived growth factors has led to the demonstration that BMSC can very efficiently migrate toward experimental gliomas for instance, making these cells a good candidate for cellular carrier systems of anti-glioma therapy (34, 35). Among the circulating chemotactic mediators, chemokines play key roles in haematopoietic stem cell trafficking (36), while circulating lipid mediators such as S1P have emerged as one of the most potent *in vitro* BMSC chemotactic agent (13, 14). Recent findings, in fact, demonstrated that S1P is a tumorigenic growth factor likely produced by tumor cells themselves that could be targeted by lipidomic-based cancer therapeutics (37). In line with this evidence, an original MT1-MMP/S1P signalling axis has been recently proposed to regulate chemotaxis in BMSC (13, 14) as well as angiogenic functions in endothelial cells (38). Our current study reveals that MT1-MMP functions in ECM degradation and BMSC mobilization also regulate cell survival potentially under the metabolic control of a microsomal G6PT.

Recently, a new and underestimated function for G6PT as a potential regulator of cells chemotaxis was reported (19). Indeed, besides its classical role in regulating the rate limiting step of G6P transport and subsequent hydrolysis by the glucose-6-phosphatase (G6Pase) catalytic subunit into glucose and phosphate, its specific functional inhibition by chlorogenic acid led to a significant decrease in ERK-mediated intracellular signaling (19), potentially through modulation in calcium mobilization. This exciting new role for G6PT in regulating cell mobilization may also impact on events known to regulate cell proliferation, cell cycle division, ECM degradation, and response to growth factors. The latter cellular events have, interestingly, been also shown to involve MT1-MMP functions such as in cell migration (39), and in ERK and RhoA/ROK signalling (14, 15, 40). More recently, we have revealed several of these MT1-MMP roles in regulating the angiogenic and chemotaxis properties of BMSC in response to hypoxia (41) and to brain tumor-derived U-87 glioma cells *in vitro* and *in vivo* (42). In fact, tissue necrosis is a characteristic feature of malignant gliomas, in particular glioblastoma, and is most likely the consequence of rapidly increasing tumor mass that is

inadequately oxygenized by the preexisting vasculature (43). Interestingly, hypoxia was shown to both increase MT1-MMP levels in BMSC (41) and to lead to cell death (44). Whether a role and regulation of G6PT in these conditions also concomitantly occurs is currently under investigation.

Since its discovery, MT1-MMP has been extensively investigated and is now one of the best characterized MMPs. MT1-MMP has been implicated in various physiological and pathological processes such as wound healing, bone development, angiogenesis, inflammation, and cancer invasion and metastasis (45). Because MT1-MMP acts on the cell surface, it modifies the immediate environment of the cell resulting in alteration of signaling from the ECM adhesion molecules (15, 46), or in response to chemokines thereby influencing cellular functions (47). In our study, we show that maintenance of cytoarchitecture is required for cell survival, since its perturbation by cytochalasin-D- or ConA-mediated MT1-MMP mechanisms downregulated cell survival and were associated with proMMP-2 activation (29, 30, our study). In fact, we show that silencing of the MT1-MMP gene prevents ConA from inducing cell death and from activating proMMP-2. Conclusions regarding impaired ECM degrading capacity were also reached when BMSC were isolated from MT1-MMP$^{-/-}$ mice and which failed to respond to IL-1/TNFα stimulation to degrade collagen (48). Moreover, we show that inhibiting the catalytic function of MT1-MMP only partially reverses the lethal effect of MT1-MMP, while the intracellular domain of MT1-MMP is, in contrast, absolutely required in transducing the intracellular signaling that leads to cell death. In fact, a caspase-dependent mechanism has recently been associated to MT1-MMP functions in endothelial cells morphogenic differentiation (24). This suggests that MT1-MMP acts as a potential cell death sensor/effector that signals ECM degradation processes to be activated. The fact that G6PT overexpression inhibited ConA-induced proMMP-2 activation, but not cell death, further suggests that complex differential regulation takes place and confirms the pleiotropic intracellular functions of G6PT.

Interestingly, our data are consistent with some of the abnormal polymorphonuclear neutrophils (PMN) phenotype observed in GSD type 1b, a clinical condition where the G6PT gene and/or protein activity is defective (23, 49). In fact, induction of cell apoptosis (23), and diminution in several processes such as mitochondrial respiration, chemotaxis, phagocytosis and calcium signaling (50-52) was observed when G6PT functions were inhibited with

analogs of chlorogenic acid, the most potent inhibitor of G6PT (53). Chlorogenic acid cellular targeting was recently found to inhibit human hepatocellular carcinoma cell line proliferation (54) and to induce apoptosis of chronic myelogenous leukemia (CML) cell lines and primary cells from CML patients (55). Alterations in several biochemical parameters - glucose phosphorylation, calcium mobilization, and hexose uptake and transport - have also been described as possible mechanisms through which the G6PT functional defects may be involved *in vivo*. More recently, G6PT involvement in chemotaxis was also evidenced in $G6PT^{-/-}$ mice (21). That study demonstrated that neutropenia is caused directly by the loss of G6PT activity, and that chemotaxis and calcium flux were defective in $G6PT^{-/-}$. Since cells such as PMN or BMSC have no detectable G6Pase activity or expression, G6PT must play a role different from that exerted in the liver, for instance, where it is functionally coupled to the G6Pase enzyme. In fact, it has been hypothesized that G6PT might function as a G6P receptor/sensor (49) or that it could favor calcium sequestration in the endoplasmic reticulum lumen (56). A potential mechanism may involve Ca^{2+} influx regulation which is thought to inhibit MT1-MMP proteolytic processing independently from ConA action (57). Since furin enzymes are calcium dependent (58) and have been implicated in MT1-MMP activation, one can further hypothesize that overexpression of G6PT leads to increased sequestration of an intramicrosomal pool of Ca^{2+} that would then not be available for cytosolic enzymes regulation. Moreover, such mechanism may potentially hamper rapid ConA-induced trafficking of MT1-MMP to the plasma membrane, a pre-requisite for cell surface proteolytic activity to occur (28). It thus becomes tempting to suggest that such intracellular metabolic control may, in part, contribute to regulate BMSC chemotaxis and cell mobilization.

In conclusion, our study provides the first molecular link between the ECM degrading functions of MT1-MMP and the microsomal G6PT functions, which would collectively regulate BMSC chemotaxis and cell survival. In line with our current results, high glucose concentration, a condition that is known to upregulate the G6PT expression (59), was found to antagonize the ConA-induced activation of proMMP-2 (60). Altogether, the metabolic control of BMSC response to tumor-derived growth factors will not only increase our understanding of their mobilization in pathological conditions (61), but also contribute in understanding BMSC chemotactic mechanisms involved in tissue regeneration therapeutic modalities (62-65).

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FOOTNOTES

The abbreviations used are : CHL, chlorogenic acid; ECM, extracellular matrix; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P transporter; GSD, glycogen storage disease; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP; PI, propidium iodide; siRNA, small interfering ribonucleic acid; S1P, sphingosine-1-phosphate

Fig.1

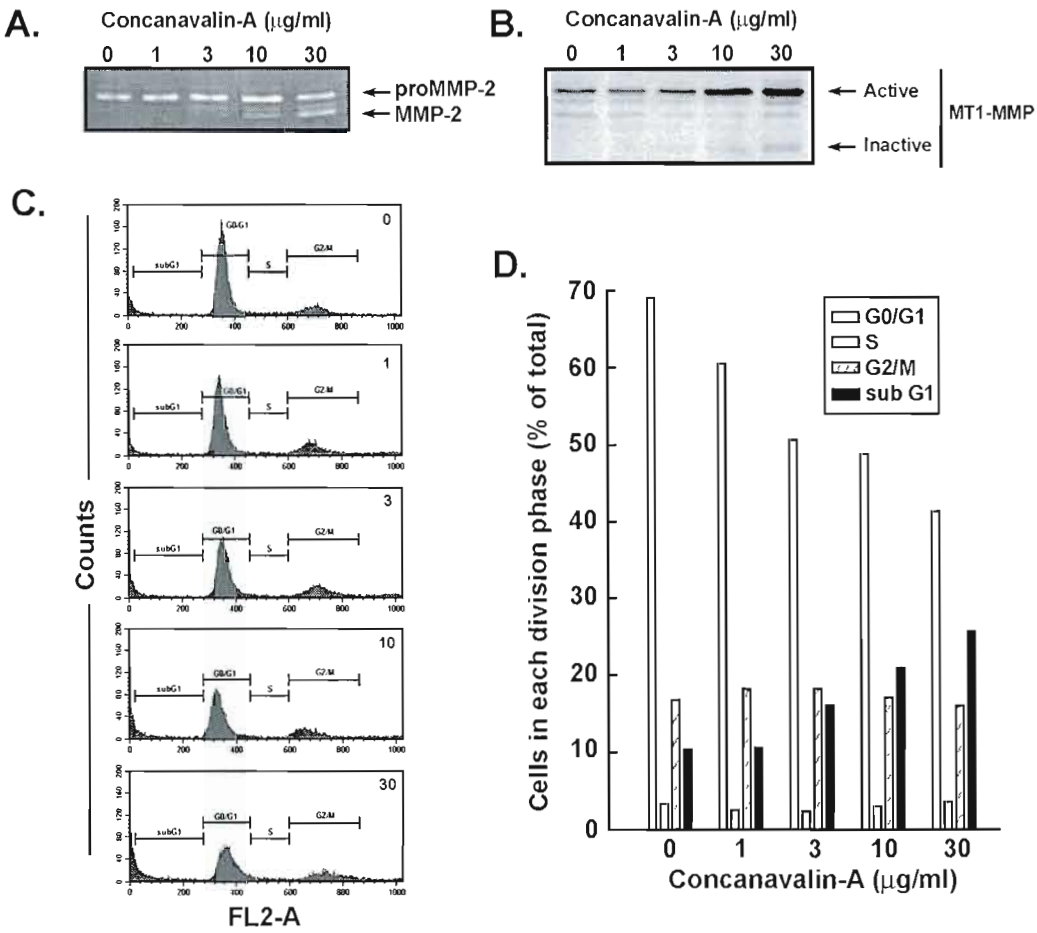

Figure 1 : *MT1-MMP-mediated activation of latent proMMP-2 by concanavalin-A correlates with a decrease in G0/G1 and an increase in subG1 cell cycle phases.* BMSC were serum-starved for 24 hours then treated with increasing concentrations of concanavalin-A for 18 hours. (A) Conditioned media was harvested and 20 µl was used to assess proMMP-2 activation using gelatin zymography. (B) BMSC were treated as above and cell lysates used to perform immunodetection of the MT1-MMP proteolytic form (active ~60 kDa, inactive ~43 kDa). (C) Cells were treated as above and cell cycle was analyzed by flow cytometry as described in the Methods section. (D) A representative experiment of cells present in each division phase was quantified and expressed as percent of total.

Fig.2

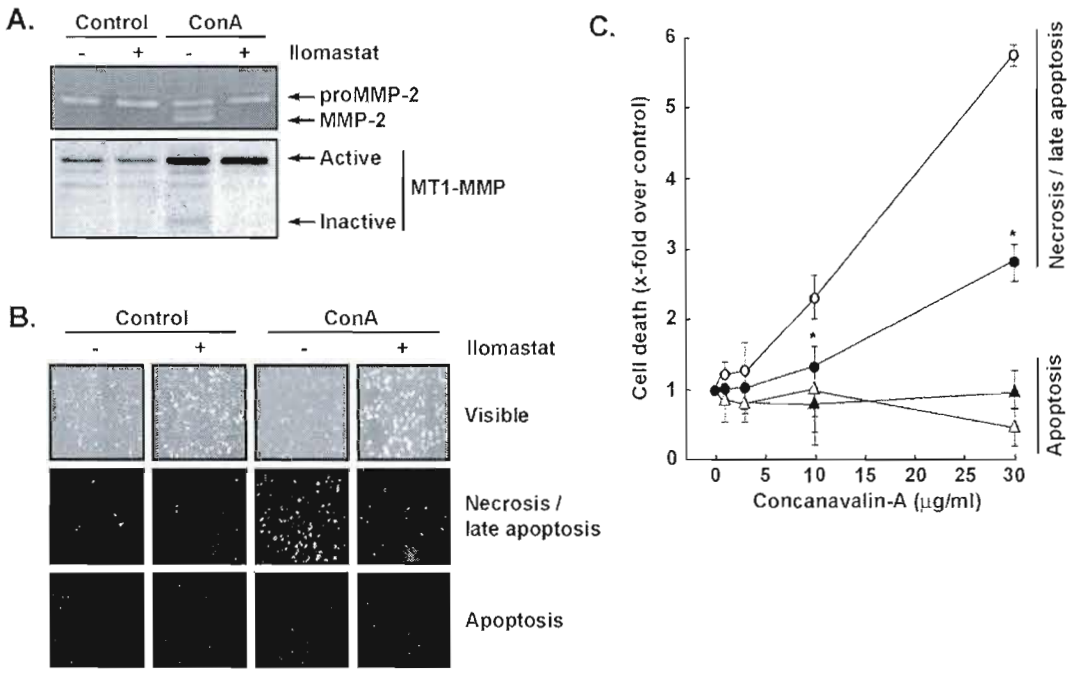

Figure 2 : Ilomastat inhibits concanavalin A-induced pro-MMP-2 activation and cell necrosis. BMSC were serum-starved for 24 h then treated with 30 μg/ml of concanavalin A in the presence or absence of 10 μM Ilomastat for 18 h. *A*, conditioned medium was harvested, and 20 μl was used to assess pro-MMP-2 activation using gelatin zymography (*upper panel*). The corresponding cell lysates were used to immunodetect MT1-MMP proteolytic processing (*lower panel*). *B*, BMSC were cultured and treated as described above. Hoechst (apoptosis) and propidium iodine (necrosis/late apoptosis) staining was then performed and visualized using fluorescence microscopy. *C*, BMSC were treated with increasing concentrations of concanavalin A as in Fig. 1 in the presence (*black symbols*) or absence (*open symbols*) of Ilomastat. Cell apoptosis (*triangles*) and necrosis (*circles*) were analyzed by flow cytometry after double staining with annexin-V and propidium iodine as described under "Experimental Procedures." Probability values of less than 0.05 were considered significant, and an *asterisk* identifies such significance in Ilomastat-treated cells against untreated control.

Fig.3

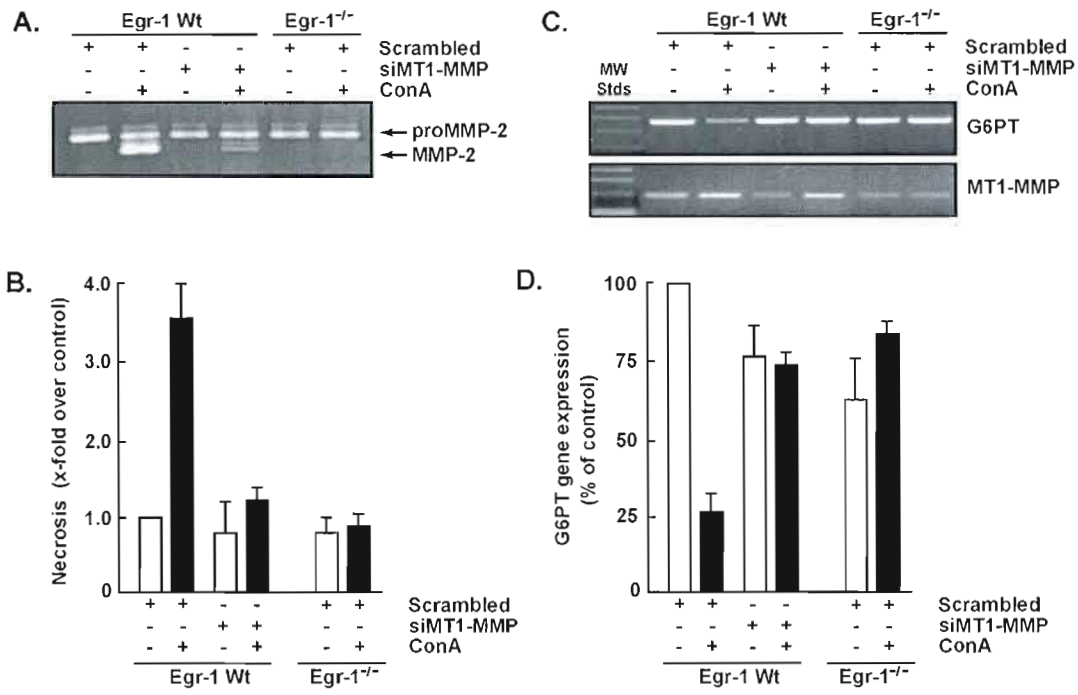

Figure 3 : *Gene silencing of MT1-MMP diminishes concanavalin-A ability to induce cell necrosis*. BMSC (*Egr-1 Wt*) transfected with either scrambled mismatched siRNA oligonucleotids (*Scrambled*) or with the siRNA against MT1-MMP (*siMT1-MMP*) or BMSC isolated from Egr-$1^{-/-}$ mouse were serum-starved in the presence or absence of 30 µg/ml concanavalin A. *A*, conditioned medium was harvested, and 20 µl was used to assess pro-MMP-2 activation using gelatin zymography. *B*, cell death was assessed in BMSC treated as above and then stained with annexin-V and propidium iodine as described under "Experimental Procedures." *C*, total RNA was isolated and RT-PCR used to assess the gene expression levels of G6PT and MT1-MMP. *D*, semiquantitative evaluation of G6PT gene expression was performed using scanning densitometry and values expressed as percent of untreated mock-transfected cells.

Fig.4

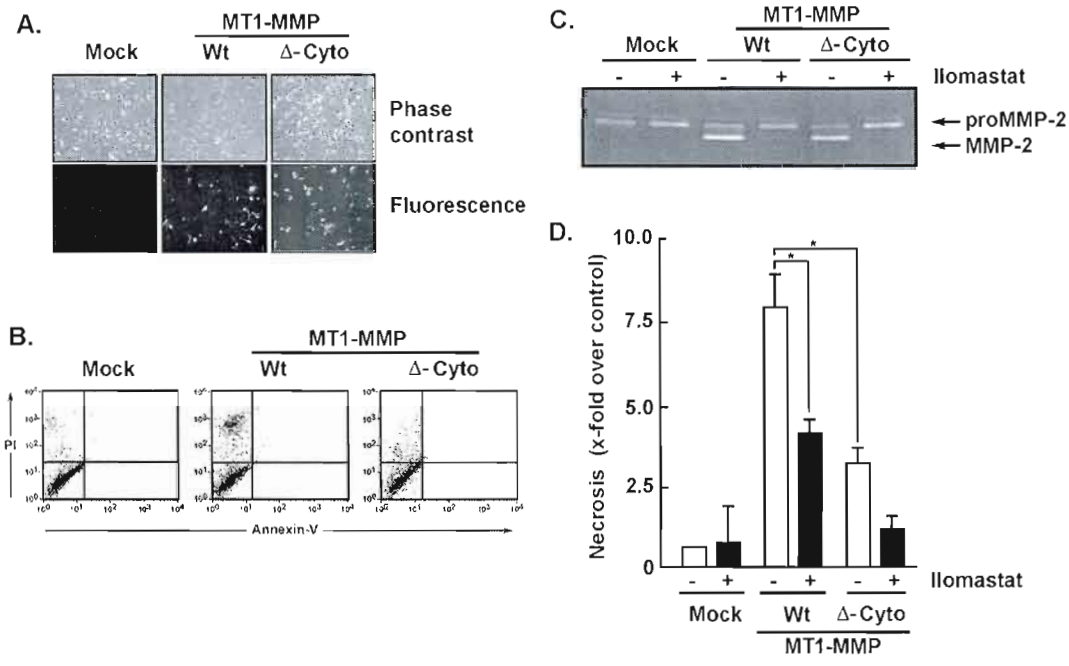

Figure 4: *Induction of necrosis by MT1-MMP requires intracellular signalling through its intact intracellular domain.* BMSC were transiently transfected with empty plasmid cDNA or cDNA encoding the full-length (*Wt*) or cytoplasmic truncated (Δ-*Cyto*) GFP-fused MT1-MMP. *A*, representative fluoromicrographs showing transfection efficiency and expression of the GFP-MT1-MMP recombinant proteins. *C*, because of the staining with annexin-V-fluorescein isothiocyanate, BMSC were transfected with plasmids cDNA encoding the non-GFP recombinant Wt and Δ-Cyto MT1-MMP. BMSC were then serum-starved for 18 h in the presence or absence of 10 μM Ilomastat and conditioned medium harvested to assess pro-MMP-2 activation using gelatin zymography. *B* and *D*, cell death was then assessed in transfected BMSC and quantified in the presence (*black bars*) or absence (*white bars*) (D) of Ilomastat through staining with annexin-V and propidium iodine as described under "Experimental Procedures."

Fig.5

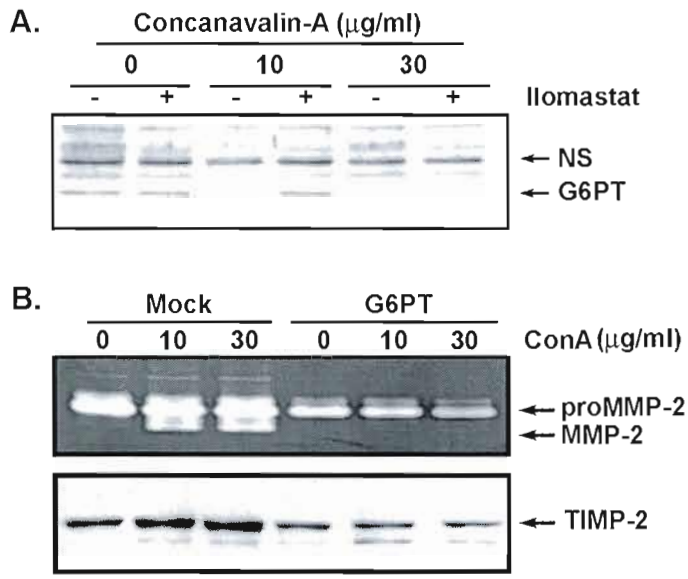

Figure 5 : *Concanavalin-A inhibits G6PT gene and protein expression.* BMSC were treated as in Fig.1 in the presence of increasing doses of concanavalin-A in the presence or not of 10 μM Ilomastat. (A) Cell lysates were used to immunodetect the microsomal glucose-6-phosphate transporter (G6PT) at ~43 kDa (NS : non-specific immunoreactive protein). (B) BMSC were treated as above in the presence or not of 10 μM Ilomastat, and conditioned media harvested to assess proMMP-2 activation using gelatin zymography.

Fig.6

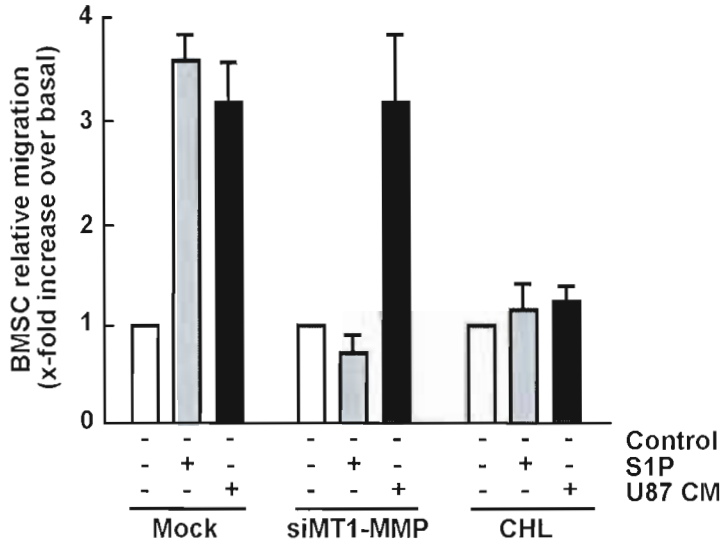

Figure 6 : *MT1-MMP and G6PT gene silencing abrogates BMSC chemotaxis in response to sphingosine-1-phosphate*. BMSC were transfected with scrambled mismatched siRNA oligonucleotids (*Mock*) or with siMT1-MMP or treated with 100 μM chlorogenic acid, then seeded (20,000 cells/filter) on top of gelatin-coated modified Boyden chambers and left to migrate for 18 h in response to brain tumor-derived growth factors from U-87 glioma cells (*U-87 CM*, *black bars*) or for 48 h in response to 1 μM S1P (*gray bars*).

3.2

Tetra- and hexavalent mannosides inhibit the pro-apoptotic, antiproliferative and cell surface clustering effects of concanavalin-A: Impact on MT1-MMP functions in marrow-derived mesenchymal stromal cells

From

Fortier, S., Touaibia, M., Lord-Dufour, S., Galipeau, J., Roy, R. and Annabi, B. (2008). Tetra- and hexavalent mannosides inhibit the pro-apoptotic, antiproliferative and cell surface clustering effects of concanavalin-A: impact on MT1-MMP functions in marrow-derived mesenchymal stromal cells. Glycobiology 18(2): 195-204

Tetra- and hexavalent mannosides inhibit the pro-apoptotic, anti-proliferative and cell surface clustering effects of concanavalin-A : Impact on MT1-MMP functions in marrow-derived mesenchymal stromal cells

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Running title : Mannosides antagonize MT1-MMP functions

Key words : Mesenchymal stromal cells / Concanavalin-A / Mannosides / MT1-MMP / Cancer

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Simon Fortier a réalisé la totalité des expériences biologiques, analysé les résultats et participé à la rédaction de l'article. Simon Lord-Dufour a été stagiaire avec Simon Fortier durant l'élaboration de certaines expériences. Mohamed Touaibia a synthétisé les molécules et analysé leur affinité par densité optique (figure 1). Jacques Galipeau a fourni les MSC. René Roy et Borhane Annabi sont les directeurs des laboratoires impliqués

ABSTRACT

Mesenchymal stromal cells (MSC) mobilization and recruitment by experimental vascularizing tumors involves membrane type 1-matrix metalloproteinase (MT1-MMP) functions. Given that the mannose-specific lectin Concanavalin-A (ConA) induces MT1-MMP expression and mimics biological lectins/carbohydrate interactions, we synthesized and tested the potential of 11 mannoside clusters to block ConA activities on MSC. We found that tetra- and hexavalent mannosides reversed ConA-mediated changes in MSC morphology and antagonized ConA-induced caspase-3 activity and proMMP-2 activation. Tetra- and hexavalent mannosides also inhibited ConA- but not the cytoskeleton disrupting agent Cytochalasin-D-induced MT1-MMP cell surface proteolytic processing mechanisms, and effects on cell cycle phase progression. The anti-proliferative and pro-apoptotic impact of ConA on the MT1-MMP/glucose-6-phosphate transporter signalling axis was also reversed by these mannosides. In conclusion, we designed and identified glycocluster constructions that efficiently interfered with carbohydrate-binding proteins (lectins) interaction with oligosaccharide moieties of glycoproteins at the cell surface of MSC. These glycoclusters may serve in carbohydrate-based anticancer strategies through their ability to specifically target MT1-MMP pleiotropic functions in cell survival, proliferation, and extracellular matrix degradation.

INTRODUCTION

Bone marrow-derived mesenchymal stromal cells (MSC) are a population of pluripotent adherent cells residing within the bone marrow microenvironment and have an ability to differentiate into many mesenchymal phenotypes (Prockop, 1997; Horwitz *et al.*, 2005). Interestingly, the recruitment of MSC by experimental vascularizing tumors has resulted in the incorporation of MSC in the tumor architecture (Studeny *et al.*, 2004), and implies that these cells must respond to tumor-derived growth factor cues (Annabi *et al.*, 2004; Birnbaum *et al.*, 2007). Membrane type 1-matrix metalloproteinase (MT1-MMP), an important protein related to tumor growth and angiogenesis, is expressed on malignant tumor cells and in activated endothelial cells (Sato *et al.*, 2005). Among its known activities, MT1-MMP is involved in cell migration, extracellular matrix (ECM) degradation and endothelial cell tubulogenesis (Genis *et al.*, 2006). Detailed studies by RNA interference recently revealed that MT1-MMP similarly controls human MSC mobilization and homing processes which also require invasion through ECM barriers (Ries *et al.*, 2007). Among the signaling molecules thought to regulate MT1-MMP biological functions, Egr-1 and Wnt regulate MT1-MMP gene expression in chemotaxis and in the invasion capacity of MSC out of the bone marrow (Neth *et al.*, 2006; Annabi *et al.*, 2003a; Currie *et al.*, 2007). On the other hand, increased expression of MT1-MMP correlated with decreased cell survival in part attributable to a decrease in expression of an endoplasmic reticulum-embedded glucose-6-phosphate transporter (G6PT) (Currie *et al.*, 2007; Belkaid *et al.*, 2007). While the activities of MT1-MMP in tumorigenesis are well documented, its potential role in cell-cell interaction and in MSC engraftment processes remains poorly understood and new strategies to target MT1-MMP-mediated processes needed to be investigated.

We have previously shown that MSC chemotaxis and cell survival are regulated, in part, by MT1-MMP (Currie *et al.*, 2007). Because of MT1-MMP's ability to promote directed cell migration across reconstituted basement membranes both in metastasis and in tumor angiogenesis processes, newly developed strategies, including specific immunoliposomal anticancer MT1-MMP targeting (Atobe *et al.*, 2007) and interference RNA

technology (Arroyo *et al.*, 2007) are currently envisioned. The design, use, and evaluation of these approaches have however been more complex than expected. Although tumor endothelial cells are increasingly accepted as a valid target for cancer therapy, since angiogenesis is a critical event for the maintenance, proliferation and metastasis of tumors (Brandwijk *et al.*, 2007), none of the above strategies has looked at the impact of MT1-MMP targeting on circulating MSC in response to tumor growth factors.

Given the known tendency of transformed cells to express selective carbohydrate motifs in the form of glycoproteins or glycolipids, the design of carbohydrate-based anticancer vaccines has recently found therapeutic applications and was developed around the multivalent or cluster effect concept designed to interfere with carbohydrate molecular recognition (Danishefsky *et al.*, 2000; Roy, 2004; Verez-Bencomo *et al.*, 2004). Interactions between carbohydrate-binding proteins (lectins) and the oligosaccharide moieties of glycoproteins at the cell surfaces are, in fact, involved in extensive cellular recognition processes including development, differentiation, morphogenesis and cell migration. The lectin from *Canavalia ensiformis* (Concanavalin-A, ConA), one of the most abundant lectins known (Lin and Levitan, 1991), thus enables one to mimic biological lectin/carbohydrate interactions that regulate ECM protein recognition and, as such, is routinely used to trigger MT1-MMP-mediated activation of latent proMMP-2 (Yu *et al.*, 1997; Zucker *et al.*, 2002; Lafleur *et al.*,2006). ConA was also found to increase the sub-G1 cell cycle phase as well as cell death in MSC; indicative of a potential role in cell surface clustering that affects cell survival (Currie *et al.*, 2007). Furthermore, MT1-MMP gene silencing significantly abrogated MSC chemotaxis in response to the tumorigenic growth factor sphingosine 1-phosphate in MSC, suggesting a crucial role for that MMP in signaling cell mobilization (Currie *et al.*, 2007; Annabi *et al.*, 2003b).

In this study, generation of several oligomannoside clusters was performed in order to optimize mannan's ability to inhibit ConA actions using synthesis strategies detailed previously (Touaibia *et al.*, 2007a; Touaibia *et al.*, 2007b; Roy and Touaibia, 2007).

Structure-function studies were initiated to evaluate their biological abilities to inhibit ConA-induced MT1-MMP-mediated proMMP-2 activation, cell death, and anti-proliferative property in MSC. As model substances for effectively functioning as biological response modifiers, the cell-binding capacity of lectins, such as ConA, enables the dissection of cellular processes involved in morphogenesis, ECM degradation and cell death. We show that specific tetra- and hexavalent mannoside clusters very effectively inhibit a spectrum of MT1-MMP-mediated cell responses that could be potentially transposed to target tumor promoting processes.

RESULTS

Chemical structures of the mannosides tested. Pentaerythritol and bis-pentaerythritol scaffolds were previously used toward the preparation of a series of tri- to hexa-clusters bearing α-D-mannopyranoside residues assembled using single steps "Sonogashira coupling" and "click chemistry". The carbohydrate precursors were built with either para-iodophenyl, propargyl, or 2-azidoethyl aglycones while the pentaerythritol moieties were built with terminal azide, propargyl or para-iodophenyl groups (Touaibia *et al.*, 2007a; Touaibia *et al.*, 2007b; Roy and Touaibia, 2007). These clusters were initially chosen for their ability to cross-link Concanavalin-A (ConA), and have already shown to be 1000 times more potent than mannose for their capacity to inhibit the binding of fimbriated *E. coli* expressing mannoside binding pili lectin to erythrocytes *in vitro* (Touaibia *et al.*, 2007b). Whether any of these clusters could further compete against the cellular and molecular effects of ConA was assessed below. The chemical structure of the different mannosides is depicted in figure 1.

Mannan and mannosides DM58, DM54, and DM75 prevent Concanavalin-A-induced changes in cell morphology. Disruption of the cytoskeleton architecture is among the first cellular events known to be affected upon treatment with ConA (Belkaid *et al.*, 2007; Lin and Levitan, 1991; Allenberg *et al.*, 1994; Zanetta *et al.*, 1994). MSC were serum-starved and treated with ConA in the presence or absence of the different mannosides shown in figure 1 as described in the Methods section. Phase contrast pictures were taken and cell aggregation was noticed in ConA-treated MSC (Figure 2A, control). Mannan, DM58, DM54, and DM75 were the only mannosides to significantly prevent the ConA-induced aggregating effect, suggesting that these four molecules may efficiently inhibit cell surface ConA-binding biological effects. The potency to inhibit ConA-induced hemagglutination of erythrocytes was performed with serial 2-fold dilutions of the synthesized molecules (Dam *et al.*, 2998). The lowest concentration of the molecules required to completely inhibit ConA-induced red blood cells agglutination was determined visually, and clearly demonstrates the high agglutination inhibitory effect of DM58 in comparison to all other molecules tested, which was ~8-times more potent than DM71 and DM75, and ~16-times more potent than DM54

(see squared wells, Figure 2B). In order to further assess the direct lectin binding ability of Mannan, DM58, DM54, and DM75 molecules (DM71 was discarded because of its inability to reverse ConA-induced cell aggregation), ConA was treated with each of the clusters at 1 mg/ml in microtiter plates using the polysaccharide yeast mannan as positive control. Rapidly and within five minutes, insoluble crosslinked complexes were formed as judged by the cloudiness within the wells (not shown). The optical density (O.D.) was measured at 490 nm as previously described (Touaibia *et al.*, 2007b), and demonstrated a significant ConA binding activity of the synthetic glycoclusters (Figure 2C). The results clearly illustrate the rapid preference of tetraaryl mannoside DM58, obtained via the Sonagashira coupling, to form a cross-linked lattice toward ConA, over the alternate functional isomer DM54 or hexamer DM75. The latter two possessed however comparable lectin binding activities to the reference yeast mannan.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced proMMP-2 activation. Cytoskeleton disrupting agents ConA and Cytochalasin-D (CytoD) trigger latent proMMP-2 activation into its active MMP-2 form (Gingras *et al.*, 2000). Serum-starved MSC were treated with either ConA or CytoD in the presence or absence of the different mannosides as described in the Methods section. Conditioned media were then harvested and assessed for latent proMMP-2 and active MMP-2 content by gelatin zymography. While none of the mannosides was able to antagonize CytoD-induced proMMP-2 activation (Figure 3A), the mannosides DM58, DM54 and DM75, in contrast, very efficiently (better even than mannan) inhibited ConA-induced proMMP-2 activation (Figure 3B). A dose response analysis of the inhibitory effect of the best mannosides against ConA-induced proMMP-2 activation was assessed by gelatin zymography. Each of the mannan, DM58, DM54, and DM75 mannosides dose-dependently inhibited proMMP-2 activation by ConA, resulting in the progressive disappearance of the active MMP-2 form concomitant to the reappearance of the latent proMMP-2 form (Figure 4A). The anti-ConA effects of the mannosides tested enabled us to calculate the IC_{50} values for each of them as follows : Mannan 8.2 ± 0.9 µg/ml, DM58 1.3 ± 0.4 µg/ml, DM54 2.6 ± 0.2 µg/ml, and DM75 5.1 ± 1.1 µg/ml, establishing DM58 ~8-times more potent than mannan for inhibiting ConA-induced proMMP-2 activation and confirming the turbidimetric data obtained in figure 2B.

These results suggest that efficient inhibition of proMMP-2 activation by mannosides may account for decreased ECM degradation capacity and subsequent mobilization of MSC.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced caspase-3 activity. ProMMP-2 activation has recently been correlated to cell death (Hinoue *et al.*, 2005; Preaux *et al.*, 2002). Since ConA-induced proMMP-2 activation is thought to be mediated through a MT1-MMP-dependent process in MSC (Currie *et al.*, 2007), we further investigated the ability of the above identified best four mannosides to antagonize ConA- and CytoD-induced cell death. Serum-starved MSC were thus treated as described above in the presence or absence of mannan, DM58, DM54, and DM75 mannosides. Cells were then harvested as described in the Methods section in order to assay caspase-3 activity. We found that ConA and CytoD significantly induced cell death and caspase-3 activity (Figure 5) as previously documented (Currie *et al.*, 2007; Belkaid *et al.*, 2007; Annabi *et al.*, 2003b). Intriguingly, while mannan and the mannosides DM58, DM54, and DM75 completely prevented caspase-3 activation in ConA-treated MSC (Figure 5, black bars), only DM58 was efficient at doing so in CytoD-treated MSC (Figure 5, grey bars). Because CytoD directly inhibits intracellular actin polymerization (Lambert *et al.*, 2001; Sanka *et al.*, 2007; Yourek *et al.*, 2007), this observation suggests that, except for DM58, the mannosides tested specifically antagonize ConA cell surface clustering in MSC. Collectively, the inhibition of ConA-induced cell morphology, proMMP-2 activation, and cell death by the mannosides prompted us to further explore the involvement of mannosides against MT1-MMP functions.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced cell surface MT1-MMP proteolytic processing and affect the MT1-MMP/G6PT signaling axis. Among the early events that take place upon cytoskeleton disorganization is the activation of proMMP-2 by MT1-MMP. Given evidence that endoplasmic reticulum (ER) dysfunction is often linked to cytoskeleton perturbations and to cell death signaling, a molecular signaling axis between MT1-MMP and the ER-embedded glucose-6-phosphate transporter (G6PT) was highlighted in MSC (Currie *et al.*, 2007). We thus sought to

investigate whether the different mannosides generated were able to regulate ConA-induced MT1-MMP cell surface processing, and if the MT1-MMP/G6PT signaling axis was affected. We found that MSC treated with either ConA or CytoD exhibited an increase in the active 55 kDa form of MT1-MMP and in the appearance of its 43 kDa inactive proteolytic fragment (Figure 6, upper panels). When MSC were co-treated with mannan, DM58, DM54 or DM75, ConA-induced MT1-MMP proteolytic processing was inhibited while it remained unaffected in CytoD-treated cells (Figure 6, upper panels). These observations are in agreement with those obtained with proMMP-2 activation using gelatin zymography (Figure 3). When the MT1-MMP/G6PT signaling axis was investigated, we found that ConA, but not CytoD, inhibited G6PT expression (Figure 6, lower panels). While mannan, DM58, DM54 and DM75 reversed the G6PT inhibition, they had no effect on G6PT expression in CytoD-treated cells (Figure 6, lower panels). Expression of the extracellular signal-regulated protein kinase (ERK) was found constant throughout the experimental conditions and used as an internal control. Collectively, the mannosides tested efficiently re-established the MT1-MMP/G6PT intracellular signaling axis that was disrupted upon ConA treatment.

The pro-survival effects of Mannoside DM58 prevent the induction of sub-G1 by ConA and CytoD, and prevent the decrease in G0/G1 cell cycle phases by Concanavalin-A. The pro-survival impact of mannoside DM58 was further investigated in terms of cell cycle progression. Because proMMP-2 activation is thought to interfere with cell survival and proliferation (Hinoue *et al.*, 2005; Preaux *et al.*, 2002), we also sought to investigate the effect of ConA and CytoD on BMSC cell cycle progression. Cells were treated with ConA or CytoD in the presence or not of DM58, staining was performed with propidium iodine (PI), and flow cytometry was used to assess the proportion of cells found in the G0/G1, S, G2/M, and sub-G1 cell cycle phases (Figure 7A). While DM58 itself had no effects on cell cycle phases, ConA triggered a significant decrease in G0/G1 and CytoD treatment resulted in a combined decrease in G0/G1 and increase in G2/M cell cycle phases (Figure 7A), and that was concomitantly observed with an increase in sub-G1 (Figure 1D). Both ConA and CytoD triggered the appearance of a sub-G1 cell population in the absence of mannosides (Figure 7B, white bars) indicative of cell death and consistent with their pro-apoptotic effects (Figure

5). In accordance with its pro-survival property, the only mannoside DM58 that was able to protect from ConA- or CytoD-induction of caspase-3, was able to reduce the sub-G1 cell population (Figure 7B, black bars) that appeared upon ConA or CytoD treatment confirming its more complex mechanistic actions.

DISCUSSION

Cell surface carbohydrate structures acting as ligands for tissue specific mammalian lectins have long been recognized in the regulation of cell-cell interactions, particularly in processes such as lymphocyte homing to specific tissues (Stoolman and Rosen, 1983), homing of hematopoietic stem cells (Aizawa and Tavassoli, 1988), and tumor cell metastasis (Kannagi *et al.*, 2004). As such, lectins on microvascular endothelial cells have also been shown to contribute to retention and secondary tumor formation of blood-borne tumor cells (Cornil *et al.*, 1990). Recent evidence from our laboratory indicates that circulating vascular progenitors derived from murine MSC are recruited by vascularizing tumors (Annabi *et al.*, 2004) and that a hypoxic environment, such as that encountered within tumor masses, regulates MSC angiogenic properties (Annabi *et al.*, 2003a). *In vivo*, subcutaneous co-injection of MSC with U-87 glioma cells into nude mice resulted in the formation of highly vascularized tumors, where differentiated MSC localized at the lumen of vascular structures (Annabi *et al.*, 2004). Although the specific implications of MT1-MMP in cell-ECM and cell-cell interactions that would promote engraftment and potential tumor development remain to be addressed *in vivo*, these data suggest that MSC can be recruited at the sites of active tumor neovascularization and prompted us to investigate new routes to the use of MT1-MMP functions as a therapeutic target by carbohydrate scaffolds.

The present study revolves around the well documented ability of the lectin ConA to efficiently trigger MT1-MMP-mediated proMMP-2 activation and cell death signalling. As such, silencing of the MT1-MMP gene prevented ConA-mediated proMMP-2 activation and cell death in MSC (Currie *et al.*, 2007; Belkaid *et al.*, 2007) demonstrating the crucial role that MT1-MMP plays in transducing cell signaling upon cytoskeleton remodeling. MT1-MMP can further function as a signalling molecule as it cooperates with tumor-derived growth factors to induce actin stress fibers and to trigger MSC migration (Meriane *et al.*, 2006). Among the signal transduction events reported, tyrosine phosphorylation is considered a major event upstream of MT1-MMP induction upon ConA cell surface clustering in platelets (Torti *et al.*, 1995), monocytes (Matsuo *et al.*, 1996), neutrophils (Ohta *et al.*, 1992)

and breast carcinoma cells (Yu *et al.*, 1997), while activation of the ERK cascade by MT1-MMP represents an important downstream event (Gingras *et al.*, 2001). Additionally, MT1-MMP-mediated intracellular ERK phosphorylation was found crucial in regulating cell-ECM interaction through CD44, thereby regulating cell homing and engraftment (Annabi *et al.*, 2004; Krause *et al.*, 2006). The cytoskeleton also plays important roles in cell morphology, growth, and signaling during MSC differentiation (Yourek *et al.*, 2007). Changes in the cytoskeleton allow the cell to migrate, divide, and maintain its shape in response to external mechanical stimuli (Hayakawa *et al.*, 2001). Maintenance of cytoarchitecture is, on the other hand, also required for cell survival, since its perturbation by CytoD- or ConA-mediated MT1-MMP mechanisms diminished cell survival and was correlated to proMMP-2 activation (Hinoue *et al.*, 2005; Preaux *et al.*, 2002; this study).

As model substances for inducing cell aggregation and adhesion, considerable literature on the binding capacity of aromatic or clustered glycosides to ConA have enabled to dissect signalling cascades relevant to numerous cellular processes (Dam *et al.*, 2000; Jain *et al.*, 2000; Dam and Brewer, 2003; Li *et al.*, 2004;). Among others, these include activation of immune cells, modulation of cytokine secretion and induction of cell apoptosis (Andre *et al.*, 2001; Timoshenko *et al.*, 2000). In fact, the role of cell surface carbohydrates in ConA-mediated cytoskeletal changes and induction of apoptosis was documented in fibroblasts (Kulkarni and McCulloch, 1995). In the present study, the use of cytoskeleton disrupting agents ConA and CytoD effectively triggered MT1-MMP-mediated cell death. Interestingly, while DM58, DM54, and DM75 mannosides antagonized ConA induction of caspase-3 activity, only DM58 was able to reverse CytoD-induced caspase-3, suggesting a direct and more complex prosurvival effect of that mannoside which remains to be investigated. Accordingly, cytoskeleton disorganization is considered an early step in the activation process of proMMP-2 by MT1-MMP, but is also associated with ER dysfunction and subsequent cell death. Given evidence that the ER-embedded G6PT regulates cell survival and that impaired chemotaxis was recently observed in bone marrow cells isolated from a $G6PT^{-/-}$ mouse model (Kim *et al.*, 2006), a MT1-MMP/G6PT signalling axis was recently shown to link MSC survival, ECM degradation and mobilization (Currie *et al.*, 2007).

Important progress has been made in the development of carbohydrate scaffolds for drug discovery. Carbohydrates have been proven as valuable scaffolds to display pharmocophores and the resulting molecules have demonstrated useful biological activity towards various targets including the somatostatin receptors, integrins, HIV-1 protease, MMP, multidrug resistance-associated protein, and as RNA binders, and have shown antibacterial and herbicidal activity (Becker *et al.*, 2006). In our study, we used carbohydrates as scaffolds to display chemical functionalities that have the potential to interact with carbohydrate-recognizing receptors. We found that the spatial arrangement of the clusters having the triazole or the phenyl rings appeared to be crucial for their affinity against ConA activities, thus illustrating the influence of multivalency on this scaffold. The introduction of four or six mannopyranoside moieties using extended precursors and 1,3-cycloaddition had a minor effect on the relative affinity, since compounds DM13, DM6, DM80 and DM65 having respectively three, four and six mannoside residues, were almost equipotent (Figure 1A). Compounds DM58, DM54 and DM75, to which a mannoside moiety having a phenyl ring was introduced by Sonogashira coupling, in contrast, showed significant potency. The position of the phenyl ring appeared to be rather important with regard to modulating the activity of DM58 and which differs from DM54 only by the relative positioning of the phenyl ring (Figure 1B).

In conclusion, we report the biological evaluation and the identification of specific mannosides which appear to play an important role against the interaction between lectins and carbohydrate glycoconjugates present at the cell surface of MSC. The specific action of these mannosides revolves around their capacity to antagonize MT1-MMP-mediated events induced by ConA and that involve cell death signalling and activation of latent proMMP-2 in MSC. While novel approaches to the inhibition of MT1-MMP activity are explored (Arroyo *et al.*, 2007), our observations may, thus, find broader therapeutic implications than only in MSC recruitment, cell surface glycoprotein-mediated cell-cell contacts and engraftment within a tumor environment. Increased expression of stem cell markers in malignant melanoma including cell surface glycoproteins such as the activated leukocyte adhesion molecule (CD166) and prominin-1 (CD133) have recently been revealed as potential new

prognostic markers (Klein *et al.*, 2007). Given the non-cytotoxic effects of the mannosides tested in our study (Figure 2, see vehicle panels), it can now be envisioned to test those mannosides *in vivo* against experimentally implanted tumors. Based on our results, it is further tempting to suggest that the specific targeting of cell surface glycoproteins by those mannosides may, in part, be directed against MT1-MMP-mediated processes that regulate cancer cells survival, metastasis, and endothelial cell-mediated angiogenesis. As such, efficient delivery of those mannosides may achieve both direct tumor cell killing and indirect tumor cell killing via the destruction of tumor-associated endothelium or recruitment/engraftment of circulating cells.

MATERIALS AND METHODS

Materials. Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Cell culture media were obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against MT1-MMP (AB815) and ERK were respectively from Chemicon (Temecula, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA), while the polyclonal antibody against G6PT was generated against the 5-GYGYYRTVIFSAMFGGY-21 peptide derived from the human G6PT primary sequence (accession # AAD19898) (Chen *et al.*, 2002) at the Biotechnology Research Institute (Montreal, QC). All other reagents were from Sigma-Aldrich Canada.

Cell culture : Bone marrow-derived mesenchymal stromal cells (MSC) were isolated from the whole femur and tibia bone marrow of C57BL/6 female mice and cells were cultured and characterized as previously described (Meriane *et al.*, 2006). Analysis by flow cytometry, performed at passage 14, revealed that MSC expressed CD44 yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3) and Tie2 (angiopoietin receptor) (data not shown).

Hemagglutination inhibition assay : Hemagglutination assays were carried out in round-bottomed microtitre plates. A total volume of 100 µL was used in each well: 25 µL of ConA (0.1 mg/ml) was added to 25 µL aliquots of serial 2-fold dilutions of the different mannosides synthesized (0.5 mg/ml stock solutions) and 50 µL of 3% (v/v) rat erythrocyte suspension in saline HEPES buffer (0.1 M HEPES, 0.15 M NaCl, 1 mM $CaCl_2$, and 1mM $MnCl_2$, pH 7.2). The microtitre plate with the 100 µL erythrocyte suspension, containing the serial double dilutions of the tested sugars, was incubated for 3 hours at room temperature.

The lowest concentration of the sugars required to completely inhibit red blood cells agglutination by ConA was determined visually (Dam *et al.*, 1998).

Gelatin zymography : Gelatin zymography was used to assess the extent of latent and proMMP-2 and active MMP-2 activity. 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 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.

Fluorimetric caspase-3 activity assay : MSC were grown to about 80% confluence in 6-well dishes and treated for 16-18 hrs with ConA or CytoD either lacking, or in combination with, mannan, DM58, DM54 or DM75 mannosides. After treatment, cells were collected and washed in ice-cold PBS pH 7,0. Cells were lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 1 hr at 4°C and the lysates were clarified by centrifugation at 16,000 g for 20 min. Caspase-3 activity was determined by incubation with 50 µM 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 min at 37°C on a fluorescence plate reader (Molecular Dynamics, Sunnyvale, CA) (λ_{ex}=400nm, λ_{em}=505nm).

Immunoblotting procedures: Proteins from control and treated MSC were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 hr

at room temperature 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 in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

Analysis of cell cycle by flow cytometry: Distribution of BMSC throughout the cell cycle was assessed by flow cytometry (Currie *et al.*, 2007). Serum-fasting preparation was performed prior to analysis and therefore the cell populations were synchronous. Cells were harvested by gentle scraping, pelleted by centrifugation, washed with ice-cold PBS/EDTA (5 mM), then resuspended in 1 volume PBS/EDTA and fixed with 100% ethanol overnight. Three volumes of staining solution, containing propidium iodine (PI, 50 µg/ml) and DNAse-free RNAse (20 µg/ml), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a Becton Dickinson FACS Calibur flow cytometer equipped with CellQuest Pro software. In particular, the characteristics of cell distribution in the subG1 region were studied on the DNA histogram.

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ABBREVIATIONS

MSC, mesenchymal stromal cells; ConA, concanavalin-A; CytoD, cytochalasin-D; ECM, extracellular matrix; ER, endoplasmic reticulum; G6PT, glucose-6-phosphate transporter; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP

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Fig.1

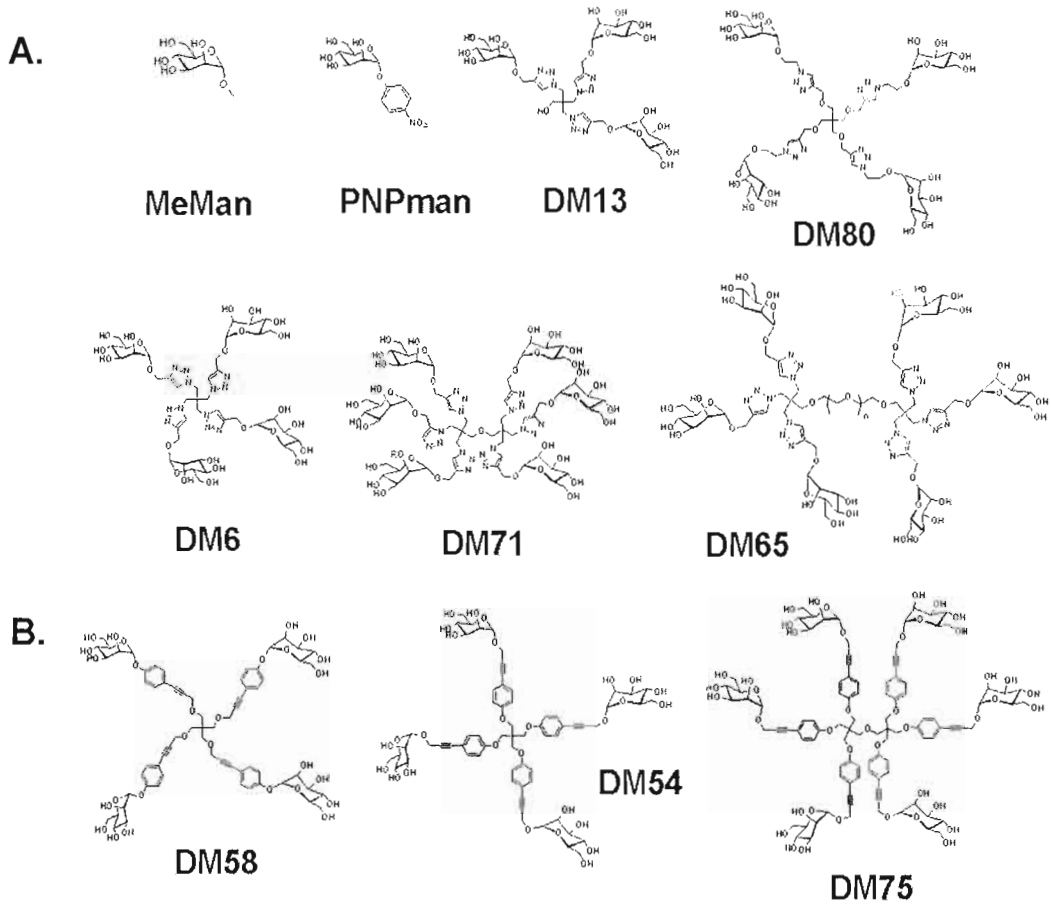

Figure 1: *Chemical structures of the mannosides tested against Concanavalin-A.* (A) MethylMannose (MeMan), *p*-nitrophenylmannoside (PNPMan) and mannoside clusters synthesized by Cu(I)-catalyzed [1,3]-dipolar cycloadditions using pentaerythritol scaffolds bearing either alkyne or azide functionalities are represented. (B) Mannoside clusters synthesized by palladium-catalyzed Sonogashira reactions using pentaerythritol scaffolds bearing either alkyne or p-iodophenyl functionalities are depicted.

Fig.2

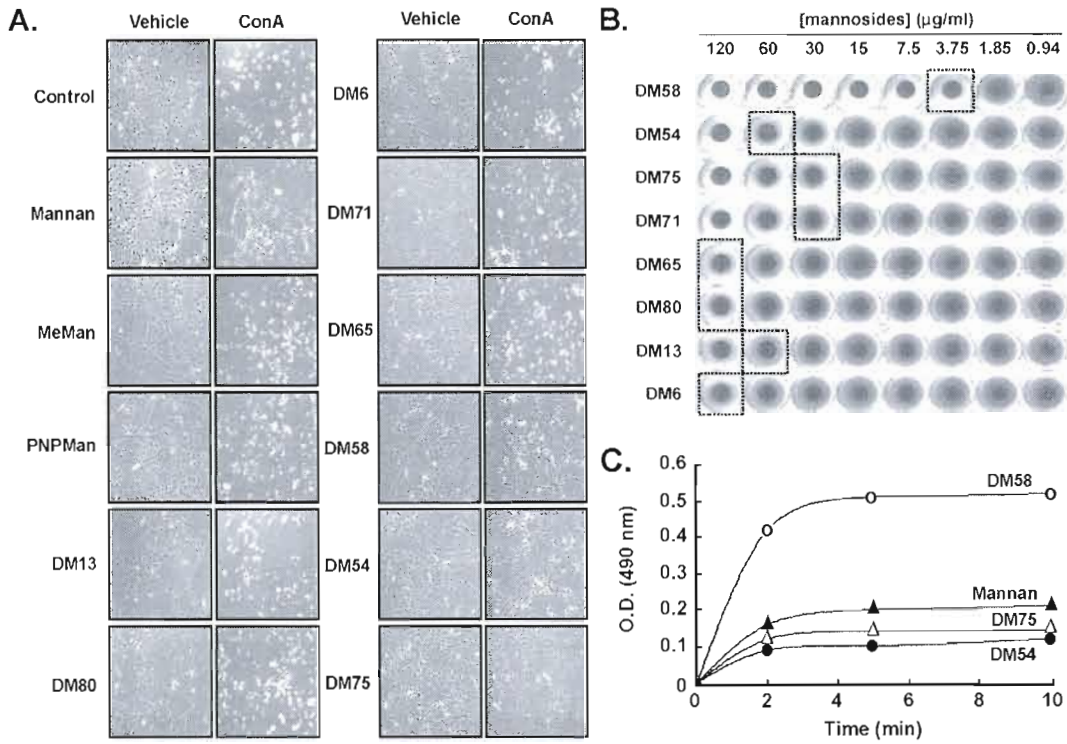

Figure 2 : Mannan *and mannosides DM58, DM54, and DM75 prevent Concanavalin-A-induced changes in cell morphology through direct cross-linking properties*. (A) Sub-confluent MSC were serum-starved and treated with 10µg/ml ConA or vehicle (water) in the presence or absence of 10 µg/ml of the different mannosides described in figure 1 for 18 hrs at 37°C. Phase contrast pictures were taken and cell aggregation was reflected by rounding in the cell morphology. (B) Inhibition of ConA-mediated hemagglutination of erythrocytes potency was tested as described in the methods section for all the mannosides synthesized. Squared wells show the minimum concentration required for inhibition from a representative experiment (n=3). (C) The classical time course of the microtiter plate turbidimetric analysis (micro-precipitation) of ConA was performed as described previously (Touaibia *et al.*, 2007b) in the presence of mannosides DM58 (open circle), DM54 (closed circle), DM75 (open triangle) and yeast mannan (closed triangle) as positive control. Measurements were performed in PBS at 1 mg/ml ConA using an ELISA plate reader at 25°C. Optical density (O.D.) was read at 490 nm and data are the average of triplicate values.

Fig.3

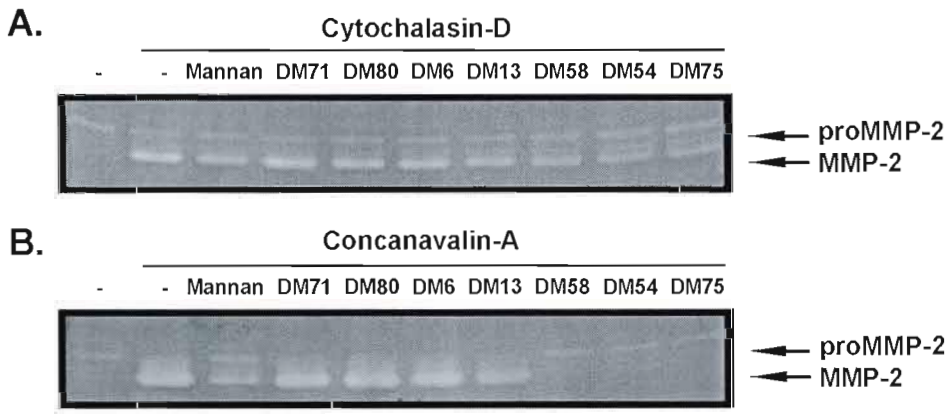

Figure 3 : Mannan *and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A- but not Cytochalasin-D-induced proMMP-2 activation.* Sub-confluent MSC were serum-starved and treated with either (A) 1 μM CytochalasinD (CytoD) or (B) 10 μg/ml ConA in the presence or absence of 10 μg/ml of the different mannosides described in figure 1 for 18 hrs at 37°C. Conditioned media were then harvested and gelatin zymography performed as described in the Methods section in order to monitor the extent of latent proMMP-2 activation into MMP-2.

Fig.4

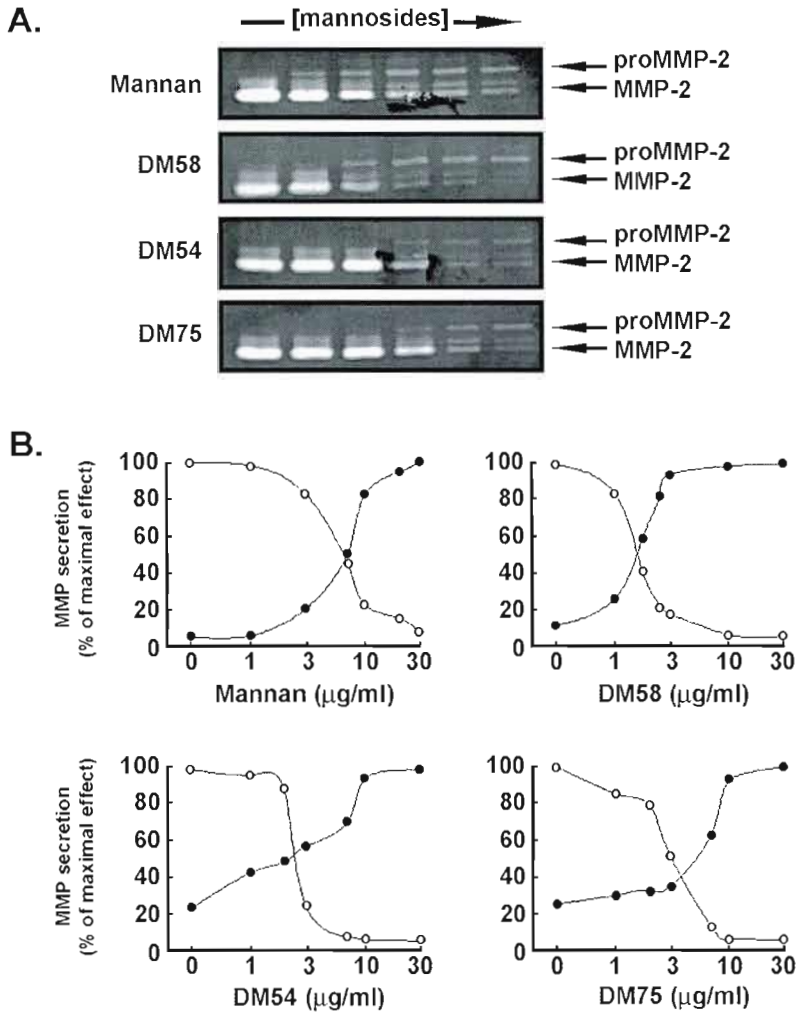

Figure 4 : *Differential potencies of mannan and mannosides DM58, DM54, DM75 for inhibiting Concanavalin-A-induced proMMP-2 activation.* Sub-confluent MSC were serum-starved and treated with increasing concentrations (0-30 μg/ml) of mannan, DM58, DM54 and DM75 in the presence of 10 μg/ml ConA for 18 hrs at 37°C. (A) Conditioned media were then harvested and gelatin zymography performed as described in the Methods section in order to monitor the extent of latent proMMP-2 activation into MMP-2. (B) Scanning densitometry of a representative experiment (out of three) was used to quantify the extent of gelatin hydrolysis by the latent proMMP-2 (closed circles) and by the active MMP-2 form (open circles).

Fig.5

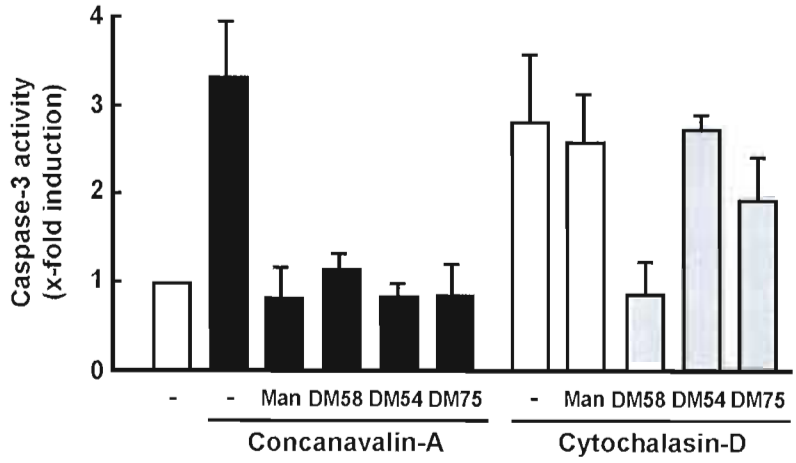

Figure 5 : *Mannan and mannosides DM58, DM54 and DM75 inhibit Concanavalin-A-induced caspase-3 activity.* Sub-confluent BMSC were serum-starved and treated with 10µg/ml of either mannan, DM58, DM54 or DM75 in the presence of 10 µg/ml ConA (black bars) or CytoD (grey bars) for 18 hrs at 37°C. Caspase-3 activity is shown from a representative experiment and was measured as described in the Methods section in triplicate.

Fig.6

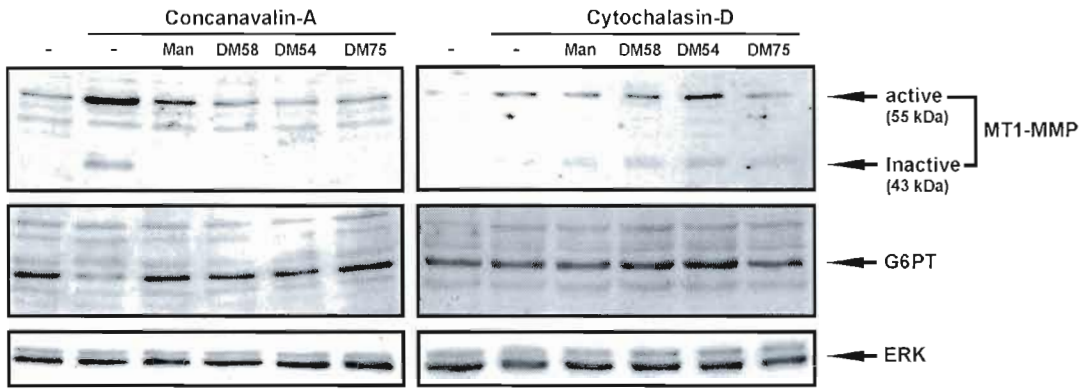

Figure 6 : *Mannan and mannosides DM58, DM54 and DM75 inhibit Concanavalin-A-induced cell surface MT1-MMP proteolytic processing and affect the MT1-MMP/G6PT signaling axis.* Sub-confluent MSC were serum-starved and treated with 10 μg/ml of either mannan, DM58, DM54 or DM75 in the presence of 10 μg/ml ConA (left panel) or CytoD (right panel) for 18 hrs at 37°C. Cell lysates were isolated from each condition and Western blotting followed by immunodetection were performed with anti-MT1-MMP, anti-G6PT and anti-ERK antibodies as described in the Methods section.

Fig.7

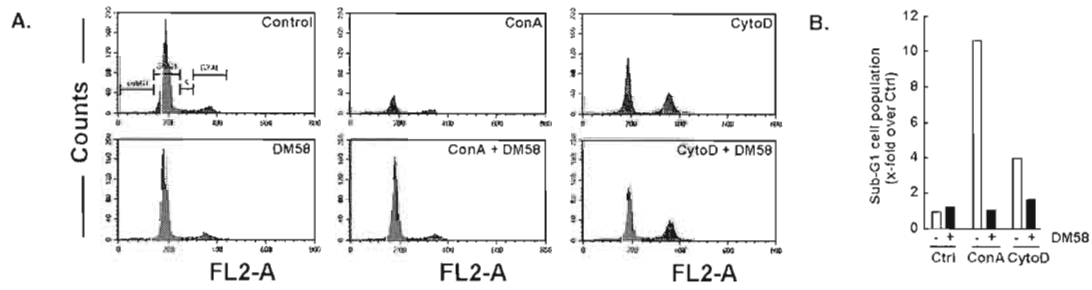

Figure 7 : *The pro-survival effects of Mannoside DM58 prevent the induction of sub-G1 by ConA and CytoD, and prevent the decrease in G0/G1 cell cycle phases by Concanavalin-A.* BMSC were serum-starved for 24 h and cell cycle phases synchronized. (**A**) Cells were treated with 10 mg/mL ConA, or 1 μM CytoD, in the presence or not of 10 μg/mL DM58, and cell cycle was analyzed by flow cytometry as described in the Materials and methods section. A representative experiment out of three shows cells present in each division phase. For the sake of clarity, gating for the sub-G1, G0/G1, S, and G2/M cell cycle phases are shown in the Control (Ctrl) picture. (**B**) A representative sub-G1 cell population is depicted upon ConA or CytoD treatments in the absence (white bars) or presence (black bars) of DM58.

3.3

Silencing of the MT1-MMP/ G6PT axis suppresses calcium mobilization by sphingosine-1-phosphate in glioblastoma cells

From

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Silencing of the MT1-MMP/ G6PT axis suppresses calcium mobilization by sphingosine-1-phosphate in glioblastoma cells

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Running title : MT1-MMP/G6PT axis regulates calcium flux

Keywords: MT1-MMP; S1P; Glucose-6-phosphate transporter; Calcium mobilization; Cell migration

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Simon Fortier a réalisé la majorité des expérimentations et traitements cellulaires, analysé les résultats et participé à la rédaction de l'article. Dominique Labelle a réalisé l'évaluation du flux calcique par microscopie confocale. Asmaa Sina était stagiaire durant les expérimentations. Robert Moreau et Borhane Annabi sont les directeurs des laboratoires impliqués.

ABSTRACT

The contributions of membrane type-1 matrix metalloproteinase (MT1-MMP) and of the glucose-6-phosphate transporter (G6PT) in sphingosine-1-phosphate (S1P)-mediated Ca^{2+} mobilization were assessed in glioblastoma cells. We show that gene silencing of MT1-MMP or G6PT decreased the extent of S1P-induced Ca^{2+} mobilization, chemotaxis, and extracellular signal-related kinase phosphorylation. Chlorogenic acid and (-)-epigallocatechin-3-gallate, two diet-derived inhibitors of G6PT and of MT1-MMP, respectively, reduced S1P-mediated Ca^{2+} mobilization. An intact MT1-MMP/G6PT signalling axis is thus required for efficient Ca^{2+} mobilization in response to bioactive lipids such as S1P. Targeted inhibition of either MT1-MMP or G6PT may lead to reduced infiltrative and invasive properties of brain tumour cells.

INTRODUCTION

Glioblastoma multiform is the most commonly occurring primary brain tumor in adults and is highly malignant, displaying increased vascularization, aggressive growth and invasion into surrounding brain tissue [1]. Among the serum-derived lipid and growth factors that exhibit chemotactic influences towards glioblastoma cells, sphingosine-1-phosphate (S1P) is a bioactive lipid that stimulates growth and invasiveness of glioblastoma cell and that signals through a family of five G-protein-coupled receptors termed S1PR(1-5) [2]. The S1PR contribution to intracellular calcium (Ca^{2+}) homeostasis and stimulation of glioblastoma cell proliferation correlated with activation of extracellular signal-regulated protein kinase (ERK) MAP kinase [3, 4]. Among the two sphingosine kinase (SphK) isoforms, SphK-1 correlates with short survival of glioblastoma patients [5, 6], and is overexpressed in brain tumor-derived endothelial cells [7]. Consequently, the generation of S1P is hypothesized to contribute to the acquisition and the maintenance of the multidrug resistance phenotype in brain tumors as well as to exert chemotactic migration effects in numerous types of cells including ovarian cancer cells [8], HT-1080 fibrosarcoma cells [9], U-87 glioblastoma cells [10] and mesenchymal stromal cells [11]. The molecular players that link the control of S1P-mediated intracellular Ca^{2+} mobilization to cell migration and to extracellular matrix (ECM) degradation remain to be investigated.

Recent evidence from our laboratory identified a crucial signaling axis composed of a cell surface membrane-bound matrix metalloproteinase (MT1-MMP) and of an endoplasmic reticulum (ER)-embedded glucose-6-phosphate transporter (G6PT) which functions as a bioswitch in the regulation of glioblastoma cell survival and migration [12]. Interestingly, genetic deficiencies in G6PT cause a clinical condition where patients have impaired neutrophil chemotaxis and Ca^{2+} flux [13]. These findings, along with G6PT's endoplasmic/sarcoplasmic reticulum Ca^{2+} sequestration function [14], demonstrate that G6PT is not just a G6P transport protein but may also potentially contribute to intracellular Ca^{2+} homeostasis. This explains, in part, why chlorogenic acid (CHL), among the most potent functional inhibitor of G6PT [15], inhibited S1P-induced glioblastoma cell migration as well as the rapid, S1P-induced ERK phosphorylation in glioblastoma cells [16].

In this study, we specifically assessed whether gene silencing of MT1-MMP or G6PT may affect S1P-mediated intracellular Ca^{2+} mobilization and glioblastoma cell migration in response to S1P. We report that an intact, functional MT1-MMP/G6PT signalling axis is required for efficient Ca^{2+} mobilization in response to circulating bioactive lipids such as S1P, suggesting that targeted inhibition of either MT1-MMP or G6PT function may lead to reduced infiltrative and invasive properties of brain tumour cells.

MATERIALS AND METHODS

2.1. Materials

Sodium dodecylsulfate (SDS), bovine serum albumin (BSA), PD98059, U73122, Y27632, sphingosine-1-phosphate (S1P), thapsigargin and verapamil were purchased from Sigma (Oakville, ON). TRIzol reagent for total RNA extraction was from Life Technologies. The anti-phospho-ERK antibody was from Cell Signaling Technology (Beverly, MA) while the polyclonal anti-ERK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and used for immunoblotting procedures as described previously [16].

2.2. Transfection method and RNA interference

The U-87 glioblastoma cells were transiently transfected with the cDNA constructs encoding either MT1-MMP [17] or G6PT [18], or with 20 nM siRNA (see below) using Lipofectamine 2000 (Invitrogen). The occurrence of MT1-MMP or G6PT specific gene knockdown was evaluated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) with the One-Step RT-PCR Kit (Invitrogen) and validated by assessing MT1-MMP-mediated proMMP-2 activation with concanavalin-A using gelatin zymography of the conditioned media [11]. Mock transfections of U-87 cultures with pcDNA (3.1+) were used as controls. Small interfering RNAs against MT1-MMP (siMT1-MMP) and G6PT (siG6PT), and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield) and annealed to form duplexes. The sequence of the siMT1-MMP and siG6PT used in this study are as follows : siMT1-MMP : 5'-CCAGAAGCUGAAGGUAGAAdTdT-3' (sense) and 5'-UUCUACCUUCAGCUUCUGGdTdT-3' (antisense); siG6PT : 5'-CGAAACAUCCGCACCAAGAdTdT-3' (sense) and 5'-UCUUGGUGCGGAUGUUUCGdTdT-3' (antisense) [12, 16].

2.3. Calcium mobilization assay

U-87 glioblastoma cells were cultured in 4-well Labtek chambers (Nalge Nunc). Culture media was then changed to HEPES-buffered saline solution (HBSS) (mM: 121 NaCl, 5.4 KCl, 0.8 Mg_2SO_4, 25 HEPES, 1.8 $CaCl_2$ and 6.0 $NaHCO_3$ at pH 7.3) and loaded with 2 μM Fluo-3 AM (Molecular Probes) with an equivalent volume of 20% Pluronic F127 and 5 μM Verapamil for 45 min at 37 °C in the dark. Thereafter, cells were washed with HBSS and the loaded dye was allowed to deesterify for 45 min at room temperature in the dark. Following transfer to a Ca^{2+}-free solution (HBSS without $CaCl_2$), additions were made in an open chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad) Nikon TE300 microscope with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488 nm and emission was collected with a 515 nm filter. Data were analyzed with Laser Sharp 2.1T, Time Course 1.0 software. The individual fluorescence intensities of 20-30 cells per field were used to obtain a mean fluorescence for each experiment.

2.4. Analysis of U-87 glioblastoma cell migration

U-87 cell migration was assessed using modified Boyden chambers. The upper surfaces of Transwell inserts (8-μm pore size; Costar, Acton, MA) were pre-coated with 0.2% gelatin. The Transwells were then assembled in a 24-well plate and the lower chamber was filled with serum-free MEM or MEM supplemented with either 1 μM S1P or 20 ng/ml basic fibroblast growth factor (bFGF). Cells were collected by trypsinization, washed and resuspended in serum-free medium; 10^5 cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37°C in 5% CO_2/95% air, and migration left to proceed for 6 hours. Cells that had migrated to the lower surfaces of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet-20% methanol (v/v). Images of at least five random fields per filter were digitized (100X magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging Inc). Migration data are expressed as a mean value derived from at least four independent experiments.

RESULTS

3.1. Pharmacological inhibition of sphingosine-1-phosphate-induced calcium mobilization

ERK phosphorylation represents, among the early intracellular Ca^{2+}-dependent events, an efficient means by which to examine sphingosine-1-phosphate (S1P) effects. U-87 glioblastoma cells were thus exposed for 2 minutes to various doses of S1P (Fig.1A) and the extent of ERK phosphorylation found optimal at 1 μM S1P (Fig.1B). That S1P concentration was then used throughout the remaining study, and the contribution of the S1P G-protein-coupled receptors (S1PRs) intracellular signaling pathways involving the phospholipase C, MAPK and RhoA/ROK assessed [19]. While Ca^{2+} mobilization upon S1P treatment was rapidly and transiently induced in the control condition, that mobilization was inhibited in PD98059 (a MEK kinase inhibitor), Y27632 (a RhoA/ROK inhibitor), and in U73122 (a phospholipase C antagonist) pre-treated cells (Fig.1C). This suggested that functional signaling cascades for S1P were required to elicit proper Ca^{2+} mobilization in part through a receptor-dependent mechanism acting via G(i)/ERK pathway in conjunction with activation of Rho- and phospholipase C-mediated signals. When cells were treated with either epigallocatechin-3-gallate (EGCg) or chlorogenic acid (CHL), two diet-derived molecules shown to inhibit respectively MT1-MMP or microsomal G6P transport functions [20-22], we also observed a decrease in S1P-induced Ca^{2+} mobilization (Fig.1C). Interestingly, epigallocatechin (EGC) failed to inhibit S1P-induced Ca^{2+} mobilization suggesting a structure-function relationship of the gallate moiety previously shown to target the cooperative functions of MT1-MMP [23]. Following each of the above tested inhibitors, Ca^{2+} release from ER was induced as a control by the addition of 5 μM Thapsigargin (not shown).

3.2. Gene silencing of MT1-MMP and of G6PT impairs sphingosine-1-phosphate-mediated calcium mobilization

The relevance of each MT1-MMP and G6PT was assessed with respect to Ca^{2+} mobilization by S1P. Gene silencing strategies using specific siRNA was used as previously described [18, 24] and efficiency of gene silencing confirmed by RT-PCR (Fig.2A). MT1-MMP function

was efficiently reduced by gene silencing since concanavalin-A, a known MT1-MMP inducer and proMMP-2 activator [25], was unable to trigger proMMP-2 activation (Fig.2B, siMT1-MMP). In contrast, cells that overexpressed recombinant MT1-MMP were found to activate proMMP-2 into MMP-2 and this effect was amplified by concanavalin-A treatment (Fig.2B). Gene silencing of G6PT did not affect concanavalin-A's ability to induce proMMP-2 activation. However, transient G6PT cDNA cell transfections lead to decreased proMMP-2 activation by concanavalin-A (Fig.2B) in agreement with the prosurvival role played by G6PT [12]. When S1P-induced Ca^{2+} mobilization was assessed, both siMT1-MMP- and siG6PT-transiantly transfected cells showed a significant decrease in Ca^{2+} mobilization (Fig.2C). On the other hand, cells that overexpressed MT1-MMP demonstrated an increase in Ca^{2+} mobilization by S1P, while those that overexpressed G6PT did not exhibit any changes when compared to mock-transfected cells (Fig.2C). SiMT1-MMP and siG6PT did not block thapsigargin-induced Ca^{2+} release (not shown). Collectively, this confirms the primary cooperative role of MT1-MMP with S1P, while G6PT, because of its inability to increase S1P-mediated Ca^{2+} mobilization in G6PT-transfected cells, may only play a complementary role in the MT1-MMP/G6PT signaling axis.

3.3. Gene silencing of both G6PT and MT1-MMP suppresses ERK phosphorylation by S1P

We assessed the impact of MT1-MMP and G6PT in S1P-induced ERK phosphorylation. Cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in the Methods section. Cells were then incubated in the presence of S1P for up to 20 minutes and the extent of ERK phosphorylation was assessed by Western blotting (Fig.3A). In agreement with the Ca^{2+} mobilization effects, we found that S1P triggered a rapid but transient phosphorylation of ERK, while silencing of both MT1-MMP and G6PT genes almost completely abolished ERK phosphorylation (Fig.3B).

3.4. Gene silencing of both G6PT and MT1-MMP suppresses S1P chemotactic effects

S1P is among the most potent chemotactic agents and has been found to induce cell migration in multiple cell models [26]. Given that Ca^{2+} mobilization, as well as ERK phosphorylation, in response to S1P may in part play a role in U-87 cell migration, we assessed the impact of MT1-MMP and G6PT in cell migration. Cells were transiently transfected with siRNA specific for MT1-MMP and G6PT and assessed for migration using modified Boyden chambers (Fig.4A). While basal cell migration (Fig.4B, white bars) remained relatively unaffected, silencing of both the MT1-MMP and G6PT genes resulted in an overall 55-80% decrease in cell migration in response to S1P (Fig.4B, black bars). Interestingly, gene silencing of MT1-MMP and of G6PT did not affect U-87 cell migration in response to bFGF (Fig.4B, grey bars), suggesting that the MT1-MMP/G6PT signaling axis is specifically involved in the S1P-induced chemotaxis but not in non-lipid bFGF-mediated signaling.

DISCUSSION

The ER is primarily known as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. It is also a multifunctional metabolic compartment that controls entry and release of Ca^{2+}, sterol biosynthesis, apoptosis and the release of arachidonic acid [27]. As such, metabolic profiling of cell growth and death in cancer is already used in order to identify the changes in glucose utilization for macromolecule synthesis in cancer [28]. Release of Ca^{2+} from ER stores was observed upon S1P activation [29, this study] and was correlated with the activation of the small GTPase Rac [29]. Interestingly, the coordinate modulation of MMP-2 activity and of MT1-MMP expression/processing by Rac1 is consistent with MT1-MMP's role in cell invasion [30]. Among the several brain tumor-derived cell lines tested, G6PT expression was found to be the highest in U-87 glioblastoma cells [16]. This suggests that metabolic adaptative capacity, in part through G6PT, may regulate the invasive phenotype of aggressive cancer cells. Given the ER localization of G6PT and the crucial role that the ER fulfills as a metabolic compartment, intracellular regulation of Ca^{2+} flux, cytosolic ATP and G6P levels would be among the parameters that G6PT could modulate in the transformed proliferating cells. We thus propose that G6PT further acts as a mediator in the regulation of cancer cell survival and ECM degradation signaling. In fact, evidence that regulation of G6PT expression may function as a bioswitch enabling cells to promote either migration or cell death processes through MT1-MMP cytoplasmic domain signaling was recently provided [24].

Recent evidence has revealed a molecular link between MT1-MMP and S1P signaling in mesenchymal stromal cell and endothelial cell migration, survival, ECM proteolysis, and cytoskeletal rearrangement [19, 24, 31, 32]. We now highlight a significant and new contribution of the MT1-MMP/G6PT signaling axis that may further regulate S1P activity as a second messenger in mobilizing intracellular Ca^{2+} in tumor cells. Several explanations for this MT1-MMP/S1P cooperative effect have been forwarded. Among these, RhoA signaling, a crucial intermediate step in S1P transduction was increased in MT1-MMP-transfected cells [21]. More recently, S1P was shown to regulate endothelial cell locomotion by inducing the association of MT1-MMP with the adaptor protein p130Cas at the leading

edge of migrating cells [33]. Although the ability of S1P to increase cytosolic Ca^{2+} has been characterized in terms of the individual S1PRs involved [4], specific studies to identify the S1PR that cooperates with MT1-MMP activity may however be challenging. Ca^{2+} mobilization being a hallmark of most S1PRs [34], it can be anticipated that one S1PR would take the functional relay upon another. Design and use of specific siRNA targeting each of the S1PRs (S1P-1 to -5) should shed light on how MT1-MMP cooperates with S1PRs functions in the control of cell migration, cell survival, and ECM degradation. It is known that S1P1 and S1P2 receptors are expressed on glioblastoma cells [35], and that S1P2 is likely responsible for the effect of PLC, Ca^{2+} release and PLD [10].

Altered expression, maturation and trafficking of MT1-MMP to the plasma membrane were observed in diabetic states [36, 37], a condition known to upregulate the expression of G6PT [38]. G6PT expression was shown to be downregulated by MT1-MMP in mesenchymal stromal cells [24]. This is in agreement with the fact that cytoskeleton disorganization, an early step in the activation process of matrix metalloproteinase 2 (MMP-2) by MT1-MMP, is also associated with ER dysfunction and subsequent cell death. Furthermore, recent studies suggest that the microtubule cytoskeleton and the centrosomes (the microtubule cytoskeleton-organizing centers) are essential for the trafficking and the internalization of the membrane-bound matrix metalloproteinase MT1-MMP [39], involved in brain tumor cell invasion, ECM degradation and cell-ECM interaction [40]. Our results suggest that an intact, functional MT1-MMP/G6PT signaling axis is required for efficient Ca^{2+} mobilization processes to take place in response to circulating bioactive lipids such as S1P. Given that such axis regulates cell survival and chemotaxis, targeted inhibition of either MT1-MMP or G6PT function may lead to reduced infiltrative and invasive properties of brain tumor cells. Documenting the Ca^{2+}-mediated roles and potential contribution of G6PT in cancer cells will further help optimize or design new anti-tumor therapies.

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Fig.1

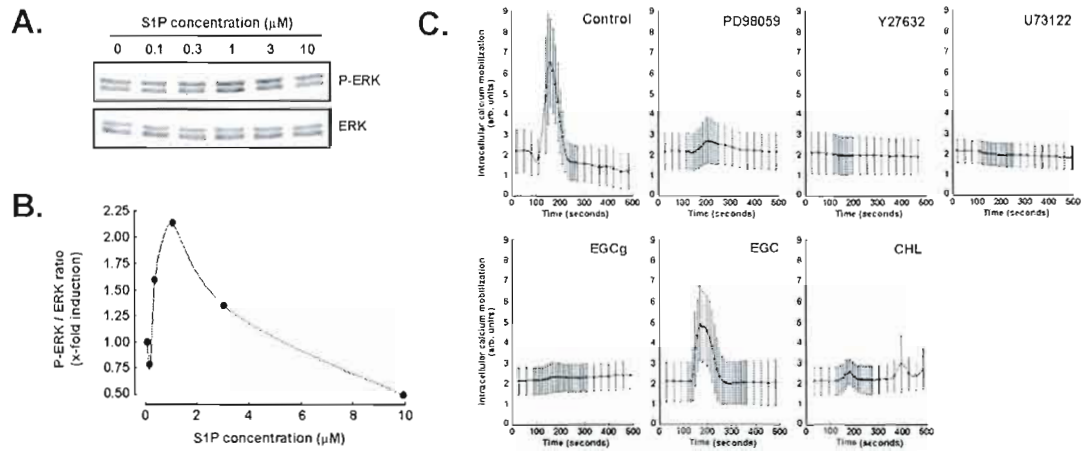

Figure 1: *Pharmacological inhibition of sphingosine-1-phosphate-induced calcium mobilization.* (A) Increasing concentrations of sphingosine-1-phosphate (S1P) were used to treat serum-starved U-87 glioblastoma cells for 2 minutes. Following treatment, 20 μg of cell lysates were loaded on SDS-PAGE and immunoblotting was performed to detect the extent of ERK phosphorylation. (B) Scanning densitometry was used to quantify ERK phosphorylation, and data are represented by a ratio of P-ERK/ERK from one representative experiment. (C) U-87 glioblastoma cell cultures and Ca^{2+} mobilization were induced with 1 μM S1P as described in the Methods section. Cells were pre-treated with either vehicle (Control) or 10 μM EGCg, EGC, CHL, PD98059, U73122, or Y27632 for 30 minutes prior to stimulation with S1P. A representative Ca^{2+} mobilization profile, out of three experiments, is shown.

Fig.2

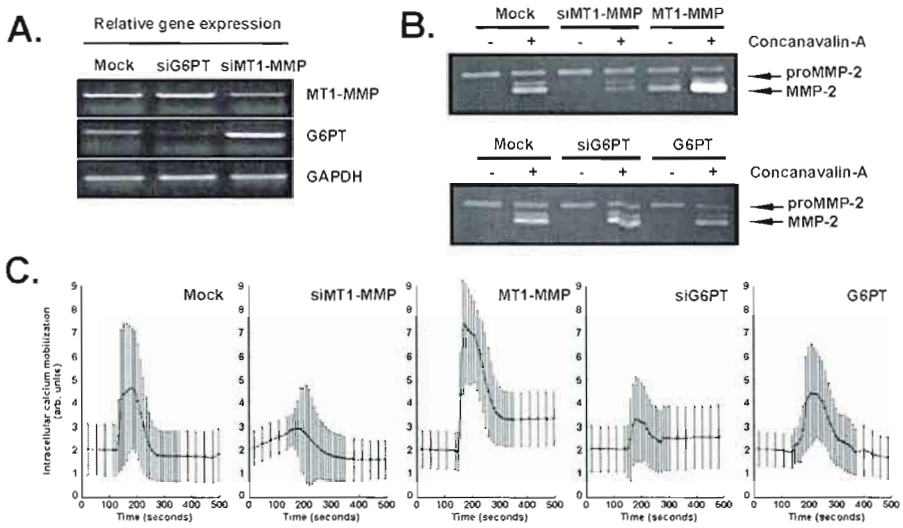

Figure 2: *Gene silencing of MT1-MMP and of G6PT impairs sphingosine-1-phosphate-mediated calcium mobilization.* (A) Total RNA was isolated from mock-, siMT1-MMP-, and siG6PT-transfected U-87 cells. The efficiency of gene silencing was assessed using RT-PCR, and cDNA amplicons were visualized on agarose gels stained with ethidium bromide. (B) Serum-starved mock-, siMT1-MMP-, MT1-MMP, siG6PT, and G6PT-transfected U-87 cells were treated in the presence or absence of 10 μM concanavalin-A for 18 hours. Conditioned media were harvested and the extent of proMMP-2 activation was assessed by gelatin zymography as described in the Methods section. (C) The above transfected U-87 glioblastoma cell cultures were assessed for Ca^{2+} mobilization induced with 1 μM sphingosine-1-phosphate (S1P) as described in the Methods section. A representative Ca^{2+} mobilization profile, out of three experiments, is shown.

Fig.3

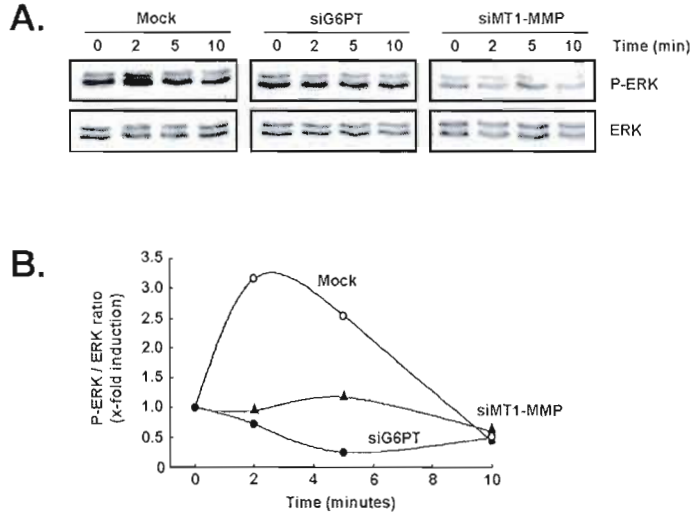

Figure 3 : *Gene silencing of both G6PT and MT1-MMP abrogates ERK phosphorylation by sphingosine-1-phosphate.* (A) U-87 glioblastoma cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in the Methods section. Cells were then incubated in the presence of sphingosine-1-phosphate (S1P) for up to 20 minutes and the expression of phosphorylated ERK (P-ERK) and of total ERK was assessed in cell lysates by Western blotting. (B) Quantification was performed using scanning densitometry, and results are expressed as the ratio of P-ERK/total ERK for each of the conditions. A representative Western blot, out of three experiments, is shown.

Fig.4

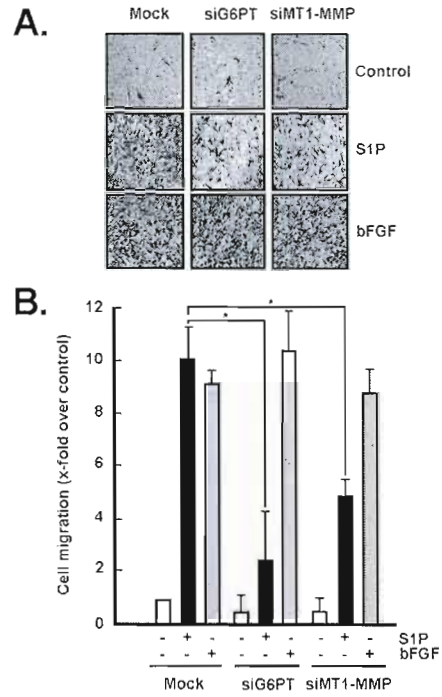

Figure 4 : *Gene silencing of both G6PT and MT1-MMP abrogates sphingosine-1-phosphate chemotactic effects.* (A) U-87 glioblastoma cells were transiently transfected with siRNA specific for MT1-MMP and G6PT as described in the Methods section. Cells were then assessed for migration using modified Boyden chambers in the absence (white bars) or the presence of 1 μM sphingosine-1-phosphate (S1P, black bars), or 20 ng/ml bFGF (grey bars). Cell migration proceeded for 6 hours and was quantified (B). Values shown represent the means ±SD of a representative experiment where 5 random fields per filter were counted for each condition. Probability values of less than 0.05 using Student's unpaired *t*-test were considered significant when S1P- or bFGF-induced cell migration was compared to mock-transfected cells, and an asterisk (*) identifies such significance.

CHAPITRE IV

Discussion et perspectives

Les travaux présentés dans ce mémoire tentent d'approfondir notre compréhension des mécanismes de signalisation régissant la chimiotaxie et la survie cellulaire. Ces processus sont d'une importance capitale dans la mobilisation des cellules et dans le développement tumoral. L'avancement de la recherche dans le domaine des cellules souches, en particulier leur implication dans le développement de cancers, a ouvert la voie à une compréhension différente du développement tumoral. Les études rapportées dans ce mémoire ont permis de mettre au jour des joueurs important qui régissent la survie et la migration des cellules souches mésenchymateuses dans un contexte tumoral.

4.1 Mobilisation et survie des MSC : l'axe MT1-MMP/G6PT

Dans un premier temps, nous avons montré que l'induction de l'expression de la MT1-MMP via des agents perturbateurs du cytosquelette comme la ConA et la cytochalasine-D (CytoD) menait à une augmentation de l'activation de la proMMP-2, menant ainsi à une augmentation de la dégradation de la MEC. De plus, cette expression accrue de MT1-MMP corrèle avec une diminution de l'expression de G6PT, de même qu'à une augmentation de la mort cellulaire. Le rôle important de l'axe MT1-MMP/G6PT dans la mort cellulaire a déjà été relevé chez les glioblastomes (Belkaid et al., 2006). Nous avons pu montrer que l'inhibition de l'activité catalytique de MT1-MMP n'arrivait pas à renverser totalement l'induction de la mort cellulaire provoquée par la ConA, mais que l'absence du domaine intra-cytoplasmique de MT1-MMP pouvait ramener le taux de mort cellulaire au niveau basal. Il s'agissait ainsi d'une première preuve de l'existence d'une cascade de signalisation impliquant MT1-MMP. Une étude récente a permis de relever le rôle important de TIMP-2 dans le processus de transduction des signaux via le domaine cytoplasmique de MT1-MMP. En effet, une interaction entre TIMP-2 et MT1-MMP est suffisante pour gérer la prolifération et la migration cellulaire, et ce, sans impliquer une activité protéolytique et

indépendamment de la dégration de la MEC, via l'activation de ERK 1/2 (D'Alessio et al., 2008).

De plus, nous avons démontré que la surexpression de la protéine G6PT était suffisante pour inhiber l'activation de la proMMP-2 par la ConA, mais non de la mort cellulaire. Il s'agit donc d'un processus de régulation plus complexe qui mérite qu'on s'y attarde plus longuement.

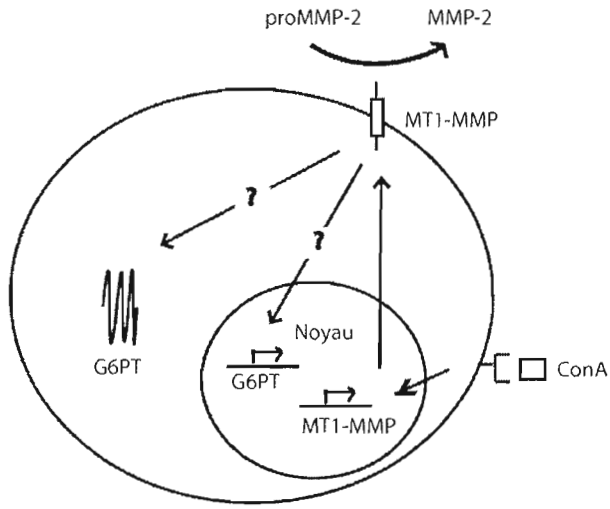

Figure 8 : *Axe de signalisation MT1-MMP/G6PT*

4.2 Utilisation de glycomimétiques pour inhiber les fonctions de MT1-MMP

Les glycoprotéines membranaires jouent un rôle clé dans les interactions cellule-cellule et cellule-MEC, tels que dans les processus métastasiques (Kannagi et al., 2004). Aussi, le design de glyco-molécules de synthèse s'avère de plus en plus populaire à des fins d'utilisation multiples, que ce soit comme véhicule spécifique pour de nouvelles drogues, comme molécule mimant la structure d'une glycoprotéine ciblée synthétisée par la cellule, ou encore comme inhibiteur des fonctions d'autres glycoprotéines (Meutermans et al., 2006). L'utilisation de lectines comme agents permettant la caractérisation des fonctions de certaines glycoprotéines membranaires est une pratique courante en recherche (Komath et al., 2006; Rudiger et Gabius, 2001). La ConA est une lectine pouvant se lier de façon réversible à certaines glycoprotéines membranaires, et par la suite induire une signalisation intracellulaire

menant à l'augmentation de l'expression de MT1-MMP (Gingras et al., 2000). De plus, l'implication de glycoprotéines membranaires dans les changements du cytosquelette et dans l'induction de l'apoptose suite à l'utilisation de la ConA a été démontrée chez des fibroblastes (Kulkarni et McCulloch, 1995). Il était alors valable d'effectuer des tests d'activité de la caspase-3, une protéine clé dans le processus d'apoptose, en clivant plusieurs protéines importantes, notamment la poly-ADP-ribose polymérase (PARP) (Cohen, 1997). Nous avons donc documenté l'augmentation de l'apoptose induite par la ConA chez les cellules souches mésenchymateuses et remarqué que cette dernière était supérieure de 3,5 fois en moyenne par rapport à la condition contrôle. Le point le plus intéressant de cette étude se situe justement dans l'évaluation de l'activité de la caspase-3. En effet, les études précédentes ont permis d'établir l'efficacité des différentes constructions à lier la ConA et ainsi déterminer leur potentiel à inhiber les fonctions de MT1-MMP. Toutefois, nous avions démontré avec les premières figures expérimentales l'incapacité des mannosides construits à inhiber l'activation de la proMMP-2 induite par la CytoD, un autre agent perturbateur du cytosquelette. Il était donc très surprenant de constater que le mannoside ayant le plus grand potentiel de liaison à la ConA, le DM58, arrivait à renverser l'augmentation de l'activité de la caspase-3 induite par la CytoD, contrairement à tous les autres construits. Le mode d'action de la CytoD est différent de celui de la ConA, bien que les effets sur le cytosquelette et l'activation de la proMMP-2 soient les mêmes. Alors que la ConA se fixe à des glycoprotéines de surface, la CytoD pénètre à l'intérieur de la cellule et inhibe la polymérisation des fibres d'actine, induisant ainsi une cascade de signalisation via une perturbation du cytosquelette. Le fait que le DM58 puisse probablement inhiber cette voie de signalisation menant à l'augmentation de l'activité de la caspase-3 implique potentiellement une liaison entre le mannoside et une glycoprotéine de surface venant interférer avec la voie de signalisation menant à l'activation de la caspase-3. Le DM58 pourrait s'avérer être une molécule intéressante pour de futures expérimentations visant dans un premier temps à identifier la ou les glycoprotéines interagissant avec ce dernier.

4.3 L'axe MT1-MMP/G6PT et la mobilisation du calcium intracellulaire

Dans un dernier temps, nous avons voulu évaluer l'implication des joueurs de l'axe MT1-MMP/G6PT sur la mobilisation du calcium intracellulaire en réponse à la sphingosine-

1-phosphate (S1P), un biolipide actif circulant sécrété par les cellules tumorales. On sait déjà que le calcium agit comme second messager dans plusieurs processus physiologiques. Il était déjà documenté que G6PT jouait un rôle dans la séquestration du calcium à l'intérieur du réticulum endoplasmique. De plus, il est connu que la S1P, via son récepteur S1PR, induit elle aussi une mobilisation du flux calcique dont la cascade de signalisation passe par l'activation de la petite GTPase Rac (Mehta et al., 2005). Cette observation corrèle en même temps avec la régulation de l'activité de la MMP-2 et celle de l'expression de MT1-MMP via la même GTPase Rac (Zhuge et Xu, 2001). Aussi, on sait que la surexpression de MT1-MMP entraîne une augmentation de l'activité de signalisation de RhoA, un processus important dans la transduction du signal via la S1P (Annabi et al., 2005). Suite aux nouvelles observations que nous avons faites sur l'axe MT1-MMP/G6PT, il allait de soi de vérifier l'impact d'une modulation de l'expression de ces deux protéines sur la propension qu'avait la S1P à induire le flux calcique. Les résultats parlent d'eux-mêmes. En effet, une diminution de l'expression génique de G6PT ou MT1-MMP entraîne une perte de la mobilisation du calcium intracellulaire suite à une stimulation à la S1P. De plus, l'utilisation d'inhibiteurs des voies de signalisation connues pour être impliquées en aval de MT1-MMP, soit la voie de MAPK, de RhoA/ROK et de la phospholipase C provoque une perturbation du flux de calcium, suggérant que ces dernières entreraient dans le processus de transduction du signal de la voie MT1-MMP/G6PT.

En plus, nous avons démontré que l'épigallocatéchine-3-gallate (EGCg), un inhibiteur potentiel de MT1-MMP issu de notre alimentation, perturbait aussi le flux calcique, alors que l'épigallocatéchine (EGC) n'avait aucun effet sur la mobilisation du calcium. Il semble donc que le groupement gallate soit important dans le processus d'inhibition de l'activité de MT1-MMP, et relève l'importance du groupement gallate dans l'activité de liaison de l'EGCg avec ses partenaires. De plus, il a récemment été démontré que le groupement gallate était aussi nécessaire à l'activité d'autres catéchines, comme l'épicatéchine-gallate (ECg) et la catéchine-gallate (Cg) (Hayes et al., 2006). Il faut de plus faire attention dans l'interprétation des résultats obtenus. On ne peut pas affirmer que l'EGCg se lie directement à MT1-MMP, puisqu'une étude récente a montré que cette catéchine

pouvait lier efficacement la MMP-2 et inhiber son activité d'hydrolyse de la gélatine lors des zymographies. En plus de ce rôle d'inhibition de la MMP-2, il a été relevé dans cette même étude que l'EGCg provoquait une augmentation de l'expression de TIMP-2, l'inhibiteur physiologique de la MMP-2 et de MT1-MMP (Cheng et al., 2005). La compréhension du mécanisme d'action de l'EGCg ne change toutefois pas le résultat final dans notre cas, c'est-à-dire une perturbation du flux calcique induit par la CytoD lors de la présence de l'EGCg.

4.4 Perspectives

L'étude des implications moléculaires de l'axe MT1-MMP/G6PT pourra permettre de mieux comprendre et de mieux cibler les phénomènes de chimiotaxie et de survie cellulaire qui en dépendent. Cet axe étant présent aussi bien chez les cellules tumorales que chez les cellules souches mésenchymateuses, on peut déjà imaginer le rôle important que ces recherches pourront avoir dans la compréhension des processus de développement, de recrutement des cellules souches et d'invasion tumoraux.

Des recherches visant à expliquer les phénomènes moléculaires impliqués dans l'axe MT1-MMP/G6PT sont déjà en cours au laboratoire. Une étude récente a relevé l'importance de l'hypoxie que l'on retrouve au sein des foyers tumoraux sur le niveau d'expression du facteur inductible hypoxique-1α (HIF-1α) et des MMP (Miyazaki et al., 2008). De plus, il est connu que les gènes régulés par HIF-1α sont impliqués dans l'érythropoïèse, l'angiogénèse, le métabolisme de l'ATP de même que dans la prolifération et la mort cellulaire (Lee et Paik, 2006; Zhang et al., 2007). L'une des principales caractéristiques des MSC est leur capacité à survivre en conditions hypoxiques (Chen et al., 2008; Mylotte et al., 2008). G6PT permet, par ailleurs, un contrôle métabolique contribuant à la mobilisation et à la survie des MSC. Les effets d'un environnement faible en oxygène (1,2% O_2) sur l'expression de G6PT sont inconnus et pourraient expliquer en partie la contribution et le recrutement des MSC au centre d'un foyer tumoral. L'environnement hypoxique ainsi que l'hypoxie artificielle recréé suite à un traitement au chlorure de cobalt ($CoCl_2$), induisent à la fois l'expression génique de G6PT, de VEGF et de HIF-1α (résultats non-publiés). L'analyse de la séquence promotrice du gène de G6PT révèle également la présence de sites potentiels de liaison pour HIF-1α. La diminution à l'aide d'un siRNA du gène encodant HIF-1α antagonise l'induction de G6PT

ainsi que de VEGF dans les MSC en conditions de cultures hypoxiques. Finalement, nous avons généré un modèle de MSC exprimant constitutivement HIF-1α, et avons observé une augmentation significative des niveaux endogènes de l'expression de G6PT, de la mobilité cellulaire ainsi que de la résistance à l'apoptose. Nos résultats démontrent donc un nouvel axe de régulation métabolique de G6PT potentiellement dépendant de HIF-1α.

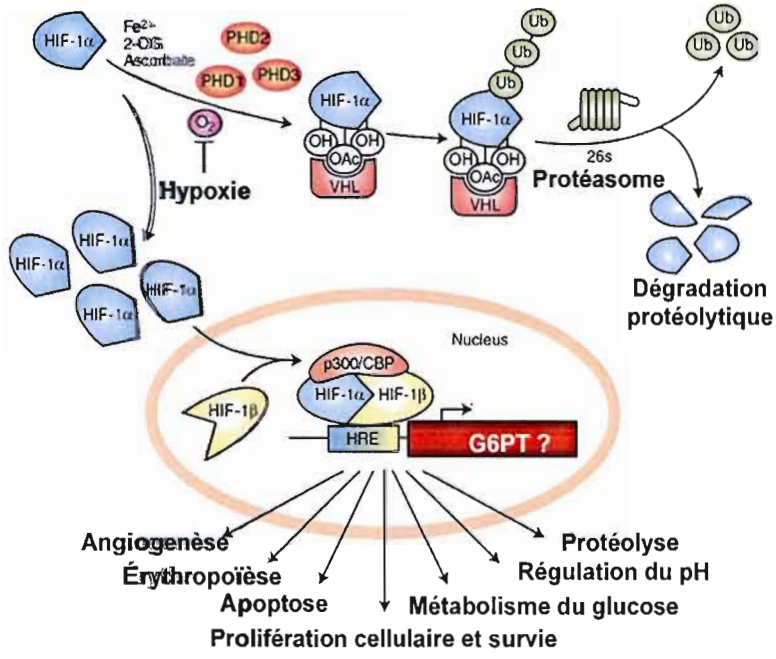

Figure 9 : *Implication possible de HIF-1α dans la régulation de G6PT*

Cet axe contribuerait à la flexibilité métabolique caractérisant les MSC et leur permettant de survivre dans des conditions telles que l'ischémie ou le développement tumoral caractérisé par l'hypoxie et la privation en nutriments. Ces récentes découvertes permettent la mise à jour d'un nouveau joueur dans l'axe de signalisation MT1-MMP/G6PT, soit HIF-1α.

Conclusion

Les connaissances actuelles sur les cellules souches se multipliant à une vitesse incroyable, la caractérisation de leurs propriétés fondamentales et l'évaluation de leur contribution au développement tumoral permettra sans doute l'élaboration de nouvelles stratégies thérapeutiques. En effet, le chemotaxisme important des cellules souches mésenchymateuses en réponse aux facteurs de croissance tumoraux permet de penser que ces dernières sont avidement recrutées aux foyers tumoraux, laissant présager leur possible utilisation en tant que véhicules pouvant transporter d'une façon spécifique des molécules thérapeutiques anticancéreuses. De plus, la mise au jour des cellules souches cancéreuses pave la voie à des thérapies ciblant spécifiquement ces cellules plus résistantes et ayant la capacité à donner naissance à de nouveaux foyers tumoraux.

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