UNIVERSITÉ DU QUÉBEC À MONTRÉAL

NOUVEAU RÔLE D'UN TRANSPORTEUR MICROSOMAL DE GLUCOSE-6-PHOSPHATE DANS LA RÉGULATION DU POTENTIEL INVASIF DE CELLULES DÉRIVÉES DE GLIOBLASTOMES HUMAINS

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ADN	acide désoxyribonucléique
ADNc	ADN complémentaire
ARN	acide ribonucléique
ARNm	ARN messager
bFGF	FGF basique
CD	cytochalasine D
CHL	acide chlorogénique
ConA	concanavaline A
EGCg	epigallocatéchine-gallate
EGF	facteur de croissance épidermique
EGFR	récepteur du EGF
ERK	*extra cellular signal-regulated protein kinase*
FGF	facteur de croissance fibroblastique
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G6PT	transporteur du glucose-6-phosphate
GFP	*green fluorescent protein*
Glc	glucose
GLUT	transporteur glucose
IGF	facteur de croissance analogue à l'insuline
LT	lymphotoxine
MEC	matrice extracellulaire
MMP	métalloprotéase de la matrice extracellulaire
MT1-MMP	métalloprotéase membranaire de type 1
PDGF	facteur de croissance dérivé des plaquettes
Pi	phosphate inorganique
p-ERK	ERK phosphorylé
RE	réticulum endoplasmique
RT-PCR	*reverse-transcriptase polymerase chain reaction*
S1P	sphingosine-1-phosphate
SIDA	syndrome d'immunodéficience acquise
TAF	facteur d'angiogenése tumorale
TGF-β	facteur de croissance transformant bêta
TIMP	inhibiteur tissulaire des métalloprotéases
TNF	facteur de nécrose tumorale

RÉSUMÉ

Les glioblastomes sont, de par leur caractère invasif et infiltrant, des tumeurs cérébrales extrêmement résistantes aux thérapies classiques. Nous avons étudié les propriétés anti-cancérigènes de polyphénols extraits de produits naturels, notamment l'acide chlorogénique (CHL). Le CHL est un puissant inhibiteur fonctionnel du transporteur microsomale de glucose-6-phosphate (G6PT) responsable de l'étape limitante de la conversion du glucose-6-phosphate en glucose (Glc) et en phosphate inorganique par la glucose-6-phosphase (G6Pase) lors de la glyconéogenèse et de la glycogénolyse. En émettant l'hypothèse selon laquelle le système G6Pase se localise exclusivement dans les tissus glyconéogéniques tels que le foie et les reins, nos travaux ont alors pour objectif d'élucider le rôle de G6PT dans des tissus non producteurs de Glc tels les cellules gliales, et particulièrement dans la progression tumorale des glioblastomes. Au cours de notre étude, nous avons testé l'impact de l'inhibition de G6PT soit fonctionnelle par le CHL, soit génique par un siRNA spécifique, sur différents processus impliqués dans la survie et l'invasion tumorale tel que la migration cellulaire et la dégradation de la matrice extracellulaire (MEC) par les métalloprotéases (MMPs).

Expérimentalement, les mesures des niveaux d'ARNm par RT-PCR, démontrent que l'expression génique de G6PT est plus élevée dans les glioblastomes U87, que dans toute autre lignée tumorale cérébrale testée. Le CHL inhibe la sécrétion de la MMP-2, et la migration cellulaire des U87, deux pré-requis pour l'invasion tumorale. La migration induite par G6PT recombinant suite à une transfection des cellules à l'aide du cDNA codant pour ce transporteur est aussi inhibée par le CHL. De plus, le CHL inhibe la migration cellulaire et la phosphorylation de ERK induite en réponse à la sphingosine-1-phosphate, un lysophospholipide abondant dans la MEC cérébrale. Nous démontrons, de plus, que le 2-deoxy-D-Glc et le 5-thio-Glc, deux analogues non métabolisables du Glc et menant à la déplétion de l'ATP intracellulaire, inhibent la sécrétion de MMP-2. Par ailleurs, l'inhibition de G6PT par un siRNA induit une mort cellulaire par apoptose détectée par cytométrie de flux. Une surexpression de la métalloprotéase membranaire de type 1 (MT1-MMP) conduit à une diminution de l'expression génique de G6PT. De manière globale, nos résultats suggèrent que G6PT régulerait certaines fonctions invasives des cellules cancéreuses, et pourrait avoir une implication dans la signalisation intracellulaire de ces dernières, d'où son potentiel comme nouvelle cible pour les thérapies anti-cancer.

Mots-clés : Glucose-6-phosphase, Acide chlorogénique, Glioblastomes.

CHAPITRE I

INTRODUCTION

1. Cancer

1.1 Incidence du cancer

Chaque année, près de 10 millions de nouveaux cas de cancers sont diagnostiqués à travers la planète. Selon l'OMS, cette valeur devrait augmenter à plus de 15 millions de nouveaux cas d'ici 2020. D'après les statistiques canadiennes, environ 38% des Canadiennes et 41% des Canadiens seront atteints d'un cancer au cours de leur vie. Malgré les progrès énormes et les milliards de dollars consacrés à la recherche, à la prévention et à la lutte contre le cancer, certains types de cancers, notamment les tumeurs cérébrales, provoquent le décès de 70% des patients (statistiques Canada). La recherche sur les tumeurs cérébrales demeure d'actualité et s'avère donc une nécessité afin de développer de nouvelles thérapies.

C'est au niveau de l'organisation cellulaire que les activités vitales ont lieu et que les processus pathologiques trouvent leur origine. Le cancer requiert des changements dynamiques au génome qui, pris individuellement, permettent à une cellule d'acquérir des avantages de croissance et de survie en comparaison aux autres cellules de l'organisme. C'est l'accumulation de ces avantages au sein d'une cellule qui mène à sa transformation en cellule cancéreuse. Il en résulte une croissance incontrôlée et la formation d'un néoplasme que l'on nomme aussi tumeur, qui peut croître et s'étendre à d'autres sites pour la formation de métastases (Marieb, 1993).

Les cellules cancéreuses diffèrent souvent des cellules saines avoisinantes par une série de changements phénotypiques telle qu'une division accélérée et incontrôlée, l'invasion de nouveaux territoires, un métabolisme plus intense, l'expression de nouveaux antigènes membranaires, des différences morphologiques (Marieb, 1993). Cette augmentation du nombre de cellules due à une division cellulaire plus fréquente est appelée hyperplasie. Les cellules malignes ressemblent en général à des cellules immatures ou indifférenciées. Il est clair que des facteurs régulant le cours normal de la différenciation y ont subi des altérations et tout l'intérêt du problème réside dans la compréhension des principales causes et mécanismes de transformation des cellules normales en cellules cancéreuses. Les scientifiques avancent plusieurs raisons, toutefois les agents liés à l'environnement et les virus sont les plus susceptibles d'être responsables de cette transformation. D'autres causes reliées au mode de vie ont leurs part de responsabilité dans l'apparition du cancer notamment l'alimentation, le tabagisme actif ou passif, le manque d'exercice physique, l'alcool, les contaminants chimiques (Horwitz, 1999).

1.2 Classification des tumeurs malignes cérébrales

En raison de la variabilité d'action, de la diversité et de la rapidité d'invasion de certains types tumoraux malins, un système de classification s'avère essentiel afin de pouvoir communiquer l'information la plus précise possible, planifier les traitements et tenter de prédire la progression de la tumeur. Plusieurs systèmes de classification ont été crées et utilisés. Cependant, celui de classification de l'OMS demeure l'un des plus utilisés par les cliniciens et les chercheurs. Ce système contient quatre paliers de classification, représentant l'augmentation croissante de la malignité. Cette classification est basée sur six principaux critères : la similarité de la cellule cancéreuse avec la cellule saine (atypie), la mitose, l'observation de croissance

incontrôlée, la présence de nécrose cellulaire, l'invasion ou infiltration cellulaire et la vascularisation tumorale (American Bain Tumor Association, 2004).

1.3 Description des glioblastomes humains

Les astrocytomes sont la forme la plus fréquemment observée des tumeurs cérébrales. D'un point de vue clinique, les astrocytomes de type IV ou glioblastomes, représentent plus de 50% de toutes les tumeurs cérébrales primitives de l'adulte. Le traitement standard de ces tumeurs gliales malignes repose sur la chirurgie de réduction tumorale, lorsqu'elle est possible, et la radiothérapie focalisée délivrant 54 à 60 Gy (Gray : mesure la quantité d'énergie absorbée par la matière ou dose absorbée, 1Gy = 1J/Kg) en 27 à 33 fractions selon la taille et le volume de la cible et la proximité d'organes à risques (Kristiansen et al., 1981 ; Walker et al., 1978). Le glioblastome est une tumeur radiorésistante d'où l'amélioration de l'efficacité de la radiothérapie a été recherchée en contraignant l'hypoxie, un facteur connu de radiorésistance. De plus, il a été rapporté que ce type tumoral était l'un des plus vascularisé chez l'humain avec un taux de mitose très élevé et une masse importante de cellules nécrosées (Lemeke et al., 2004).

Comme toutes les tumeurs, les glioblastomes sont classés en deux catégories : les glioblastomes primaires qui sont des tumeurs qui apparaissent de *novo* en grade IV, et les glioblastomes secondaires, qui sont des tumeurs préexistantes de grade inférieur progressant ultérieurement en grade IV (Aldape et al., 2003). La survenue de certains avènements moléculaires est typique des glioblastomes, comme l'amplification de l'expression de gènes codants pour les récepteurs au PDGF (PDGFR) et à l'EGF (EGFR), les phosphatases, et des homologues de tensines, ou bien au contraire, l'altération de certains gènes suppresseurs de tumeurs tel que P53,

PTEN et rétinoblastomes (Brat et al., 2002). Afin de développer des traitements efficaces contre les glioblastomes, il est primordial de mieux comprendre les phénomènes aboutissants à la tumorigenèse des glioblastomes.

1.4 L'angiogenèse tumorale

L'angiogenèse se définit comme étant la formation de nouveaux vaisseaux sanguins à partir de ceux préexistants (Figure 1), sous l'influence, directe ou indirecte, d'un foyer tumoral (Flamme et al., 1997; Risau, 1998). On réserve le terme angiogenèse aux processus qui se caractérisent par la formation de nouveaux réseaux vasculaires à partir de vaisseaux déjà existants; et le terme vasculogenèse à ceux caractérisant la formation directe de vaisseaux à partir d'un foyer mésenchymateux (Hanahan et Folkman, 1996). L'angiogenèse tumorale ne représente donc qu'un aspect des processus angiogéniques observés dans l'organisme. En effet, elle est nécessaire à de nombreux processus physiologiques humains tels l'implantation et le développement embryonnaire, l'organogenèse et la cicatrisation. Elle l'est également pour des processus pathophysiologiques comme la croissance et l'invasion tumorale (locale et métastatique), les rétinopathies, les hémangiomes infantiles, l'arthrite rhumatoïde, le psoriasis, les ulcères duodénaux, la fibrose etc, ... (Hanahan et Folkman, 1996). Lorsque les cellules tumorales forment une masse de plus d'un millimètre cube, l'apport en nutriments et en oxygène par les vaisseaux environnants n'est plus suffisant, il y a hypoxie. En fait, il est clairement établi que l'hypoxie, phénomène cellulaire caractérisé par une concentration en oxygène déficiente, entraîne l'augmentation de l'expression des métalloprotéases matricielles (MMP) qui dégradent la matrice extracellulaire (MEC) (Lelievre et al., 1996; Ingber et Folkman, 1989) permettant la migration et la formation de nouveaux capillaires (Beck et al., 1997; Yancopoulos et al., 1998).

1.4.1 Interactions entre les cellules cancéreuses et les cellules endothéliales

Durant les dernières années, la compréhension des mécanismes de l'angiogenèse a permis d'améliorer l'approche thérapeutique dans le traitement du cancer. Les cellules endothéliales sont parmi les plus stables de l'organisme, leur demi-vie excède 500 jours, mais cette dernière peut être réduite à quelques jours seulement lors des processus angiogéniques. La conversion des cellules endothéliales (latentes) en cellules endothéliales (actives) implique leurs stimulations par des facteurs angiogéniques, les plus couramment observés étant le bFGF et le VEGF. Ce dernier stimule les collagénases et les activateurs du plasminogène (Hanahan, Folkman, et al., 1996).

Les facteurs de croissance, tels que : les facteurs de croissance épidermiques (EGF), les facteurs de croissance de l'endothélium vasculaire (VEGF), les facteurs de croissance des fibroblastes (FGF), les facteurs de croissance dérivés des plaquettes (PDGF), les facteurs de croissance des nerfs (NGF), les facteurs d'angiogénèse tumorale (TAF), les facteurs de croissance analogues à l'insuline (IGF) et les cytokines (lymphokines et monokines), stimulent et assurent le fonctionnement normal des cellules (Yoshida et al., 1990 ; Kermorgant et al., 1998 ; Aparicio et al., 1998). Les cytokines comprennent les interleukines (IL-1 à IL-13), le facteur de nécrose tumorale (TNF), le facteur de croissance transformant bêta (TGF-β), les interférons gamma, la lymphotoxine (LT). Suite à la stimulation par ces divers stimuli, les cellules endothéliales peuvent produire de la sphingosine-1-phosphate (S1P). Cette production est catalysée par la sphingosine kinase et est le résultat d'une dégradation ou d'une déphosphorylation de la palmitaldéhyde et/ou de la phosphoéthanolamine.

La S1P est un lysophospholipide secrété lors de la stimulation des plaquettes, de macrophages, et aussi de nombreux types de cellules tumorales. Il a été récemment

démontré que ce composé stimulait la migration des cellules endothéliales ainsi que la cicatrisation cellulaire. Les mécanismes moléculaires impliquant les réponses angiogéniques induites par le S1P ne sont pas très bien compris. Cependant un mécanisme impliquant l'action du S1P est clair, il s'agit de son interaction à la membrane plasmique avec des récepteurs couplés aux protéines G permettant la différenciation de certains gènes responsables de la formation de vaisseaux sanguins (Hla et al., 2000).

1.4.2 La matrice extracellulaire

La matrice extracellulaire (MEC) est un élément essentiel du micro-environnement cellulaire, qui régule les interactions entre les épithéliums et leurs stromas. La MEC permet le maintien de l'intégrité structurale des organismes vivants puisqu'elle sert de charpente stabilisant la structure physique des tissus. Cette matrice n'est pas une structure inerte et stable, mais plutôt en équilibre dynamique entre la synthèse et la dégradation de ses composantes.

Dans cette partie on s'intéresse particulièrement à la MEC gliale, entourant les cellules tumorales cérébrales. En plus d'être impliquée dans le développement cérébral, elle est indissociable des phénomènes de différenciation, de migration neuronale et de formation synaptique. Son espace extracellulaire est principalement rempli par un enchevêtrement complexe de macromolécules. La glie renferme les collagènes (type I, II, et III), la laminine, la fibronéctine, et une variété de protéoglycanes. L'augmentation de ces composantes peut se produire par l'accroissement de la synthèse et/ou par une réduction de la dégradation des protéines. La dégradation des protéines de cet environnement cellulaire fait intervenir une cascade enzymatique impliquant successivement plusieurs familles de protéases : les cystéines-protéases (Cathepsine B, Cathepsine L), les aspartates-protéases

(Cathepsines D), les serines-protéases et finalement les métalloprotéases matricielles (MMP) (Cuvelier et al., 1997).

1.4.3 Les métalloprotéases

Lors des phénomènes d'invasion, les cellules tumorales secrètent des MMP afin de dégrader la MEC. Les MMP sont une grande famille d'endopeptidases qui peuvent dégrader tous les types de MEC. A ce jour, 28 membres ont été identifiés, dont au moins 22 chez l'humain. Le tableau 1 illustre quelques unes d'elles avec leurs principaux substrats. Il existe deux types majeurs de MMP: celles qui sont solubles ou sécrétées dans le milieu extracellulaire et celles qui sont ancrées à la membrane cellulaire (Wossner, 1998). Nos études ont principalement porté sur le rôle de deux MMP : la MMP-2 (Gélatinase A) et la MT1-MMP (MMP-14) dont la structure moléculaire est schématisée dans la figure 2.

La MMP-2 est sécrétée sous forme de zymogène (pro-MMP-2) et possède par le fait même 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 (Brikedal-Hansen et al., 1993). Cette enzyme hydrolyse préférentiellement la gélatine et le collagène de type IV, mais peut aussi cliver les collagènes de type I, II, VII et X, l'élastine, la décorine, la fibronectine et les protéoglycanes du cartilage (Lehti et al., 2002).

Le mode d'activation de la pro-MMP-2, nécessite la liaison de la pro-enzyme à un complexe formé de la MT1-MMP/TIMP-2 (inhibiteur tissulaire de métalloprotéase) à la surface des cellules (Polette et al., 1998). TIMP-2 se lie via son domaine N-terminal au domaine catalytique de la MT1-MMP, inhibant l'activité de cette dernière. Par la suite, la pro-MMP-2 se lie à l'extrémité C-terminale de la TIMP-2 via son domaine hémopéxine localisé au niveau de la membrane cellulaire,

résultant en la formation d'un complexe tri-moléculaire concentré à la surface (Atkinson et al., 1995). Une MT1-MMP adjacente et libre peut alors initier le clivage de la pro-MMP-2 entre l'Asn 37 et la Leu 38, générant la forme intermédiaire de la MMP-2 à 64 KDa. Ce clivage initial déstabilise la structure du propeptide, entraînant l'autoprotéolyse entre l'Asn 90 et la Tyr 91 de la MMP-2 et la libération du peptide ainsi que la MMP-2 complètement active (Strongin et al., 1995). L'activation de la pro-MMP-2 est également associée avec une augmentation d'ARNm de la MT1-MMP (Yu et al., 1998). Plusieurs agents sont reconnus pour induire une augmentation du niveau d'ARNm de la MT1-MMP tel que le TNF-α, le bFGF et la concanavaline A (ConA) (Murphy et al., 1999). La ConA est une lectine induisant l'activité de la MT1-MMP et par le fait même l'activation de la pro-MMP-2. Elle semble agir principalement en augmentant la production de l'ARNm de la MT1-MMP. Certaines études ont démontré l'apparition d'une forme de la MT1-MMP à 43 kDa suite à l'activation de la pro-MMP-2. Cette forme est inactive, puisque le domaine catalytique est clivé, suggérant un mécanisme d'auto-régulation de la MT1-MMP. En effet, l'équipe de Yu a observé que l'augmentation du calcium intracellulaire avec l'ionomycine ou la thapsigargine inhibe l'effet d'activation de la pro-MMP-2 induite par la ConA. Ces inhibiteurs bloquent le clivage de la MT1-MMP à 43 kDa sans affecter le niveau d'ARNm suggérant que le clivage de la MT1-MMP à 43 kDa est requis pour sa fonctionnalité (Yu et al., 1998). La proMT1-MMP est activée à l'intérieur de la cellule par une protéinase de la famille des furines, une pro-protéine convertase présente dans l'appareil de Golgi (Sato et al., 1997). Des études ont démontré que l'activation de la MT1-MMP par la furine n'est pas un pré-requis pour l'activation de la Pro-MMP-2 (Murphy et al., 1999).

1.4.4 La migration cellulaire lors de l'invasion tumorale

La migration cellulaire représente un processus essentiel pour une variété d'évènements biologiques. Il a été établi que l'invasion tumorale requérait le

remodelage de la MEC suite à l'activation des protéases à sérine ou des MMPs (Forget et al., 1999). La migration des cellules endothéliales avoisinantes à la tumeur est induite par la sécrétion des facteurs de croissances cités ultérieurement et requiert également l'action des MMP. Lors de la migration cellulaire en général, et celle des glioblastomes en particulier, la cellule fait preuve d'une grande souplesse due à un synchronisme, et une coordination entre trois évènements majeurs :

1. L'adhésion intercellulaire par des complexes formés par les récepteurs d'intégrines et des cadhérines.
2. L'adhésion substrat-cellule qui dépend de la réorganisation du cytosquelette.
3. La dégradation de la MEC par les MMPs (Lefranc-Brotchi et al., 2005).

2. Contrôle du métabolisme glucidique

De récentes études ont démontré une augmentation du métabolisme en général, et de la glycolyse en particulier, dans les cellules cancéreuses comparativement à des cellules saines (Timperley et al., 1980). La cause majeure de l'augmentation du métabolisme glucidique est un besoin urgent en ATP pour subvenir aux besoins de la masse tumorale croissante, et contrer la diminution en oxygène causée par un manque de vascularisation. Il faut noter que l'accroissement du métabolisme glucidique diffère d'un type de tumeur à un autre, mais ce qui demeure commun à tous les types tumoraux est la surexpression des récepteurs au glucose (GLUT) à la surface des cellules, et l'augmentation de l'activité des hexokinases, deux étapes cruciales dans la production d'ATP. Le tableau 2 présente la surexpression de ces dernières dans plusieurs types tumoraux. (Moreno-Sanchez et al., 2007).

Dans un corps sain, le foie et le cortex rénal contribuent au maintien d'un niveau de Glc sanguin stable (± 5 mM) (Gerin et al., 2001). En période de jeûne, le Glc sanguin dont la capture est facilitée par des transporteurs spécifiques (GLUT1 et

GLUT2 pour le foie, GLUT1 et GLUT4 pour le muscle), est phosphorylé par une glucokinase dans les hépatocytes pour former du glucose-6-phosphate (G6P). Ce dernier est ensuite métabolisé en empruntant la voie glycolytique menant à la formation de pyruvate, ou bien la voie glycogénique pour former du glycogène. Le G6P est aussi formé à partir du glycogène par la voie glycogénolytique, et/ou à partir du pyruvate par la voie néoglucogenique, qui peut être par la suite, hydrolysé par la glucose-6-phosphatase (G6Pase) avant d'être libéré dans la circulation sanguine. Ainsi, le système G6Pase joue un rôle clé dans l'homéostasie du Glc (Figure 3).

2.1 Localisation tissulaire du système G6Pase

Par des techniques de Northern Blot et de RT-PCR (reverse transcription polymerase chain reactions), l'ARNm des composantes du système G6Pase a pu être détecté. Des niveaux élevés d'expression de ce dernier sont retrouvés dans le foie et le rein, en raison de leur activité glyconéogenique (Hers, de Duve, et al., 1950). Dans un état de jeûne ou de diabète, d'autres cellules semblent aussi pouvoir exprimer la G6Pase telles que les astrocytes, les cellules musculaires, les cellules β des îlots pancréatiques. Cependant l'activité dosée dans des microsomes de ces tissus est négligeable comparée à celle mesurée dans le foie et le rein.

2.2 Le système G6Pase

Deux théories sont proposées en ce qui concerne l'organisation moléculaire du système G6Pase dans la membrane du réticulum endoplasmique (RE). D'une part, un modèle conformationnel qui implique la reconnaissance et la liaison du substrat à la G6Pase sur la surface externe de la membrane microsomale et mène à son hydrolyse et à la libération des produits de la réaction dans la lumière microsomale (Gerin et al., 2001). D'autre part, le modèle de transport du substrat qui fut confirmé avec le temps par des études cinétiques et des techniques de clonage moléculaire.

2.2.1 Le modèle de transport de substrat

En 1975, Arion et ces collaborateurs, proposèrent un nouveau modèle pour expliquer les fonctions du système G6Pase (Arion, Wallin, et al., 1975). Arion postule que G6Pase est une phosphatase associée avec un complexe de protéines spécifiques, comprenant une unité catalytique orientée vers la lumière du RE, un transporteur réversible du G6P cytosolique vers le RE (G6PT, T1), un transporteur de phosphate inorganique (T2) et un autre de Glc (T3). Plusieurs études ont confirmé le modèle d'Arion, les plus pertinentes sont les suivantes :

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 de T1.
3. L'existence de maladie (Glycogénose Type 1b) due à une déficience de T1.
4. La démonstration que le produit d'hydrolyse du G6P par la G6Pase sont les premiers à être libérés dans la lumière microsomale.

Cette théorie attribue donc à T1 un rôle clé dans l'étape limitante de la conversion du G6P en Glc et Pi (Van Schaftingen et al., 2002).

2.3 Identification et caractéristiques des composantes du système G6Pase

Selon ce qui a été cité auparavant, la G6Pase est un système composé de plusieurs protéines, une phosphotransférase (G6PT, T1), une phosphohydrolase et deux translocases T2 et T3, toutes les quatre, localisées dans la membrane du RE (Figure 4). La théorie d'Arion repose essentiellement sur le transporteur microsomal de G6P (T1, G6PT). G6PT est codé par un seul gène de 5.3Kb, contenant 9 exons localisés sur le chromosome 11q23 (Annabi et al., 1998; Baochuan et al., 1998). Plusieurs groupes de recherche ont mis au point des méthodes pour caractériser cette protéine. Tout d'abord une méthode chimique, proposée par le groupe d'Arion qui compare les concentrations du G6P et Man-6-P intramicrosomal après l'incubation de

microsomes hépatiques avec ces produits et suivie de centrifugation sur couche de silicone contenant du $HClO_4$. Mais cette méthode reste controversée à cause d'une grande probabilité d'avoir des concentrations négatives. (Ballas, Arion, 1977). L'existence d'inhibiteurs spécifiques au transporteur de Glc-6-P est une preuve de plus de son existence, en effet le groupe de Karnovski (Zoccoli et al., 1980) a utilisé le 4.4'-di-isothiocyanostilbene-2-2'-disulphonate (DIDS) un inhibiteur de transporteurs d'ions. L'incubation du [^{3}H]DIDS avec des microsomes de foie forme un lien covalent avec un polypeptide de 54 KDa, qui peut être renversé par le Glc-6-P, suggérant ainsi que la protéine marquée de 54 KDa est le transporteur de Glc-6-P (Zoccoli, et al., 1982). Plusieurs études ont suivi et pu déterminer par des inhibiteurs spécifiques, l'existence de T1 avec une masse apparente de 46 kDa et qui migre à un poids de 37 kDa dans un gel SDS/Page (Herling et al., 1999 ; Parker et al., 1998 ; Kramer et al., 1999). Par la suite le groupe de Chou a cloné le cDNA du G6PT issu de 3 espèces différentes (humain, murin, rat) en 1998. Ils ont déduit après le séquençage du gène du G6PT (Figure 5 et 6), que ce dernier est une protéine de 429 acides aminés; l'analyse algorithmique de la séquence prédit l'existence de 9 à 10 domaines transmembranaires (Hoffman et al., 1993). Des études en cinétique enzymatique ont confirmé l'existence d'un transporteur (T2) commun pour le Pi, le pyrophosphate inorganique (Ppi) et le carbamyl phosphate, distinct du T1 (Arion et al., 1980). De récents résultats suggèrent que T1 est un pore traversant la membrane du RE de part et d'autre et non pas un transporteur, étant donné que son fonctionnement demande moins d'énergie que celui d'un transporteur (Gerin et al., 2001). Bien qu'au départ T2 ait été identifié à l'aide d'anticorps dirigés contre un transporteur mitochondrial de Pi/OH^- (Gibb et al., 1986). En 1980, Arion et ces collaborateurs ont expliqué la sortie du Glc du RE hépatique après hydrolyse du G6P par un transporteur de Glc (T3).

2.4 Contrôle nutritionnel et hormonal du système G6Pase

L'activité physiologique de la G6Pase est régulée par la concentration du substrat, en effet le Km de la G6Pase pour le G6P (2-3 mM) est plus élevé que le contenu intracellulaire en G6P (0.05-1 mM). Dans des hépatocytes purifiés, Ichai et ses collaborateurs ont observé que le glucagon augmente les niveaux de Glc mais diminue la concentration intracellulaire du G6P, (Ichai et al., 2001). Par ailleurs, le Glc peut inhiber le système G6Pase à court terme. Ceci peut être expliqué par le fait qu'à de fortes concentrations, le Glc induit la glycogenèse (synthèse de glycogène), et diminue la concentration de G6P afin de réduire la formation du Glc par le système G6Pase (Hers, 1976). A long terme, une concentration de glucose de 27.5 mM provoque une augmentation de la synthèse d'ARNm de la G6Pase. Ceci indique l'importance des métabolites de la glycolyse et la glycogénogenèse dans l'expression de la G6Pase. Cet effet peut aussi être observé en réponse à d'autres substrats tel que le xylitol, le fructose, le mannose, et le glycérol, qui sont aussi capables d'entrer dans les voies de la glycolyse et la glycogénogenèse à différents niveaux. L'effet du Glc est exercé à un niveau transcriptionnel et en partie par la stabilisation de l'ARNm de la G6Pase (Massillon, 2001).

Un diabète (type 1 ou 2), ou un état de jeûne sont responsables d'une augmentation de 2-3 fois de l'activité de la G6Pase hépatique. Ce qui corrèle avec la diminution de l'activité de la G6Pase *in vivo* observée à des niveaux élevés d'insulinémie. L'effet inhibiteur de l'insuline sur la G6Pase *in vivo* étudié sur des hépatocytes en culture, confirme l'existence d'un mécanisme d'inhibition passant par des promoteurs et des protéines spécifiques (Streeper et al., 1997; Barthel et al., 2001; Schmoll et al., 2000; Dickens et al., 1998; O'brien et al., 2001). L'administration de glucocorticoïdes à des rats augmente, quant à lui, la G6Pase dans les microsomes de foie dont l'effet est complètement inhibé par l'insuline (Hemmerle et al., 1997).

L'augmentation de l'expression de la G6Pase induite par l'AMPc s'exerce principalement à un niveau transcriptionnel (Chatelain et al., 1998).

2.5 Déficience du système G6Pase

Les maladies du métabolisme du glycogène peuvent être héréditaires et la conséquence d'une déficience de certaines enzymes spécifiques impliquées dans la synthèse ou la dégradation du glycogène. Les glycogénoses constituent un groupe de maladies génétiques affectant les voies métaboliques de mise en réserve de glucose sous forme de glycogène et l'utilisation du glycogène pour le maintien de la glycémie et la fourniture d'énergie. Il existe sept types de glycogénose (GSD) avec des caractéristiques biochimiques et cliniques bien distinctes. On s'intéressera à la glycogénose de type I, causée par une défaillance du système G6Pase.

La GSD de type I ou maladie de Gierke est une maladie autosomique récessive. Les patients présentent les signes cliniques suivants : une hépatomégalie et une néphromégalie dues à une accumulation de glycogène dans le foie et les reins, et les signes biochimiques suivants : une acidose lactique, une hypoglycémie, une hyperlipidémie, et une hyperurecémie (Van Schaftingen et al., 2002). Deux formes majeures sont décrites, la GSD de type Ia et Ib.

2.5.1 La GSD type Ia

C'est la forme la plus fréquente de GSD type I (80%), causée par une déficience de l'activité catalytique de la G6Pase. L'abolition de l'activité de la G6Pase est due à des mutations localisées dans le gène codant pour cette enzyme (Lei et al., 1995 ; Rake et al., 2000). L'équipe de Chou et Mansfield a répertorié 31 mutations dont la plupart sont localisées dans des régions non codantes, d'autres sont des insertions ou des délétions avec déplacement du cadre de lecture. Des transfections transitoires de cellules COS ont démontré que ces mutations réduisent complètement l'activité de la G6Pase (Lei et al., 1993 ; Lei et al., 1994; Kishnan et al., 1997). Un modèle transgénique de souris G6Pase$^{-/-}$ a été généré par recombinaison homologue. Les souris présentent les mêmes symptômes que la GSD type I avec un retard de croissance et un niveau élevé de lactate sanguin. De plus, l'hypoglycémie des animaux atteints de GSD type Ia n'est pas normalisable à la suite de l'administration d'adrénaline ou de glucagon. Certaines études ont rapporté l'usage de thérapie rétrovirale comme nouvelle alternative de traitement de la GSD type Ia (Zingone et al., 2000).

2.5.2 La GSD type Ib

A la fin des années 50, on a identifié pour la première fois la GSD de type Ib chez des patients qui présentaient les mêmes symptômes que ceux de la GSD type I, mais avec une activité de la G6Pase hépatique normale. L'identification du cDNA muté dans la GSD type Ib confirme que cette pathologie est due à une défaillance du transporteur T1. Soixante-neuf différentes mutations ont été observées chez des sujets avec la GSD type Ib, dont la majorité sont des substitutions dans des régions conservées, générant une perte complète de l'activité de G6PT (Chen, Lin, et al., 2000). Chen et ces collaborateurs ont étudié des mutations dans des régions

différentes du gène. Quelques unes sont répertoriées dans le tableau 3. La mutation W393X qui est responsable d'une modification au milieu de l'hélice 10, diminuerait complètement l'expression de G6PT, contrairement à la substitution R415X, qui affecte la dernière boucle cytosolique de la protéine, en diminuant l'expression de 70%, tout en conservant l'activité du transporteur. Alternativement, la GSD type Ib ne dépend pas seulement de l'activité de T1 ou de son expression, mais aussi de son mode d'insertion dans la membrane du RE (Chen et al., 2000).

2.5.3 La GSD type Ic

La glycogénose de type Ic est due à une déficience du transporteur de phosphate et pyrophosphate (T2). Toutefois, le diagnostique doit être posé avec précaution, car on a observé une latence dans l'activité de la G6Pase, qui peut être causé par l'accumulation du Pi intramicrosomal (Nordlie et al., 1983 ; Nordlie et al., 1992). Le groupe de Chou a toutefois montré l'absence de mutations au niveau des gènes codant pour T1 et l'unité catalytique, chez les patients atteints de cette forme de GSD. (Lei et al., 1995; Lin et al., 1999).

2.6 Les inhibiteurs de T1

L'existence d'inhibiteurs spécifiques du G6PT est une preuve de plus pour confirmer le modèle de transport du substrat d'Arion. Le DIDS, cité au paragraphe 2.3, est parmi les premiers inhibiteurs de T1 qui a été découvert. L'acide 3-mercaptopicolinique, connu pour inhiber la phosphoénolpyruvate carboxykinase, semble également avoir une action inhibitrice sur T1 (Foster et al., 1994). Une nouvelle génération de produits avec une action spécifique contre T1, sont synthétisés ou extraits à partir de plantes. Parmi ceux-ci, l'acide chlorogénique (CHL), un inhibiteur de T1 dans des microsomes intacts (Ki : 0.5 mM) (Arion et al., 1997) est

disponible commercialement. Il est considéré comme un hypoglycémiant, tout en générant une accumulation du glycogène hépatique et rénal (Hemmerle et al., 1997). Récemment, des nouveaux inhibiteurs ont été identifiés. Le Kodaistain A et C ont été isolés d'*Aspergillus Terreus* et ont respectivement des Ki de 80 et 130 nM. Par ailleurs, le Mumbaistatin, isolé de *Streptomyces,* avec un Ki de 5nM est considéré parmi les plus puissants inhibiteurs de G6PT (Vertesy et al., 2001).

En conclusion, ce chapitre est un résumé de la bibliographie des deux thématiques sur lesquels s'est basée notre recherche : l'invasion tumorale et le transporteur de G6P. Les chapitres II et III regrouperont les prémisses de nos travaux ainsi que les manuscrits publiés respectivement.

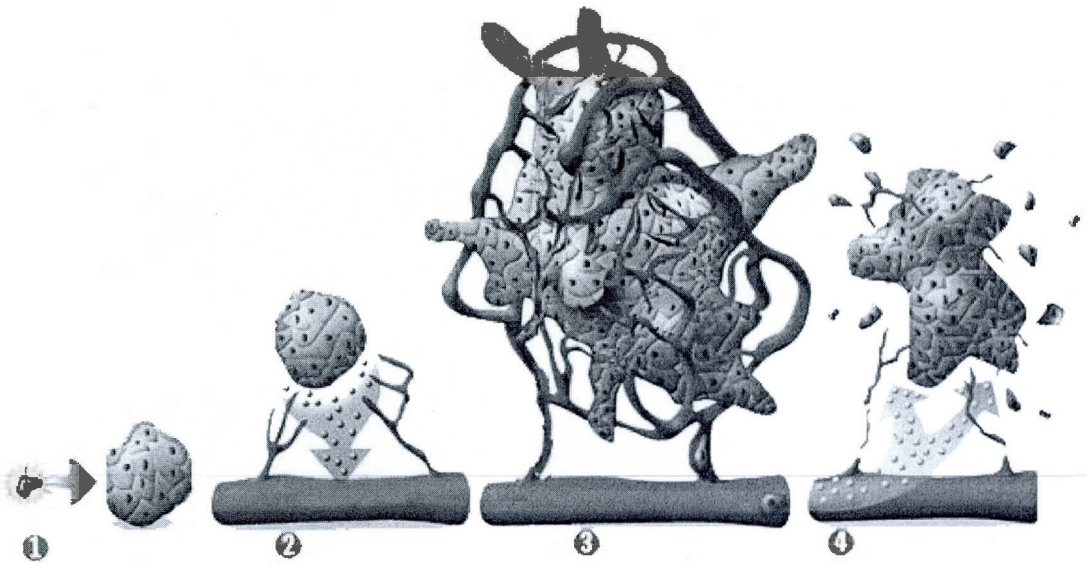

Figure 1. *L'angiogénèse tumorale.* Les cellules tumorales situées à distance d'un vaisseau sanguin sont en hypoxie (1) et sécrètent des facteurs angiogéniques (2). En réponse à ces facteurs, la tumeur se vascularise, et l'apport adéquat en oxygène et en nutriments lui permet alors de croitre (3). Un nombre de ces cellules va se détacher et passer dans la circulation sanguine pour former des métastases. L'inhibition de la vascularisation peut s'avérer efficace pour diminuer le volume de la masse tumorale (4).

Tableau 1 . *Liste des différentes métalloprotéases.*

Groupe	Membres	Nomenclature des MMPs	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	Pas connu
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
Autres			
	Métalloélastase	MMP-12	Élastine
	Énamélysine	MMP-20	Pas connu
	MMP *xénopus*	MMP-18	Pas connu
	Pas connu	MMP-19	Aggrecan
	Pas connu	MMP-23	Pas connu

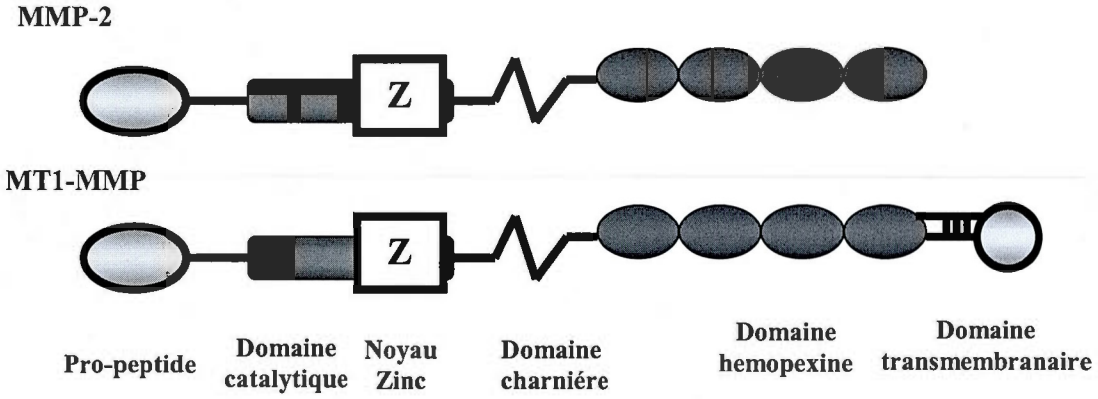

Figure 2. *Structure moléculaire de la MMP-2 et de la MT1-MMP*. La MMP-2 et la MT1-MMP sont des métalloprotéases retrouvées sous forme de propeptides. Le clivage du prodomaine permet leur activation. Dans le cas de MT1-MMP, un domaine membranaire lui permet de s'ancrer dans la membrane plasmique.

Protéines surexprimées	Type de cellules tumorales
Transporteurs de glucose (GLUT1,2,3)	HepG2 carcinomas, tumeurs cérébrales, cancer du sein, leucémie, tumeurs gastriques, cutanées et oesophagiénnes.
Hexokinase (HK)	Carcinome HeLa, ependymomes, astrocytomes.
Phosphofructokinase type 1 (PFK-1)	HL-60, leucemies myeloides, lymphomes et gliomes.
Phosphofructokinase type 2 (PFK-2)	Carcinome de Lewis, HeLa, HepG2, cancer du sein et leucemie K562.
Toutes ces protéines sont exprimées simultanément	HeLa, **glioblastomes (U87)**, cancer de la prostate et tumeurs rénales.

Tableau 2. *L'expression des protéines du métabolisme glucidique dans les différents types tumoraux.* GLUT, HK, PFK-1, sont toutes des enzymes ou protéines cruciales dans le métabolisme glucidique. Leur surexpression est associée à un mauvais pronostic. Ce qui est important à souligner, c'est la présence de ces dernières ensemble dans les glioblastomes, le cancer de la prostate des tumeurs connues pour leurs agressivités et leur résistance aux traitement de chimiothérapie (Moreno-Sanchez, et al., 2007).

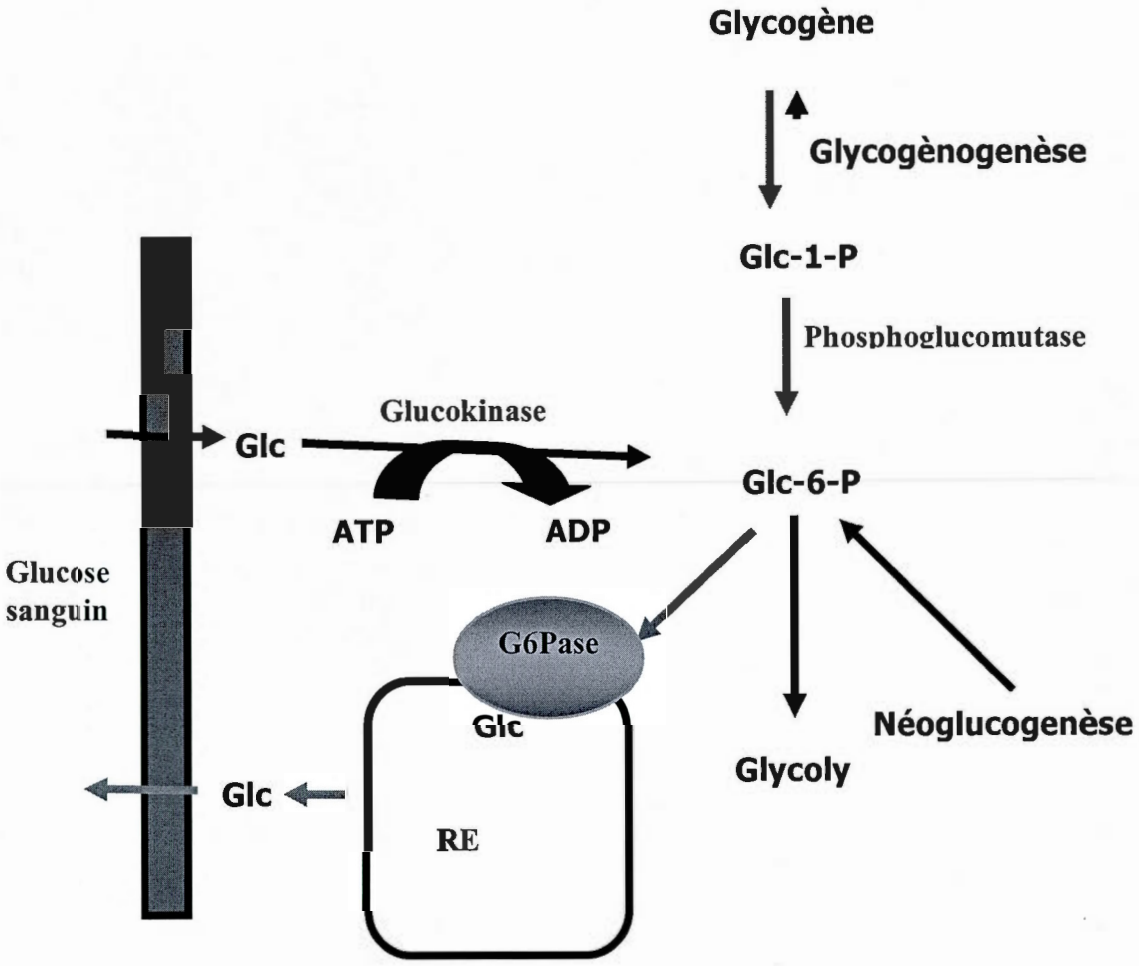

Figure 3. *Glycogénolyse et néoglucogenèse* . Le niveau de glucose sanguin est regulé par deux voies principales, la néoglucogenèse et la glycogénolyse, générant du Glc-6-P. Une seule enzyme, la G6Pase localisée dans le RE, catalyse l'étape finale de la production de glucose.

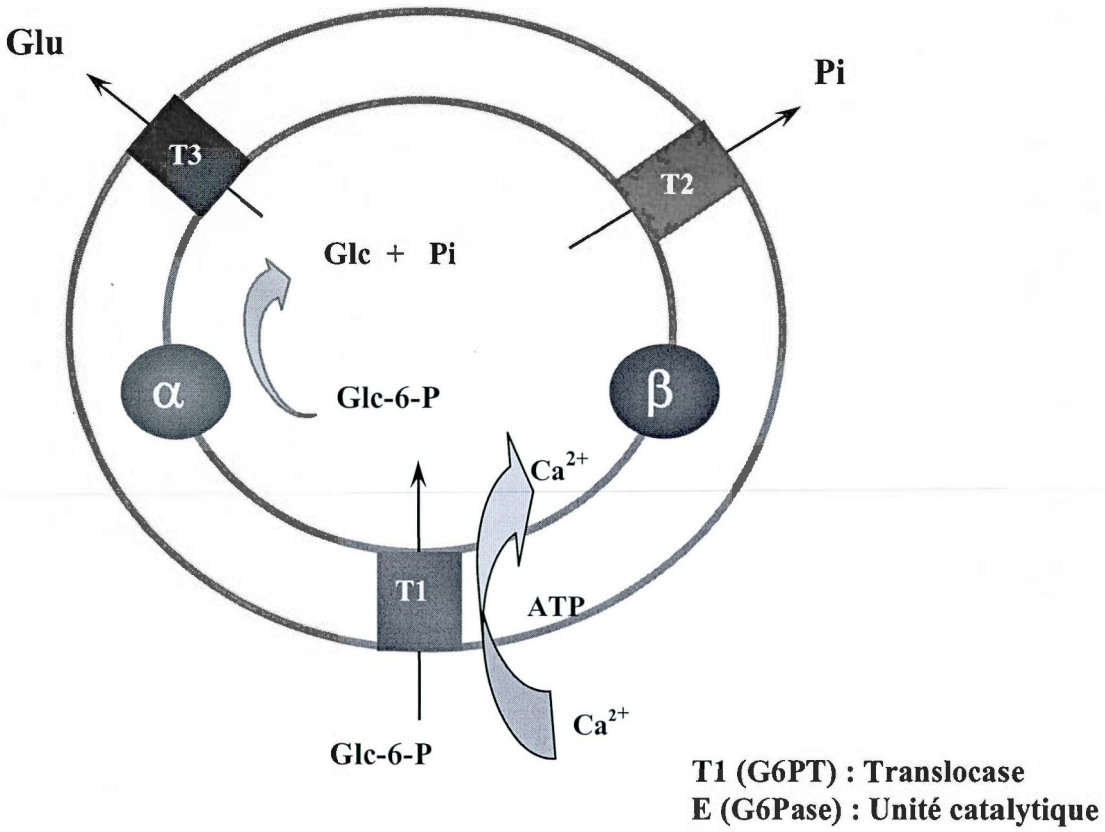

Figure 4. *Le système G6Pase microsomal.* Selon le modèle de transport de substrat, l'entrée du G6P se fait par l'intermédiaire d'un transporteur spécifique T1(G6PT). L'hydrolyse du Glc-6-P se fait par une unité catalytique (E, G6Pase). Ce sont deux protéines complètement différentes l'une de l'autre. L'évacuation des produits de la réaction se fait par deux autres transporteurs, T2 et T3.

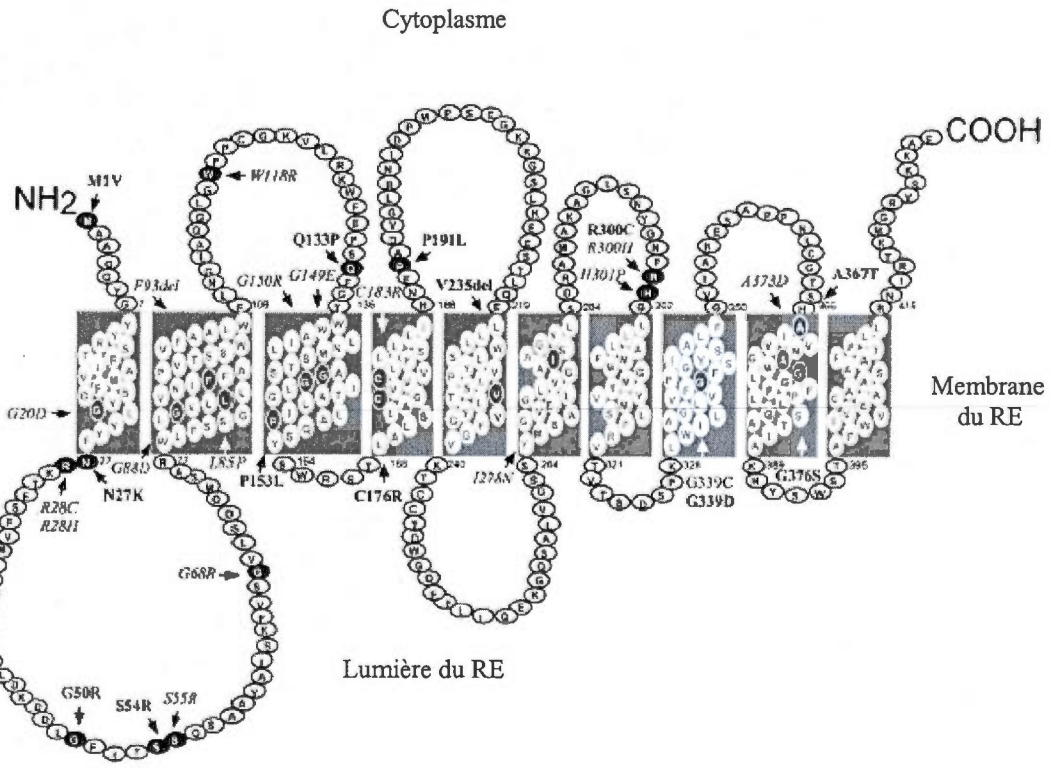

Figure 5. *Structure moléculaire du G6PT.* Représentation schématique du G6PT, avec ses 9 boucles extra membranaires et ses 10 domaines transmembranaires (parties en gris). Ces deux extrémités C- et N-terminales se trouvent à l'extérieur du RE. Les parties plus foncées des boucles et des domaines transmembranaires représentent les différentes mutations connues pour inactiver les fonctions de G6PT et qui sont nommées dans le tableau 3.

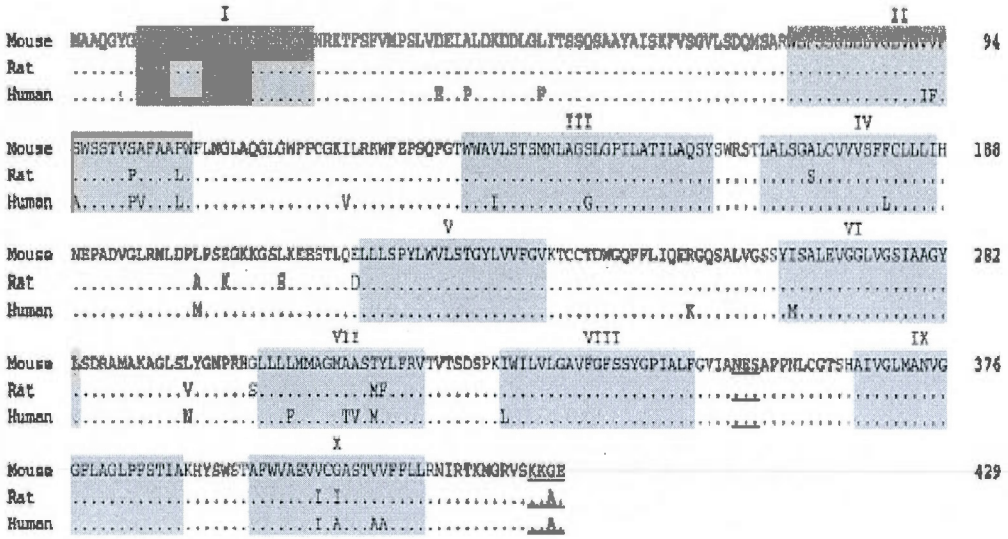

Figure 6. *Alignement en acides aminés de la protéine G6PT.* Séquence des acides aminés composants la G6PT (tirée de GeneBank) pour l'espèce humaine, murine et du rat. On peut remarquer une conservation dans les trois séquences, les parties ombragées sont les prédictions algorithmiques de Stoffel pour les domaines transmembranaires.

	Association G6PT/G6Pase	Localisation	Activité de G6PT
Mutations dans les dix hélices de G6PT	---/G6Pase		0 %
	WT/G6Pase		100 %
	G 88D/G6Pase	Heli. 2	2.2 %
	P 153L/G6Pase	Heli. 3	8.6 %
	I 278N/G6Pase	Heli. 6	10.4 %
	G 339C/G6Pase	Heli. 8	4.9 %
	A 367T/G6Pase	Heli. 9	23.1 %
	G 376S/G6Pase	Heli. 9	5.6 %
Mutations dans les parties *trans* membranaires	---/G6Pase		0 %
	WT/G6Pase		100 %
	G 68R/G6Pase	L1	8.1 %
	R 300C/G6Pase	C3	5.2 %
	R 300H/G6Pase	C3	7.1 %
	H 301P/G6Pase	C3	24.2 %
Mutations sur le C-terminal	---/G6Pase		0 %
	WT/G6Pase		100 %
	G 68R/G6Pase		15.1 %
	R 300C/G6Pase		17.4 %
	R 300H/G6Pase		16.5 %
	H 301P/G6Pase		8.7 %
Autres mutations	G 20D/G6Pase		
	R 28C/G6Pase		
	R 28H/G6Pase		0 %
	S 55R/G6Pase		Abolition de l'activité
	G 68R/G6Pase		
	L 85P/G6Pase		
	G 88D/G6Pase		
	W 118R/G6Pase		

Tableau 3. *Les mutations causant la GSD Ib*. L'activité de G6PT est mesurée après une co-transfection des cellules Cos-1 avec la forme sauvage (WT) ou mutée de G6PT et de l'unité catalytique G6Pase (Li-Yuan Chen et al., 2002). 15 mutations sont répertoriées au niveau des régions clés soit à l'extrémité C-terminal, l'une des 10 hélices, ou bien dans les parties transmembranaires, les autres sont des mutations apportées sur toutes les régions qui induisent une abolition complète de l'activité de transport de G6PT.

CHAPITRE II

PRÉSENTATION DU PROJET

1. Hypothèse de travail

Nous avons découvert l'existence de la G6PT, une protéine microsomale régulant potentiellement une partie du métabolisme glucidique des cellules dérivées de glioblastomes. Ces tumeurs cérébrales sont reconnues pour leur résistance aux traitements habituels du cancer. En utilisant l'acide chlorogénique (CHL), un polyphénol aux propriétés anti-cancérigènes et un puissant inhibiteur fonctionnel de G6PT, nous avons mis en évidence de nouvelles fonctions de G6PT, indépendantes de l'hydrolyse du G6P. C'est pourquoi, il est impératif d'élucider la pertinence de la présence de G6PT dans ce tissu non néoglucogénique. Pour ce faire nous avons tenté de répondre aux trois questions suivantes :

Question 1 : Quel est l'effet du CHL et son impact sur les fonctions de G6PT dans les processus d'invasions tumorales tels que la migration et la dégradation de la MEC ?

Question 2 : Si G6PT est impliqué dans ces mécanismes, quel est son rôle dans la survie cellulaire des cellules tumorales ?

Question 3 : Par quelles voies de signalisations, G6PT interagirait pour réguler ces processus invasifs, et G6PT agit-il lui-même comme agent de signalisation ?

2. Un nouveau rôle du transporteur microsomal de glucose-6-phosphate dans la régulation de la migration et de la dégradation de la matrice extracellulaire.

Une partie de notre étude portait sur l'implication de G6PT dans l'invasion tumorale des glioblastomes (cellules U87). Cette étude avait pour but de démontrer jusqu'à quel point G6PT pouvait influencer certains processus essentiels à l'invasion tumorale. Par la suite, nous avons tenté d'expliquer l'effet anti-cancérigène du CHL connu pour être un inhibiteur de G6PT. Nous avons donc formulé l'hypothèse que G6PT régule certaines fonctions invasives des cellules cancéreuses et peut être une nouvelle cible pharmacologique du CHL.

La littérature rapporte les effets bénéfiques de plusieurs polyphénols de l'alimentation comme le CHL et l'EGCg. Plusieurs études ont également mis à jour leurs propriétés anti-oxydantes, anti-inflammatoires, et anti-cancérigènes. Au cours de notre exploration des différents gènes du système G6Pase exprimés dans les glioblastomes, G6PT s'est avéré être significativement exprimé dans les U87 en comparaison à d'autres lignées tumorales cérébrales testées. De plus, une inhibition fonctionnelle de G6PT par le CHL diminuerait la migration des U87 et la sécrétion de la MMP-2. En plus d'identifier une nouvelle cible pharmacologique, l'originalité de cette étude est qu'elle présente les prémisses d'une implication potentielle de G6PT dans la signalisation intracellulaire qui régule la tumorigénèse des U87. Nos résultats amènent à postuler de possibles avantages de l'association du CHL en chimiothérapie pour une meilleure qualité de vie des patients. Les résultats obtenus sont présentés sous forme d'article publié dans *Cancer Cell International* (section expérimentale).

3. Rôle du transporteur microsomal de glucose-6-phosphate dans la survie cellulaire.

Après avoir exploré l'implication de G6PT dans la migration cellulaire et la dégradation de la MEC, nous avons étudié la possible contribution de G6PT dans la survie de cellules U87 dérivées de glioblastomes. En utilisant un ARN interférant spécifique à G6PT, nous avons pu constater que G6PT est indispensable à la survie cellulaire et que toute inhibition de l'expression génique de G6PT engendre la mort cellulaire. Ceci valide notre hypothèse selon laquelle G6PT aurait des implications complexes dans l'invasion tumorale, au delà de son rôle dans le contrôle du métabolisme glucidique. En plus d'interagir dans différents mécanismes d'invasion tumorale, l'inhibition de l'expression génique de G6PT pourrait être une approche thérapeutique efficace par des produits naturels ayants des propriétés anti-cancérigènes. Les résultats obtenus sont présentés sous forme d'article publié dans *FEBS Letters* (section expérimentale).

4. Rôle de l'axe moléculaire MT1-MMP/G6PT dans l'invasion et/ou la survie cellulaire.

Le dernier volet de notre recherche avait pour objectif d'explorer une possible implication de G6PT dans la signalisation moléculaire régulant le phénotype invasif des U87. Nos travaux visaient plus spécifiquement la comparaison des niveaux d'expression génique et protéique de G6PT et de MT1-MMP à différents stades de l'invasion tumorale. L'objectif étant de démontrer la possible existence d'un axe de signalisation entre MT1-MMP et G6PT dont l'équilibre serait important dans la régulation du processus invasif et de la mort cellulaire.

Diverses études ont rapporté que le dysfonctionnement du réticulum endoplasmique (RE) dans lequel G6PT est ancré, serait responsable de plusieurs maladies insulinodépendantes et de différents désordres lipidiques. L'une des fonctions principales du RE est la synthèse de protéines cytosoliques ou membranaires. Nous avons constaté, lors de nos premiers essais, que la ConA et la Cyto D, deux agents perturbateurs du cytosquelette qui activent la pro-MMP-2 via un mécanisme MT1-MMP, diminuait les niveaux d'expression géniques et protéiques de G6PT, et que toute diminution de G6PT induisait une mort cellulaire. Cette mort cellulaire, nous a permis d'établir un lien entre ces deux acteurs de l'invasion et de la survie cellulaire. Il existe donc un état d'équilibre entre MT1-MMP et G6PT dans une cellule tumorale, mais tout évènement amenant à le déstabiliser pourrait être l'élément déclencheur d'une cascade de mécanismes induisant la mort cellulaire ou l'invasion tumorale. Ces travaux nous ont permis de postuler l'existence d'un axe moléculaire entre la survie cellulaire et l'invasion tumorale. Les résultats obtenus sont présentés sous forme d'article publié dans *NEOPLASIA* (section expérimentale).

CHAPITRE III

ARTICLES

ARTICLE # 1

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The chemopreventive properties of chlorogenic acid reveal a new role for the microsomal glucose-6-phosphate translocase in brain tumor progression

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Background : Chlorogenic acid (CHL), the most potent functional inhibitor of the microsomal glucose-6-phosphate translocase (G6PT), is thought to possess cancer chemopreventive properties. Whether any G6PT functions are involved in tumorigenesis is however unknown. We investigated the effects of CHL and the potential role of G6PT in regulating the invasive phenotype of brain tumor-derived glioma cells. *Results :* RT-PCR was used to show that, among the adult and pediatric brain tumor-derived cells tested, U-87 glioma cells expressed the highest levels of G6PT mRNA. U-87 cells lacked the microsomal catalytic subunit glucose-6-phosphatase (G6Pase)-α, but expressed G6Pase-β which, when coupled to G6PT, allows G6P hydrolysis into glucose to occur in non glyconeogenic tissues such as brain. CHL inhibited U-87 cell migration and matrix metalloproteinase (MMP)-2 secretion, two prerequisites for tumor cell invasion to occur. Moreover, CHL also inhibited sphingosine-1-phosphate (S1P)-induced cell migration as well as the rapid S1P-induced extracellular signal-regulated protein kinases phosphorylation potentially mediated through intracellular calcium mobilization, suggesting that G6PT may also bear crucial functions in regulating intracellular signalling. Overexpresion of the recombinant G6PT protein induced U-87 glioma cell migration that was, in turn, antagonized by CHL. MMP-2 secretion was also inhibited by the ATP-depleting agents 2-deoxyglucose and 5-thioglucose, a mechanism that is thought to indirectly regulate G6PT functions in ATP-mediated calcium sequestration. *Conclusions :* Collectively, we highlight a new G6PT function in glioma cells that could regulate the invasive phenotype of brain tumor cells and that can be targeted by the anticancer properties of CHL.

Key words : human glioma, cell invasion, chlorogenic acid, glucose-6-phosphate translocase

INTRODUCTION

Dietary polyphenols have been widely assumed to be beneficial to human health by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity, and altering signal transduction pathways [1-3]. Epidemiological studies have also highlighted the association between the consumption of polyphenol-rich food and beverages and the prevention of various human diseases [4, 5]. Among these polyphenols, the antitumor activities of flavonoids as well as the inhibition of carcinogenesis by polyphenols has revealed properties beneficial for the use of nutraceuticals in cancer therapy [6-8]. Among the sources of these anticancer polyphenols, modern phytochemical research shows that tea contains a large number of plant secondary metabolites owning different chemical structures such as amino acids, catechins, purine alkaloids, and chlorogenic acid, and where each group of compounds has their special biological properties [9]. While green tea catechins are now well recognized for their chemopreventive effects [10, 11], the impact of chlorogenic acid (CHL), which has been attributed possible cancer chemoprevention properties is not well documented [12, 13].

Interestingly, CHL inhibition of matrix metalloproteinase (MMP)-9 secretion, which process is known to be involved in tumor cell invasion and metastasis, was recently reported [14], and this property adds up to CHL's antioxydant and anti-inflammatory properties [15, 16]. The anti-cancerous molecular mechanisms transducing CHL effects are however not well understood. CHL derivatives have mostly been known to selectively inhibit endoplasmic reticulum (ER) glucose-6-phosphate (G6P) transport, and hence microsomal glucose-6-phosphatase (G6Pase) activity in isolated microsomes [17] as well as *in vivo* [18, 19]. In intact cells, the CHL derivative and G6PT inhibitor S3483 was found to inhibit G6P transport in microsomes isolated from polymorphonuclear neutrophils (PMN) and from

differentiated promyelocytic HL-60 cells [20]. Interestingly, PMN defects in glycogen storage disease (GSD) type 1b patients, a clinical status where the G6PT gene or protein is defective [20, 21], include a reduction in several processes such as respiratory burst, chemotaxis, phagocytosis, and in calcium signaling [22-24].

Moreover, alterations of several other biochemical parameters - glucose phosphorylation, calcium mobilization, and hexose uptake and transport - have been described as the possible background of the G6PT functional defects [25-27]. Since cells such as PMN have no detectable G6Pase activity, G6PT must bear a different role than that exerted in the liver, for instance, where it is functionally coupled to the G6Pase enzyme. Moreover, G6PT functions have never been investigated in brain tumor-derived cells. It has been hypothesized that G6PT might have a sort of function as a G6P sensor [28] or that it could favor calcium sequestration in the ER lumen [29]. Such roles have not been yet proven and would not explain why other non hepatic cells expressing G6PT are apparently unaffected in GSD-1b.

In the present work two topics have been addressed. Does a functional inhibition of G6PT regulate any brain tumor-derived cells tumorigenic properties such as MMP-mediated extracellular matrix (ECM) hydrolytic activity and cell migration ? If so, can a connection be found between the G6PT functions and intracellular signaling that regulates such invasive phenotype ? The inhibition of the microsomal G6PT functions was modeled by the addition of CHL, which is a highly specific inhibitor of G6PT [30], while upregulation of G6PT was performed through cDNA transient transfection. The results demonstrate that G6PT may regulate brain tumor-derived invasive phenotype by controlling intracellular signaling that lead to cell migration. Moreover, we provide the first molecular rationale for the anticancer properties of CHL in the regulation of MMP secretion.

LIST OF ABBREVIATIONS USED

The abbreviations used are : CHL, chlorogenic acid; 2-DG, 2-deoxy-d-glucose; ECM, extracellular matrix; EGCg, epigallocatechin-(3)-gallate; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinases; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P translocase; GSD, glycogen storage disease; MMP, matrix metalloproteinase; 5-TG, 5-thioglucose.

METHODS

Materials : Agarose, (-)-epigallocatechin 3-gallate (EGCg), sodium dodecylsulfate (SDS), gelatin, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). The TriZOL reagent was from Life Technologies (Gaithersburg, MD). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC).

Cell culture and cDNA transfection method : The U-87, U-118, U-138 glioma cell lines, the DAOY medulloblastoma cell line and the HEP-G2 hepatoma cell line were purchased from American Type Culture Collection and cultured in their respective media. Specifically, U-87 cells were maintained in Eagle's Minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. The G6PT plasmid was a generous gift from Dr Christopher Newgard [35]. U-87 cells were transiently transfected with the cDNA construct using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed by western blotting and zymography. All experiments involving these cells were performed 36 hrs following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis : Total RNA was extracted from monolayer cultured U-87 cells using the TriZOL reagent. One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON). Primers for G6Pase-α (forward : 5'-TTCAGCCACATCCACAGCATC-3', reverse : 5'-

GGGGTTTCAAGGAGTCAAAGACG-3'), for G6Pase-β (forward : 5'-ACTCTTCCTGACTTCTTGTGTGCC-3', reverse : 5'-TTGCCTTTGCTCTTTGGGGG-3') and for G6PT (forward : 5'-CAGGGCTATGGCTATTATCGCAC-3', reverse : 5'-ATGGCTCAAACCACTTCCGCAG-3') were all derived from human sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal house-keeping gene control. PCR conditions were optimized so that the gene products were found to be at the exponential phase of the amplification PCR products and were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

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

Cell migration assay : Cells were dislodged after brief trypsinization, washed extensively and resuspended in DMEM at a concentration of 10^6 cells/ml [Annabi et al., 2004]. Cells ($7x10^5$) were then dispersed onto 1 mg/ml gelatin/PBS-coated chemotaxis filters (Costar; 8-μm pore size) within Boyden chamber inserts. Migration proceeded for 3 h at 37°C in 5% CO_2. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, colored with 0.1% crystal

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

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

RESULTS

U-87 glioma cells express the highest G6PT transcript levels among brain tumor-derived cell lines. Gene expression levels of the microsomal glucose-6-phosphate transporter (G6PT), as well as of the two glucose-6-phosphatase α and β isoforms were first assessed. Total RNA was extracted from HEP-G2 hepatoma and U-87 glioma cells, then RT-PCR performed as described in the Methods section. As expected for a cell line derived from a neoglycogenic tissue, we show that HEP-G2 express all three components of the G6Pase system with G6Pase-β being the predominant gene expressed (Fig.1a). RT-PCR was also performed on RNA extracted from U-87 glioma cells. In contrast to HEP-G2, only G6PT and G6Pase-β transcripts were shown to be significantly expressed with very low to undetectable G6Pase-α present (Fig.1a). This is in agreement with previous reports demonstrating the lack of G6Pase-α expression in brain-derived cells [31]. We also monitored the levels of G6PT in a medulloblastoma pediatric brain tumor cell line (DAOY), as well as in other U-118 and U-138 glioma cell lines. Interestingly, among the adult brain tumor-derived cell lines, U-87 glioma cells were the one that expressed the highest level of G6PT (Fig.1b), while DAOY cells expressed very low to undetectable G6PT transcript. This suggests that G6PT expression in brain tumor cells may be regulated throughout development.

The G6PT inhibitor chlorogenic acid reduces proMMP-2 secretion in U-87 glioma cells. We next assessed the potential functions that G6PT may regulate in the invasive phenotype of U-87 glioma cells. Based on the assumption that CHL was efficiently able to penetrate inside the cells [32], U-87 glioma cells were treated with increasing concentrations of CHL and the secretion of matrix metalloproteinase (MMP)-2 monitored by zymography. We show that CHL significantly inhibited the latent proMMP-2 secretion (Fig.2a), and that another tea-derived compound

epigallocatechin gallate (EGCg) was as efficient (Fig.2a and b). Since EGCg was also reported to inhibit the functions of a membrane type (MT)1-MMP in activating the latent proMMP-2 in glioblastoma cells [33], we further assessed the possible effect of CHL on MT1-MMP-mediated proMMP-2 activation. Cells were incubated with an exogenous source of proMMP-2 and treated or not with concanavalin-A in order to trigger proMMP-2 activation [34]. We show that EGCg indeed inhibited MT1-MMP-mediated activation of proMMP-2 while CHL could not (Fig.2c).

Chlorogenic acid inhibits G6PT-mediated U-87 glioma cells migration. G6PT function in U-87 glioma cells migration was next assessed. Cells were transfected or not (Mock) with the G6PT cDNA [35] and basal cell migration performed on gelatin-coated filters using modified Boyden chambers as described in the Methods section. The transfection of the G6PT cDNA was shown to specifically increase G6PT transcript levels, while those of the G6Pase-β remained unaffected (Fig.3a). Interestingly, the sole effect of G6PT overexpression increased the basal migration of U-87 glioma cells (Fig.3b, white bars), while CHL inhibited both basal and G6PT-induced cell migration (Fig.3b, black bars). This suggests that G6PT functions may in part regulate U-87 glioma cells invasive phenotype.

Chlorogenic acid inhibits sphingosine-1-phosphate-induced U-87 glioma cells migration. Sphingosine-1-phosphate (S1P) is a bioactive lipid which is present at high levels in brain tissue [36] and which is a potent mitogen for glioblastoma multiforme cells [37]. More recently, S1P was shown to also potently enhance the *in vitro* motility of glioblastoma cells [38]. We thus assessed whether CHL could interfere with U-87 basal and S1P-induced cell migration, and if it could antagonize the G6PT-mediated regulation of cell migration. Control (Mock) or G6PT-transfected U-87 glioma cells were harvested and seeded on top of gelatin-coated filters as described in the Methods section. Cell migration was then left to proceed in the

presence of either S1P, CHL, or a combination of both. Representative pictures of stained cells that had migrated through the filters are shown (Fig.4a). We observe that S1P induced basal cell migration by 2.6-fold, and that CHL was able to inhibit both the basal and S1P-induced cell migration (Fig.4b, Mock). When cells were transiently transfected with the G6PT cDNA, basal cell migration increased by approximately 2-fold in accordance with Fig.3b. Interestingly, the effect of S1P was additive to that of the overexpression of G6PT, where CHL was also efficiently able to antagonize that migration that was induced either by G6PT overexpression or by S1P in G6PT-transfected cells (Fig.4b, G6PT). These results suggest that G6PT potentially regulates crucial intracellular signaling that triggers basal and S1P-mediated cell migration, and that the functional inhibition of these events by CHL could potentially explain some of its chemopreventive effects at the molecular level.

Chlorogenic acid inhibits sphingosine-1-phosphate-induced ERK phosphorylation in U-87 glioma cells. S1P is thought to trigger intracellular signalling, in part, through the MAPK pathway and the release of intracellular calcium pools [37, 39, 40]. We thus monitored the extent of ERK phosphorylation that is triggered by S1P in U-87 glioma cells and whether CHL could interfere with that process. We show that S1P indeed triggered a rapid phosphorylation of ERK within the first 15 seconds of incubation (Fig.5, left panel). Interestingly, pre-incubation of the cells with 100 μM CHL for 30 minutes prevented that rapid phosphorylation of ERK and even delayed it at time 60 seconds (Fig.5, right panel). The inhibitory effect of CHL on S1P-induced ERK phosphorylation potentially suggests that intracellular inhibition of microsomal G6PT functions may antagonize crucial events taking place in the ER.

Intracellular ATP-depleting agents inhibit proMMP-2 secretion and antagonize S1P-induced ERK phosphorylation in U-87 glioma cells. Besides its

classical role in recognizing and translocating G6P from the cytoplasm to the lumen of the ER, G6PT has been attributed a potential role in regulating calcium flux responses [41, 42]. Such role in regulating intracellular calcium pools was indeed elegantly demonstrated in neutrophils isolated from G6PT-deficient mice [43]. Interestingly, a functional link has been proposed between G6P and calcium where cytoplasmic G6P is thought to enhance ATP-dependent sequestration of calcium in the ER [29]. In light of this, we sought to investigate the effects of ATP-depleting agents and synthetic analogs of glucose 5-thioglucose (5-TG) and 2-deoxy-d-glucose (2-DG) on proMMP-2 secretion and S1P-induced ERK phosphorylation. U-87 glioma cells were serum-starved and treated with increasing concentrations of 2-DG and 5-TG. Conditioned media was isolated and assessed for the hydrolytic activity levels of secreted proMMP-2. We show that proMMP-2 secretion was significantly decreased in both 2-DG- and 5-TG-treated cells (Fig.6a). Half-maximal inhibition constants (IC_{50}) were calculated and represent 2 μM and 19 μM respectively for 2-DG and 5-TG (Fig.6b). S1P-induced ERK phosphorylation was also monitored in 2-DG- and 5-TG pre-treated cells where both ATP-depleting agents were able to inhibit S1P-induced ERK phosphorylation (Fig.6c). Although indirect, the sum of these data suggests that whether functional G6P uptake inhibition by CHL or ATP depletion with 2-DG/5-TG are used, the inhibition of microsomal G6PT functions - ER G6P uptake, ATP-dependent calcium sequestration - leads to decreased proMMP-2 secretion and potential decrease in calcium-mediated intracellular signalization.

DISCUSSION

Because of their infiltrating character, malignant cerebral gliomas are the most common and aggressive primary tumors of the adult central nervous system. The prognosis of patients suffering from malignant cerebral gliomas has so far remained dismal despite many advances in surgery, radiation therapy, chemotherapy and understanding of the molecular players involved in their invasive phenotype [44, 45]. In the present work, we provide evidence for a potential and original role of G6PT in brain tumor-derived cells signalization and in tumor progression. Indeed, we show that these major molecular features characterizing high-grade radioresistant gliomas can be targeted by CHL, the most potent functional inhibitor of G6PT, and to potentially indirectly modulate G6PT functions such as in G6P recognition/uptake within the ER and in ATP-dependent calcium sequestration. Among tea constituents, CHL has been recently attributed chemopreventive properties [12-14]. Interestingly, we showed that EGCg, a green tea-derived polyphenol with potential similar anticancerous properties to those we describe for CHL, can also efficiently target brain tumors invasive phenotype by inhibiting RhoA/Rok-mediated intracellular cell signaling [46]. Moreover, in light of the recent studies which have shown that EGCg could serve as an IR enhancer on cancer cell lines [47], we have further shown that EGCg pretreatment of glioma cells prior to IR could reverse the cytoprotective effect of the prosurvival proteins such as Survivin [48]. The potential functional inhibition of G6PT by either CHL or by ATP-depleting agents 2-DG/5-TG could thus provide a molecular rational as to the role that G6PT plays in intracellular signalling regulating the glioma cells invasive phenotype.

Poorly differentiated and rapidly growing malignant tumors are generally characterized by higher rates of glucose usage and glycolysis as compared to

corresponding normal tissues [49]. These fundamental differences in the glucose metabolism of transformed and normal cells form however the basis of noninvasive detection and grading of tumors by positron emission tomography (PET) using tracer nanomolar doses of [^{18}F]-2-fluoro-deoxyglucose ([^{18}F]FDG), a nonmetabolizable analog of glucose that enters the cell by the same membrane transport mechanism as glucose does. Measurements of glucose uptake in human tumors by FDG-PET suggest that glucose uptake may directly correlate with the degree of malignancy and treatment resistance/poor prognosis [50]. The precise molecular mechanisms underlying such correlations remain yet to be elucidated. Interestingly, the use of [^{18}F]FDG was also demonstrated in the monitoring of adenovirus-mediated GSD-1a correction [51]. Therefore, similarly to the non-metabolizable glucose analogue 2-DG, one can expect pharmacological doses of FDG to block glycolysis, and to cause depletion of ATP as well as of glucose derivatives required for protein glycosylation. It was documented that cell killing of neoplastic cells caused by cytostatic drugs is associated with a decreased ATP content and FDG uptake. This indicates that FDG uptake was closely linked with ATP production, and that not only ATP but also FDG may be used to study drug effects *in vitro* [52].

In fact, one important implication of our study is the impact of targeting G6PT functions in radiotherapeutic modalities. The failure of radiotherapy in cerebral gliomas is primarily due to the diffusely infiltrating nature of the tumor and the presence of hypoxic, repair-proficient and intrinsically radioresistant subpopulation of cells. Since we show that the chemopreventive properties of both IR-cell sensitizing agent EGCg [48] and of CHL molecules [this study] are similarly and efficiently able to inhibit cell migration and MMP-secretion of glioblastoma cells, it becomes tempting to suggest that CHL may also be used in synergy with radiotherapeutic modalities. Interestingly, enhanced glucose usage *in vitro* [53] as well an *in vivo*, correlating with the degree of malignancy and poor prognosis, has been demonstrated in glioma tumors [54, 55]. Moreover, *in vitro* studies in established glioma cell lines

showed that the presence of 2-DG for a few hours after irradiation could increase radiation damage significantly and the radiosensitization was higher under conditions of reduced respiratory metabolism [56]. Finally, 2-DG is known to inhibit glycolytic energy (adenosine triphosphate [ATP]) production [57] and has been tested in multiple studies for possible application as an anticancer or antiviral therapeutic. Such observations provide another molecular rationale as to the effects of CHL, 2-DG and 5-TG that we report in the present study to inhibit G6PT functions, and that we have shown to be significantly expressed in U-87 glioblastoma cells.

In summary, we show that inhibiting G6PT functions by CHL or ATP-depleting agents in brain-tumor-derived cells may result in decreased invasive phenotype. We suggest that new cellular processes such as response to growth factors and secretion of MMP may collectively be regulated through calcium fluxes in part through functions involving G6PT at the ER level. Moreover, because cancer cells frequently display high rates of aerobic glycolysis in comparison to their nontransformed counterparts, the recently published hypoglycemic impact of CHL [58], combined to the possible applications of 2-DG in anticancer therapies [59, 60] may provide further support that inhibiting G6PT functions in cancer cells could partly decrease brain tumor progression.

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REFERENCES

1. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004 May;79(5):727-47.

2. Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El Bedoui J, Chataigneau M, Schini-Kerth VB. Vascular protection by dietary polyphenols. Eur J Pharmacol. 2004 Oct 1;500(1-3):299-313.

3. Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr. 2005;45(4):287-306.

4. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr. 2005 Jan;81(1 Suppl):317S-325S.

5. Block, G.; Patterson, B.; Subar, A. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18*, 1-30.

6. Lambert JD, Hong J, Yang GY, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am J Clin Nutr. 2005 Jan;81(1 Suppl):284S-291S.

7. Kanadaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, Lee MT. The antitumor activities of flavonoids. In Vivo. 2005 Sep-Oct;19(5):895-909.

8. Roudebush P, Davenport DJ, Novotny BJ. The use of nutraceuticals in cancer therapy. Vet Clin North Am Small Anim Pract. 2004 Jan;34(1):249-69.

9. Zhu X, Chen B, Ma M, Luo X, Zhang F, Yao S, Wan Z, Yang D, Hang H. Simultaneous analysis of theanine, chlorogenic acid, purine alkaloids and catechins in tea samples with the help of multi-dimension information of on-line high performance liquid chromatography/electrospray-mass spectrometry. J Pharm Biomed Anal. 2004 Feb 18;34(3):695-704.

10. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. Nutr Rev. 2004 May;62(5):204-11.

11. Kazi A, Smith DM, Daniel K, Zhong S, Gupta P, Bosley ME, Dou QP. Potential molecular targets of tea polyphenols in human tumor cells: significance in cancer prevention. In Vivo. 2002 Nov-Dec;16(6):397-403.

12. Mori H, Kawabata K, Matsunaga K, Ushida J, Fujii K, Hara A, Tanaka T, Murai H. Chemopreventive effects of coffee bean and rice constituents on colorectal carcinogenesis. Biofactors. 2000;12(1-4):101-5.

13. Chiang YM, Chuang DY, Wang SY, Kuo YH, Tsai PW, Shyur LF. Metabolite profiling and chemopreventive bioactivity of plant extracts from Bidens pilosa. J Ethnopharmacol. 2004 Dec;95(2-3):409-19.

14. Jin UH, Lee JY, Kang SK, Kim JK, Park WH, Kim JG, Moon SK, Kim CH. A phenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor: isolation and identification from methanol extract of Euonymus alatus. Life Sci. 2005 Oct 14;77(22):2760-9.

15. Yen WJ, Wang BS, Chang LW, Duh PD. Antioxidant properties of roasted coffee residues. J Agric Food Chem. 2005 Apr 6;53(7):2658-63.

16. Krakauer T. The polyphenol chlorogenic acid inhibits staphylococcal exotoxin-induced inflammatory cytokines and chemokines. Immunopharmacol Immunotoxicol. 2002 Feb;24(1):113-9.

17. Herling AW, Burger HJ, Schwab D, Hemmerle H, Below P, Schubert G. Pharmacodynamic profile of a novel inhibitor of the hepatic glucose-6-phosphatase system. Am J Physiol. 1998;274:G1087-G1093

18. van Dijk TH, van der Sluijs FH, Wiegman CH, et al. Acute inhibition of hepatic glucose-6-phosphatase does not affect gluconeogenesis but directs gluconeogenic flux toward glycogen in fasted rats. A pharmacological study with the chlorogenic acid derivative S4048. J Biol Chem. 2001;276:25727-25735

19. Bandsma RH, Wiegman CH, Herling AW, et al. Acute inhibition of glucose-6-phosphate translocator activity leads to increased de novo lipogenesis and development of hepatic steatosis without affecting VLDL production in rats. Diabetes. 2001;50:2591-2597.

20. Leuzzi R, Banhegyi G, Kardon T, Marcolongo P, Capecchi PL, Burger HJ, Benedetti A, Fulceri R. Inhibition of microsomal glucose-6-phosphate transport in human neutrophils results in apoptosis: a potential explanation for neutrophil dysfunction in glycogen storage disease type 1b. Blood. 2003 Mar 15;101(6):2381-7.

21. Hiraiwa H, Pan CJ, Lin B, Moses SW, Chou JY. Inactivation of the glucose 6-phosphate transporter causes glycogen storage disease type 1b. J Biol Chem. 1999 Feb 26;274(9):5532-6

22. Calderwood S, Kilpatrick L, Douglas SD, Freedman M, Smith-Whitley K, Rolland M, Kurtzberg J. Recombinant human granulocyte colony-stimulating factor therapy for patients with neutropenia and/or neutrophil dysfunction secondary to glycogen storage disease type 1b. Blood. 2001 Jan 15;97(2):376-82.

23. Beaudet AL, Anderson DC, Michels VV, Arion WJ, Lange AJ. Neutropenia and impaired neutrophil migration in type IB glycogen storage disease. J Pediatr. 1980 Dec;97(6):906-10.

24. Kilpatrick L, Garty BZ, Lundquist KF, Hunter K, Stanley CA, Baker L, Douglas SD, Korchak HM. Impaired metabolic function and signaling defects in phagocytic cells in glycogen storage disease type 1b. J Clin Invest. 1990 Jul;86(1):196-202.

25. Bashan N, Potashnik R, Peist A, Peleg N, Moran A, Moses SW. Deficient glucose phosphorylation as a possible common denominator and its relation to abnormal leucocyte function, in glycogen storage disease 1b patients. Eur J Pediatr. 1993;152 Suppl 1:S44-8.

26. Korchak HM, Garty BZ, Stanley CA, Baker L, Douglas SD, Kilpatrick L. Impairment of calcium mobilization in phagocytic cells in glycogen storage disease type 1b. Eur J Pediatr. 1993;152 Suppl 1:S39-43.

27. Potashnik R, Moran A, Moses SW, Peleg N, Bashan N. Hexose uptake and transport in polymorphonuclear leukocytes from patients with glycogen storage disease Ib. Pediatr Res. 1990 Jul;28(1):19-23.

28. Hiraiwa H, Pan CJ, Lin B, Moses SW, Chou JY. Inactivation of the glucose-6-phosphate transporter causes glycogen storage disease type 1b. J Biol Chem. 1999;274:5532-5536

29. Chen PY, Csutora P, Veyna-Burke NA, Marchase RB. Glucose-6-phosphate and Ca2+ sequestration are mutually enhanced in microsomes from liver, brain, and heart. Diabetes. 1998;47:874-881

30. Arion WJ, Canfield WK, Ramos FC, et al. Chlorogenic acid analogue S3483: a potent competitive inhibitor of the hepatic and renal glucose-6-phosphatase systems. Arch Biochem Biophys. 1998;351:279-285

31. Ghosh A, Cheung YY, Mansfield BC, Chou JY. Brain contains a functional glucose-6-phosphatase complex capable of endogenous glucose production. J Biol Chem. 2005 Mar 25;280(12):11114-9.

32. Konishi Y, Kobayashi S. Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal caco-2 cell monolayers. J Agric Food Chem. 2004 May 5;52(9):2518-26.

33. Annabi B, Lachambre MP, Bousquet-Gagnon N, Page M, Gingras D, Beliveau R. Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. Biochim Biophys Acta. 2002 Jan 30;1542(1-3):209-20

34. Annabi B, Lachambre M, Bousquet-Gagnon N, Page M, Gingras D, Beliveau R. Localization of membrane-type 1 matrix metalloproteinase in caveolae membrane domains. Biochem J. 2001 Feb 1;353(Pt 3):547-53.

35. An J, Li Y, van De Werve G, Newgard CB. Overexpression of the P46 (T1) translocase component of the glucose-6-phosphatase complex in hepatocytes impairs glycogen accumulation via hydrolysis of glucose 1-phosphate. J Biol Chem. 2001 Apr 6;276(14):10722-9

36. L.C. Edsall and S. Spiegel, Enzymatic measurement of sphingosine-1-phosphate. *Anal. Biochem.* **272** (1999), pp. 80–86.

37. J.R. Van Brocklyn, C.A. Letterle, P.J. Snyder and T.W. Prior, Sphingosine-1-phosphate stimulates human glioma cell proliferation through G_i-coupled receptors: Role of ERK MAP kinase and phosphatidylinositol 3-kinase β. *Cancer Lett.* **181** (2002), pp. 195–204

38. Van Brocklyn JR, Young N, Roof R. Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells. Cancer Lett. 2003 Sep 10;199(1):53-60.

39. Pyne S, Pyne NJ. Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. Biochim Biophys Acta. 2002 May 23;1582(1-3):121-31.

40. Young KW, Nahorski SR. Sphingosine 1-phosphate: a Ca2+ release mediator in the balance. Cell Calcium. 2002 Nov-Dec;32(5-6):335-41.

41. Gitzelmann R, Bosshard NU.Defective neutrophil and monocyte functions in glycogen storage disease type Ib: a literature review. Eur J Pediatr. 1993;152 Suppl 1:S33-8.

42. Garty BZ, Douglas SD, Danon YL. Immune deficiency in glycogen storage disease type 1B. Isr J Med Sci. 1996 Dec;32(12):1276-81.

43. Chen LY, Shieh JJ, Lin B, Pan CJ, Gao JL, Murphy PM, Roe TF, Moses S, Ward JM, Lee EJ, Westphal H, Mansfield BC, Chou JY. Impaired glucose homeostasis, neutrophil trafficking and function in mice lacking the glucose-6-phosphate transporter. Hum Mol Genet. 2003 Oct 1;12(19):2547-58.

46. Annabi B, Bouzeghrane M, Moumdjian R, Moghrabi A, Beliveau R. Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. J Neurochem. 2005 Aug;94(4):906-16. Epub 2005 Jun 30.

47. Baatout S, Jacquet P, Derradji H, Ooms D, Michaux A, Mergeay M. Study of the combined effect of X-irradiation and epigallocatechin-gallate (a tea component) on the growth inhibition and induction of apoptosis in human cancer cell lines. Oncol Rep. 2004 Jul;12(1):159-67

48. McLaughlin N., Annabi B., Bouzeghrane M., Temme A. Bahary J.P., Moumdjian R. & Béliveau R. (2005) The green tea polyphenol (-)

epigallocatechin-3-gallate inhibits the survivin-mediated radioresistant phenotype in glioblastomas *Molecular Brain Research* (in press)

49. Warburg O., Posener K., Negelein E. The metabolism of cancer cells. Biochem Zeitschr. 1934, 152:129-69

50. Kostakoglu L, Hardoff R, Mirtcheva R, Goldsmith SJ. PET-CT fusion imaging in differentiating physiologic from pathologic FDG uptake. Radiographics. 2004 Sep-Oct;24(5):1411-31.

51. Zingone A, Seidel J, Aloj L, Caraco C, Vaquero JJ, Jagoda EM, Chou JY, Green MV, Eckelman WC. Monitoring the correction of glycogen storage disease type 1a in a mouse model using [(18)F]FDG and a dedicated animal scanner. Life Sci. 2002 Aug 2;71(11):1293-301.

52. Minn H, Kangas L, Knuutila V, Paul R, Sipila H. Determination of 2-fluoro-2-deoxy-D-glucose uptake and ATP level for evaluating drug effects in neoplastic cells. Res Exp Med (Berl). 1991;191(1):27-35.

53. Timperley WR. Glycolysis in neuroectodermal tumours. In: Thomas DGT, Graham DI, eds. Brain tumours. Scientific basis, clinical investigation and current therapy. London: Butterworth's 1980:145-67

54. Di Chiro G, Brooks RA, Patronas NJ, Bairamian D, Kornblith PL, Smith BH, Mansi L, Barker J. Issues in the in vivo measurement of glucose metabolism of human central nervous system tumors. Ann Neurol. 1984;15 Suppl:S138-46.

55. Padma MV, Said S, Jacobs M, Hwang DR, Dunigan K, Satter M, Christian B, Ruppert J, Bernstein T, Kraus G, Mantil JC. Prediction of pathology and survival by FDG PET in gliomas. J Neurooncol. 2003 Sep;64(3):227-37.

56. Dwarakanath BS, Jain VK. Modification of the radiation induced damage by 2-deoxy-D-glucose in organ cultures of human cerebral gliomas. Int J Radiat Oncol Biol Phys. 1987 May;13(5):741-6.

57. Woodward GE, Hudson MT. The effect of 2-desoxy-D-glucose on glycolysis and respiration of tumor and normal tissues. Cancer Res. 1954 Sep;14(8):599-605.

58. Nicasio P, Aguilar-Santamaria L, Aranda E, Ortiz S, Gonzalez M. Hypoglycemic effect and chlorogenic acid content in two Cecropia species. Phytother Res. 2005 Aug;19(8):661-4.

59. Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL, Ravindranath T, Jain V. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol. 2005 Aug;181(8):507-14.

60. Kang HT, Hwang ES. 2-Deoxyglucose: An anticancer and antiviral therapeutic, but not any more a low glucose mimetic. Life Sci. 2005 (in press)

Fig.1

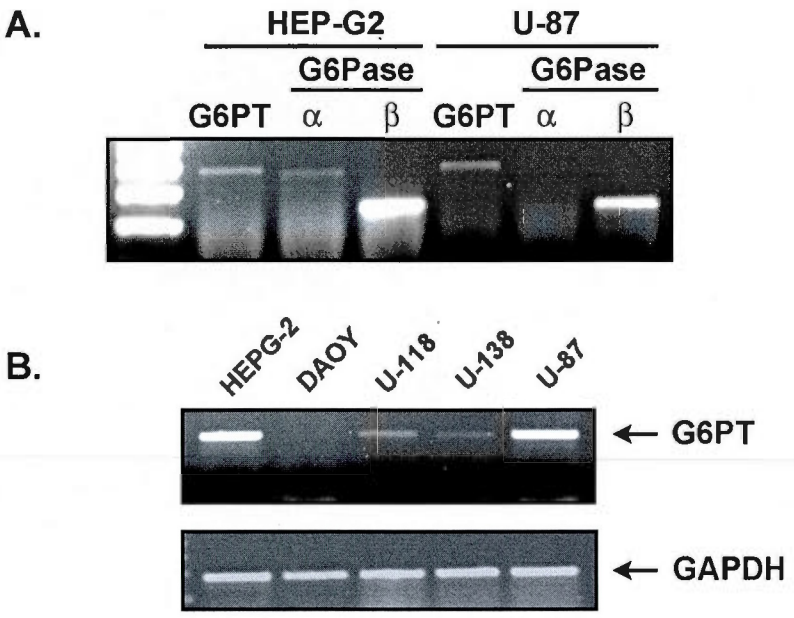

Figure 1. U-87 glioma cells express G6PT and G6Pase-β transcripts with the highest G6PT transcript levels among brain tumor-derived cell lines. Several brain tumor-derived cell lines (glioma : U-87, U-118, U-138; medulloblastoma : DAOY) were cultured until cells reached approximately 90% confluency. Total RNA was extracted and RT-PCR performed in order to assess the gene expression levels of G6PT, G6Pase-α, and G6Pase-β as described in the methods section. HEP-G2 hepatoma cells were used as positive controls for the presence of all three genes. GAPDH gene expression was used as an internal control for each cell line tested.

Fig.2

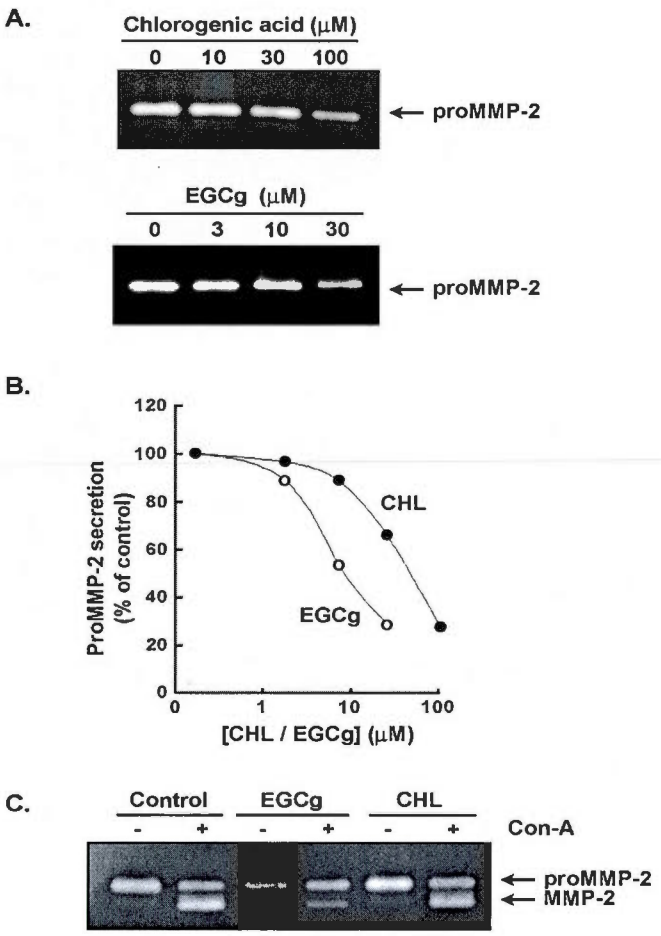

Figure 2. *The G6PT inhibitor chlorogenic acid reduces proMMP-2 secretion in U-87 glioma cells.* (A) U-87 glioma cells were serum-starved in the presence of increasing concentrations of CHL and of the green tea catechin EGCg for 18 hrs. Conditioned media was collected and proMMP-2 gelatinolytic activity assessed by zymography as described in the methods section. (B) The extent of proMMP-2 hydrolytic activity was quantified by scan densitometry and is representative of one out of three independent experiments. (C) The effect of CHL and EGCg on MT1-MMP functions was also monitored. U-87 cells were cultured in the presence of an exogenous source of proMMP-2 in the presence of concanavalin-A, a MT1-MMP-mediated inducer of proMMP-2 activation, and of EGCg or CHL. The extent of proMMP-2 activation was assessed by gelatin zymography.

Fig.3

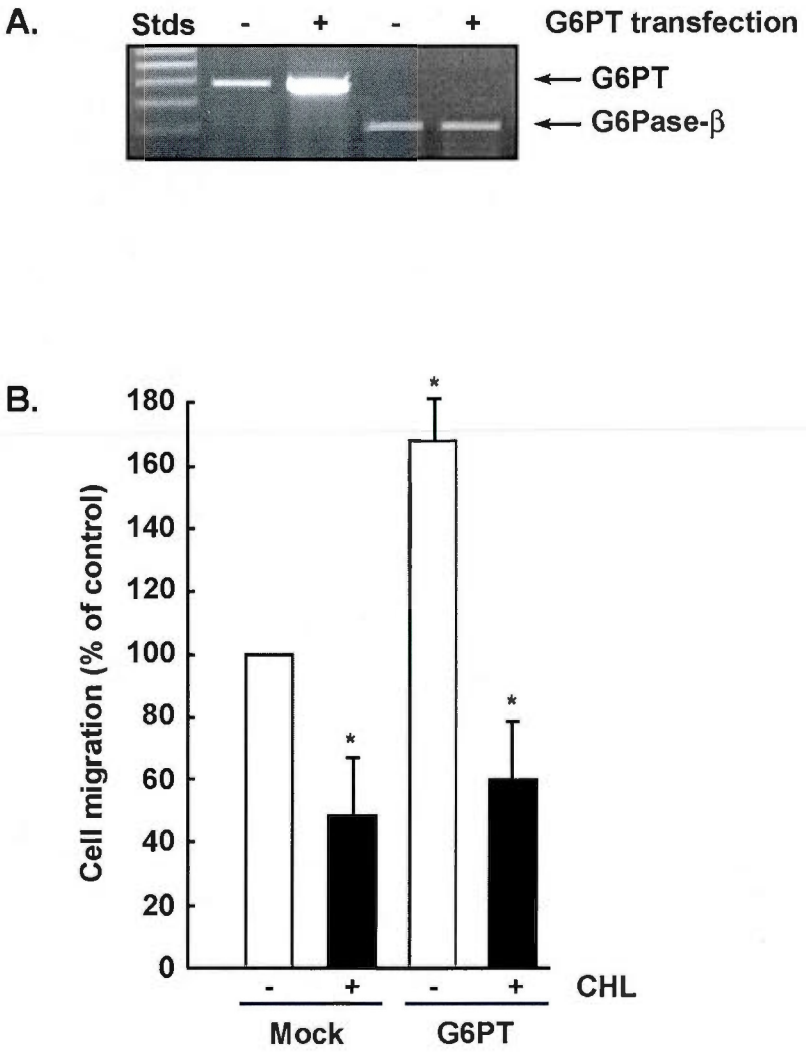

Figure 3. *G6PT regulates U-87 glioma cells migration.* U-87 glioma cells transfected with a cDNA plasmid encoding G6PT (refs) as described in the methods section. (A) Total RNA was extracted from Mock or G6PT-transfected cells (Tx) and RT-PCR performed respectively for G6PT and G6Pase-β gene expression. (B) Basal cell migration was assessed in Mock and G6PT-transfected U-87 cells as described in the methods section in the presence or not of 100 μM CHL.

Fig.4

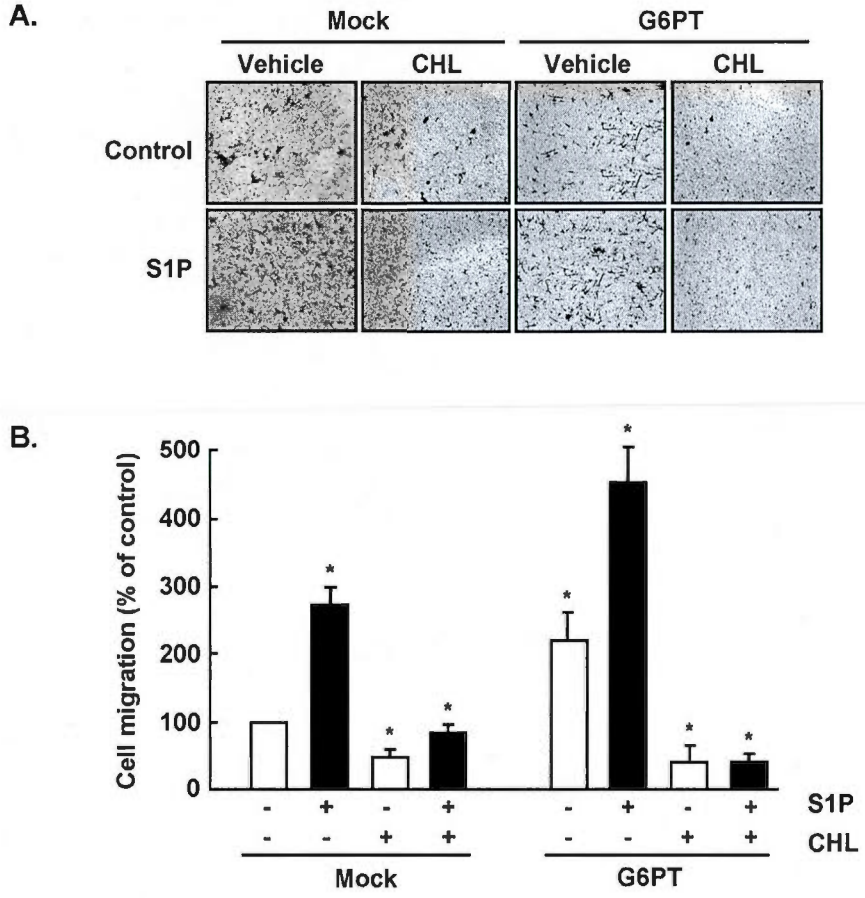

Figure 4. *Chlorogenic acid inhibits sphingosine-1-phosphate-induced U-87 glioma cells migration.* (A) Control (Mock) or G6PT-transfected U-87 glioma cells were seeded on gelatin-coated filters and cell migration assessed in the presence or not of 100 μM CHL, 1 μM S1P, or a combination of both. (B) Quantification was performed as described in the methods section and is representative of one out of three independent experiments.

Fig.5

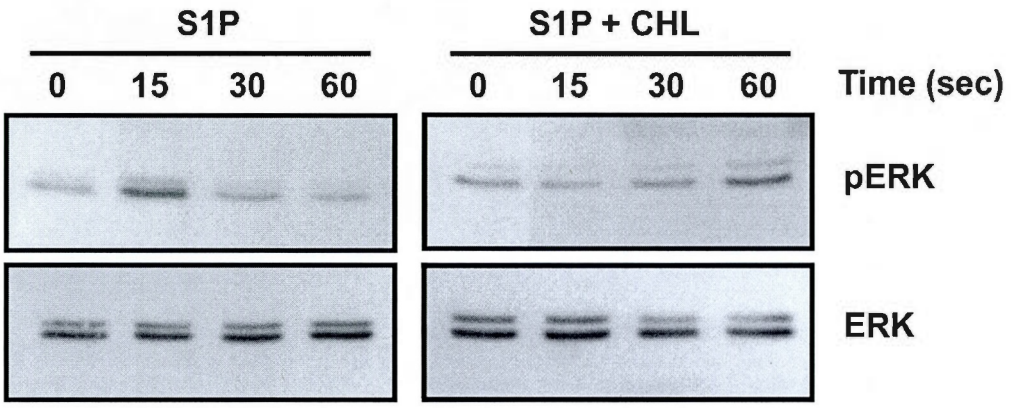

Figure 5 . *Chlorogenic acid inhibits sphingosine-1-phosphate-induced ERK phosphorylation in U-87 glioma cells.* Serum-starved U-87 glioma cells were pre-treated with for 30 minutes with 100 μM CHL. One μM S1P was then added to the media and incubation performed for 15, 30, and 60 seconds. Cells were then rapidly harvested and lysates prepared as described in the Methods section. The extent of ERK phosphorylation was monitored by immunoblotting using antibodies against p-ERK and total ERK.

Fig.6

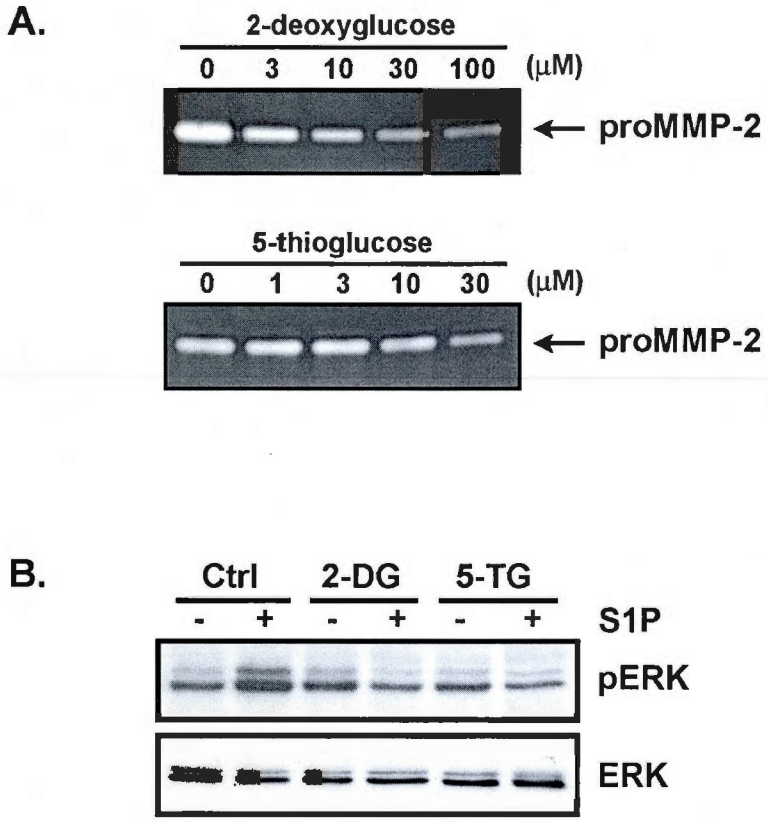

Figure 6 . *Intracellular ATP-depleting agents inhibit proMMP-2 secretion and antagonize S1P-induced ERK phosphorylation in U-87 glioma cells.* (A) Serum-starved U-87 glioma cells were treated for 18 hrs with increasing concentrations of 2-deoxyglucose and 5-thioglucose, two well established ATP-depleting agents. Conditioned media were collected and the hydrolytic activity of proMMP-2 assessed using gelatin-zymography as described in the Methods section. (B) The extent of proMMP-2 hydrolytic activity was quantified by scan densitometry and is representative of one out of three independent experiments. (C) Serum-starved U-87 glioma cells were pre-treated with for 30 minutes with 100 μM 2-deoxyglucose (2-DG) or 30 μM 5-thioglucose (5-TG). One μM S1P was then added to the media for 15 seconds. Cells were then rapidly harvested and lysates prepared as described in the Methods section.

ARTICLE # 2

Silencing of the human microsomal glucose-6-phosphate translocase induces glioma cell death : Potential new anticancer target for curcumin

Silencing of the human microsomal glucose-6-phosphate translocase induces glioma cell death : Potential new anticancer target for curcumin

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Inhibition of the microsomal glucose-6-phosphate translocase (G6PT) by chlorogenic acid induces apoptosis in neutrophils and in differentiated HL-60 leukemia cells, suggesting an important role for G6PT in cell survival. In addition, G6PT is also thought to play a crucial role in transducing intracellular signaling events regulating brain tumor-derived cancer cells invasiveness. In this report, we investigated the specific contribution of G6PT to the control of U-87 brain tumor-derived glioma cell survival using small interfering RNA (siRNA)-mediated suppression of G6PT. Three different siRNA constructs were generated against the human G6PT gene and found to suppress by up to 91% G6PT gene expression, while the glucose-6-phosphatase-β gene expression remained unaffected. Flow cytometry analysis of propidium iodide/annexin-V-stained cells indicate that silencing the G6PT gene significantly induced necrosis and late apoptosis. Increasing concentrations of the anticancer agent curcumin, inhibited G6PT gene expression by more than 90% and triggered U-87 glioma cells death. Overexpression of recombinant G6PT rescued the cells from curcumin-induced cell death. In conclusion, our data suggest that G6PT plays a key role in regulating glioma cells survival. Targeting G6PT expression and functions may provide a new mechanistic rationale for the action of chemopreventive drugs and lead to the development of new anti-cancer strategies.

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Running title : Pro-survival functions of the microsomal G6P translocase

INTRODUCTION

The incidence of malignant brain tumors is increasing in both children and in adults, and this type of cancer is often unmanageable due to its diffuse infiltrating nature.[1, 2] Although the prognosis is very grim, the standard therapies for malignant gliomas i.e. surgical resection and radiation only retard glioma growth for a short period and, paradoxically, can facilitate recurrence in the long run.[3] Hence, new approaches are needed to target the very infiltrating nature of that cancer, prevent recurrence and provide long-term management of malignant brain tumors. Lately, many dietary polyphenols have been shown to have anti-cancer properties due to their chemopreventive and anti-tumor activities.[4, 5] Among these, we have recently demonstrated that both the green tea polyphenol epigallocatechin-3-gallate (EGCg),[6, 7] as well as another polyphenol chlorogenic acid (CHA),[8] efficiently inhibited several glioblastoma cell invasive processes. Interestingly, glucose-lowering properties have also been attributed to both of these molecules, which also make them valuable anti-diabetic agents.[9, 10] Both the modulation of blood glucose levels and the chemopreventive ability of EGCg and CHA could be potentially linked to a common intracellular target, the microsomal glucose-6-phosphatase (G6Pase) system.[11, 12]

The G6Pase system catalyses the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate as a final step in both glucose-producing pathways in the liver: gluconeogenesis and glycogenolysis.[13] G6Pase is a multicomponent enzyme, consisting of three integral proteins which are located in the endoplasmic reticulum (ER): (*i*) a catalytic unit facing the lumen of the ER, (*ii*) a G6P translocase (G6PT), and (*iii*) a second translocase that mediates efflux of phosphate. While the rate-limiting step of that process is thought to be catalyzed by G6PT, its functionality in non-neoglucogenic tissues such as brain remains poorly characterized.[14] Recent

evidence, however, suggest that CHA, the most potent functional inhibitor of G6PT, triggers a host of cellular events including apoptosis in neutrophils and differentiated promyelocytic HL-60 cells,[15] and inhibition of matrix metalloproteinase (MMP) secretion in the human Hep3B hepatocellular carcinoma cell line.[16] CHA also inhibits glioma cell migration, response to chemotactic growth factors, and secretion of MMP,[8] all prerequisite processes needed for tumor growth and metastasis to occur. Whether G6PT is involved in the survival of brain tumor-derived cancer cells is currently unknown.

Aside from regulating the rate limiting step of G6P transport through the ER membrane, the specific, functional inhibition of G6PT by CHA becomes more relevant when one considers G6PT's putative role in regulating calcium-mediated signalling.[17] It has been hypothesized that G6PT might function as a G6P receptor/sensor[18] or that it could favor calcium sequestration in the ER lumen.[17] Such G6PT-mediated ER functions may collectively be responsible for crucial survival processes such as cell proliferation, cell cycle division, extracellular matrix degradation, and response to growth factors during brain tumor development.[19] Accordingly, alterations in several biochemical parameters - glucose phosphorylation, calcium mobilization, and hexose uptake and transport - have already been described as possible mechanisms through which the G6PT functional defects may be involved.[20-22]

Enhanced glucose usage *in vitro*, as well an *in vivo*, is correlated with the degree of malignancy and with poor prognosis for patients with glioma tumors.[23, 24] Interference with G6PT functions may thus be an attractive therapeutic approach to control glioma cell growth, as selective inhibition of G6PT could represent an ideal approach for metabolic regulation of brain tumor cells. Interestingly, glioma cell proliferation and survival have recently been shown to be affected by curcumin

(diferuloyl-methane), the yellow pigment found in the spice turmeric.[25, 26] Because curcumin regulates key enzymes involved in carbohydrate metabolism[27, 28] and has chemopreventive properties,[29, 30] we investigated the effects of curcumin on G6PT gene expression and on U-87 glioma cells survival.

Key words : Glioma, glucose-6-phosphate translocase, curcumin, cell death

The abbreviations used are : ATP, adenosine triphosphate; CHA, chlorogenic acid; 2-DG, 2-deoxy-D-glucose; ECM, extracellular matrix; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P translocase; GSD, glycogen storage disease; MMP, matrix metalloproteinase; PI, propidium iodide; siRNA, small interfering ribonucleic acid

MATERIALS AND METHODS

Cell culture and cDNA transfection method : The U-87 glioma cell line was purchased from American Type Culture Collection and cultured in Eagle's Minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C under a humidified atmosphere containing 5% CO_2. The G6PT plasmid was generously provided by Dr Christopher Newgard (University of Texas Southwestern Medical Center, Dallas, TX) and validated.[34] U-87 glioma cells were transiently transfected with the cDNA construct using Lipofectamine 2000 (Invitrogen, Burlington, ON). Transfection efficiency was confirmed by RT-PCR as described below. All experiments involving these cells were performed 36 hrs following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

RNA interference : RNA interference experiments were performed using Lipofectamine 2000. Three small interfering RNA (siRNA) oligonucleotides for human G6PT (gene ID : NM_001467) and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield, IN), and annealed to form duplexes. The sequences of the three siRNA used in this study are as follows : siG6PT #1 : 5'-GCACUACAGUUGGAGCACAdTdT-3' (sense) and 5'-UGUGCUCCAACUGUAGUGCdTdT-3' (antisense), siG6PT #2 : 5'-CUGUGAUCUUCUCAGCCAUdTdT-3' (sense) and 5'-AUGGCUGAGAAGAUCACAGdTdT-3' (antisense); siG6PT #3 : 5'-CGAAACAUCCGCACCAAGAdTdT-3' (sense) and 5'-UCUUGGUGCGGAUGUUUCGdTdT-3' (antisense).

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis : Total RNA was extracted from cultured monolayers of U-87 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). 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). Primers for G6Pase-α (forward : 5'-TTCAGCCACATCCACAGCATC-3', reverse : 5'-GGGGTTTCAAGGAGTCAAAGACG-3'), for G6Pase-β (forward : 5'-ACTCTTCCTGACTTCTTGTGTGCC-3', reverse : 5'-TTGCCTTTGCTCTTTGGGGG-3') and for G6PT (forward : 5'-CAGGGCTATGGCTATTATCGCAC-3', reverse : 5'-ATGGCTCAAACCACTTCCGCAG-3') were all derived from human sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal house-keeping gene control. PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification[8] and the products were resolved on 1.5% agarose gels containing 1 μg/ml ethidium bromide.

Analysis of cell death by flow cytometry: Cell death was assessed by flow cytometry in cells treated with curcumin (Sigma, Oakville, ON), as well as in untransfected (mock) cells or cells transfected with the G6PT cDNA or with siG6PT #3 oligonucleotides. Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Then, $2x10^5$ cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin-V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Mississauga, ON). 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 indicate 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 well known to translocate 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 dye 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 nonparametric one-way ANOVA with GraphPad Prism Version 4.0. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

RESULTS

Specific G6PT gene silencing in U-87 glioma cells. We first assessed microsomal G6PT gene expression, as well as the expression of the two glucose-6-phosphatase (G6Pase) isoforms α and β. Total RNA was extracted from HepG2 hepatoma and U-87 glioma cells, and then RT-PCR was performed. As it would be expected for a cell line derived from a neoglucogenic tissue, HepG2 cells expressed all three components of the G6Pase system, with a higher expression of G6Pase-β (Fig.1A). In contrast to HepG2, only G6PT and G6Pase-β transcripts were significantly expressed in U-87 glioma cells, with very low to undetectable levels of G6Pase-α (Fig.1A). This is in agreement with previous reports demonstrating a lack of G6Pase-α expression in brain-derived cells.[14] Because previous evidence demonstrated that functional inhibition of the microsomal G6PT with CHA abrogated the cell migration and the chemotactic response of U-87 cells to growth factors,[8] we have generated siRNA constructs designed to specifically downregulate G6PT gene expression in U-87 glioma cells. Three siRNA constructs were designed and cell transfection performed as described in the Methods section. RT-PCR analysis showed that G6PT transcription was specifically downregulated by all three constructs, since G6Pase-β gene expression remained unaffected by neither of them (Fig.1B). siG6PT constructs #1, #2, and #3 inhibited G6PT gene expression by 32%, 55% and 91% respectively (Fig.1C). Although treatment with a combination of the respective constructs was not tested, subsequent experiments were performed using siG6PT construct #3.

G6PT gene silencing triggers U-87 glioma cell death. To investigate the specific contribution of G6PT to cell survival processes, we used siG6PT construct #3 to downregulate G6PT gene expression. siG6PT construct #3 specifically silenced the

G6PT gene and not that of G6Pase-β and MT1-MMP, a membrane-bound matrix metalloproteinase that we have previously shown to regulate, in part, the invasiveness of U-87 glioma cells[7, 31] (Fig.2A). We next assessed cell survival using flow cytometry with propidium iodide and annexin-V staining. Our results show that, in siG6PT-transfected cells, there was an increase in overall cell death as demonstrated by a significant shift in fluorescence in cells that stained positive for necrosis (Fig.2B, upper left quadrant) as well as in late phase of apoptosis (Fig.2B, upper right quadrant). Quantification of these data shows that G6PT gene downregulation triggered a 1.5-fold increase in cell necrosis and a 2.4-fold increase in cells undergoing the late stage of apoptosis (Fig.2C). Altogether, these results suggest that G6PT is an important pro-survival protein and that any alteration in its expression could be deleterious to the cell.

The anticancer molecule curcumin inhibits G6PT gene expression in U-87 glioma cells. Since curcumin (diferuloyl-methane) has recently been attributed chemopreventive properties,[29, 30] and appears to affect glioma cells proliferation and survival,[25, 26] we investigated the effects of curcumin on G6PT gene expression in U-87 glioma cells. Cells were treated with increasing concentrations of curcumin then, total RNA was isolated to assess G6PT and G6Pase-β gene expression using RT-PCR (Fig.3A). While G6Pase-β and actin gene expression levels were not altered, 35 μM curcumin downregulated G6PT transcript levels by more than 90% (Fig.3B). These effects of curcumin suggest that, among the many intracellular proteins that it regulates, G6PT may represent a crucial target that would affect U-87 glioma cell survival.

The overexpression of recombinant G6PT rescues U-87 glioma cells from curcumin-induced cell death. We next addressed whether G6PT possesses any pro-survival functions in U-87 glioma cells. Untransfected (mock) cells or cells

transfected with an expression vector for G6PT [8, 32] were exposed to increasing concentrations of curcumin and cell death was evaluated by flow cytometry as in Fig.2. Overexpression of recombinant G6PT had no effect on cell survival in untreated cells (Fig.4A). However, curcumin dose-dependently triggered an increase in cell death (combined necrosis, early and late apoptosis) that reached an optimal effect at 25 μM (Fig.4B, white bars), and concomitantly reduced cell viability in untransfected mock cells (Fig.4B, white bars). When transient transfection of G6PT cDNA was performed, the newly expressed G6PT prevented cells from undergoing apoptosis (Fig.4A). Fig.4B shows that when G6PT was overexpressed by transient transfection, 40-55% of cells treated with 25-35 μM of curcumin were rescued from entering apoptosis. Altogether, these data strongly suggest that G6PT regulates crucial pro-survival processes in U-87 glioma cells. Our results also suggest that targeting G6PT function or gene/protein expression may permit the development of new anticancer strategies.

DISCUSSION

Glucose is absolutely essential for the survival and function of the brain since, in this tissue, there is no endogenous glucose production. Glucose availability is exclusively dependent upon blood supply which is generated in the post-prandial state by the hydrolysis of G6P through the hepatic and renal G6Pase system. Recently, a ubiquitous G6Pase-β was reported in astrocytes which represent the main reservoir of brain glycogen.[14] Interestingly, the expression and coupling of the G6Pase-β and G6PT activities enabled the formation of an active G6Pase complex, suggesting that astrocytes may provide an endogenous source of brain glucose.[14, 33] However, the physiological significance of this finding is uncertain since brain G6Pase-β has only about 12% of hepatic G6Pase-α activity. Moreover, when co-expressed with recombinant G6PT, the G6Paseβ-G6PT complex showed only ~25% of the maximal G6P accumulation activity of the liver complex.[33] Thus, the physiological roles of the native G6Pase-β remain to be confirmed as initial reports ascribed very low to no activity for this protein.[34] Our current study thus supports the possibility of alternate functions for the ubiquitously expressed G6PT in non-neoglucogenic tissues, and these functions seem to be independent from the classical G6Pase system. Our findings further provide a molecular mechanism accounting for the pro-survival effects of G6PT in brain tumor-derived cancer cells, since we show that a decrease in G6PT expression dramatically affected cell viability.

The involvement of G6PT in brain tumor-derived cell survival processes could be of major physiological significance. Consequently, specific interference with G6PT functions [8, 15] or expression [this study] could be an attractive target for therapeutic control of glioma cell growth, since selective inhibition of G6PT may provide an ideal approach for the metabolic regulation of brain tumor cells. Several

inhibitors of G6PT have been already reported, including diazobenzene sulfonate,[35] phlorizin,[36] tosyllysine chloromethyl ketone, diethyl pyrocarbonate,[37] some stilbene disulfonate derivatives,[38] and fatty acyl-CoA esters.[39] In addition, complex natural products such as ilicicolinic acid B, hericenal C, mumbaistatin, kodaistatins,[40] and derivatives of CHA[11] may have potential G6PT inhibitory activity. Aside from CHA, few of these documented G6PT inhibitors have been systematically tested for their anti-cancer properties. Although more human studies are needed to provide clear evidence between cancer risk and polyphenol consumption, *in vitro* studies however, strongly suggest that dietary polyphenols preferentially induce tumor cell cycle arrest or apoptosis.[5] Our study provides more molecular-level explanation to the chemopreventive properties of curcumin[25, 26] by targeting the pro-survival functions of G6PT in U-87 glioma cells.

Several approaches have been used to differentially modulate glucose flux and energy supply in cancer cells; hypoxic cell sensitizers are also being investigated as potential anti-cancer agents. The glucose antimetabolite, 2-deoxy-D-glucose (2-DG), a competitive inhibitor of glucose transport and phosphorylation, has long been known to block glycolytic flux, therefore modulating the synthesis of adenosine triphosphate (ATP).[41] We have recently shown that 2-DG and 5-thioglucose inhibit the secretion of MMP by U-87 glioma cells. These compounds also inhibit intracellular transduction in response to sphingosine-1-phosphate,[8] presumably, by a mechanism involving ATP-dependent calcium-sequestering activity of G6PT. Since the failure of radiotherapy in cerebral gliomas is primarily due to the diffuse infiltrating nature of the tumor, the abrupt changes in glycolytic energy demands of the brain tumor-derived cells may trigger growth arrest and/or cell death.[42] Thus, an important implication of the current study is the potential therapeutic impact of targeting G6PT functions as part of a radiotherapeutic regimen. Therefore, it is tempting to speculate that strategies aiming at the inhibition of G6PT would be beneficial in conjunction with radiotherapeutic modalities. In support to that

hypothesis, *in vitro* studies performed in established glioma cell lines show that exposure to 2-DG for a few hours after irradiation significantly increased radiation-induced cellular damage,[43] and that cancer radiotherapy was optimized in 2-DG dose escalation studies.[44]

Deficiency in G6PT function has long been recognized to cause glycogen storage disease type 1b (GSD-1b).[45, 46] Indeed, at least sixty-nine distinct mutations in the *G6PT* gene, which either greatly reduce or completely abolish G6PT function, have been identified in GSD-1b patients.[45, 47] These defects, when not rapidly identified, lead to premature death. Over the last few years though, other unrecognized functions of G6PT have been identified. For instance, polymorphonuclear leukocytes from GSD-1b patients exhibit impaired mobility, chemotaxis, and Ca^{2+} flux responses.[47] In addition, their respiratory burst, pentose phosphate shunt, glycolytic activity and phagocytotic activity are also diminished. Consequently, inhibition of G6PT functions in human neutrophils results in apoptosis, and this may explain the dysfunctional activity of neutrophils from GSD-1b patients.[15] These observations strongly support a crucial role for G6PT in keeping optimal cellular functions. While the role of G6PT in carbohydrate metabolism is well understood, its roles in alternate mechanisms such as in immune deficiency or in cancer are relatively unknown. In conclusion, our data suggest that G6PT plays a central role in regulating glioblastoma cell survival and invasiveness. Strategies aiming at the inhibition of G6PT functions with anticancer agents, such as the naturally occurring curcumin, may provide a new mechanistic rationale for the action of chemopreventive drugs and lead to the development of new anticancer strategies.

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REFERENCES

1. Baldwin RT and Preston-Martin S (2004) Epidemiology of brain tumors in childhood--a review. Toxicol Appl Pharmacol. 199: 118-131

2. Lemke DM (2004) Epidemiology, diagnosis, and treatment of patients with metastatic cancer and high-grade gliomas of the central nervous system. J Infus Nurs. 27: 263-269

3. Demuth T and Berens ME (2004) Molecular mechanisms of glioma cell migration and invasion. J Neurooncol. 70: 217-228

4. Kanadaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT and Lee MT (2005) The antitumor activities of flavonoids. In Vivo. 19: 895-909

5. Manson MM (2005) Inhibition of survival signalling by dietary polyphenols and indole-3-carbinol. Eur J Cancer. 41: 1842-1853

6. Annabi B, Lachambre MP, Bousquet-Gagnon N, Page M, Gingras D and Beliveau R (2002) Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. Biochim Biophys Acta. 1542: 209-220.

7. Annabi B, Bouzeghrane M, Moumdjian R, Moghrabi A and Beliveau R (2005) Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-

mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. J Neurochem. 94: 906-916

8. Belkaid A, Currie JC, Desgagnés J and Annabi B (2006) The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression. Cancer Cell International 6: 7

9. Nicasio P, Aguilar-Santamaria L, Aranda E, Ortiz S and Gonzalez M (2005) Hypoglycemic effect and chlorogenic acid content in two Cecropia species. Phytother Res. 19: 661-664

10. Kao YH, Chang HH, Lee MJ and Chen CL (2006) Tea, obesity, and diabetes. Mol Nutr Food Res. 50: 188-210

11. Hemmerle H, Burger HJ, Below P, Schubert G, Rippel R, Schindler PW, Paulus E and Herling AW (1997) Chlorogenic acid and synthetic chlorogenic acid derivatives: novel inhibitors of hepatic glucose-6-phosphate translocase. J Med Chem. 40: 137-145

12. Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M and Granner DK (2002) Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. J Biol Chem. 277: 34933-34940

13. van Schaftingen E and Gerin I (2002) The glucose-6-phosphatase system. Biochem J. 362: 513-532

14. Ghosh A, Cheung YY, Mansfield BC and Chou JY (2005) Brain contains a functional glucose-6-phosphatase complex capable of endogenous glucose production. J Biol Chem. 280: 11114-11119

15. Leuzzi R, Banhegyi G, Kardon T, Marcolongo P, Capecchi PL, Burger HJ, Benedetti A and Fulceri R (2003) Inhibition of microsomal glucose-6-phosphate transport in human neutrophils results in apoptosis: a potential explanation for neutrophil dysfunction in glycogen storage disease type 1b. Blood. 101: 2381-2387

16. Jin UH, Lee JY, Kang SK, Kim JK, Park WH, Kim JG, Moon SK and Kim CH (2005) A phenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor: isolation and identification from methanol extract of Euonymus alatus. Life Sci. 77: 2760-2769

17. Chen PY, Csutora P, Veyna-Burke NA and Marchase RB (1998) Glucose-6-phosphate and Ca2+ sequestration are mutually enhanced in microsomes from liver, brain, and heart. Diabetes. 47: 874-881

18. Hiraiwa H, Pan CJ, Lin B, Moses SW and Chou JY (1999) Inactivation of the glucose 6-phosphate transporter causes glycogen storage disease type 1b. J Biol Chem. 274: 5532-5536

19. Thorsen F and Tysnes BB (1997) Brain tumor cell invasion, anatomical and biological considerations. Anticancer Res. 17: 4121-4126

20. Potashnik R, Moran A, Moses SW, Peleg N and Bashan N (1990) Hexose uptake and transport in polymorphonuclear leukocytes from patients with glycogen storage disease Ib. Pediatr Res. 28: 19-23

21. Bashan N, Potashnik R, Peist A, Peleg N, Moran A and Moses SW (1993) Deficient glucose phosphorylation as a possible common denominator and its relation to abnormal leucocyte function, in glycogen storage disease 1b patients. Eur J Pediatr. 152: S44-S48

22. Korchak HM, Garty BZ, Stanley CA, Baker L, Douglas SD and Kilpatrick L (1993) Impairment of calcium mobilization in phagocytic cells in glycogen storage disease type 1b. Eur J Pediatr. 152: S39-S43

23. Timperley WR (1980) Glycolysis in neuroectodermal tumours. In: Thomas DGT, Graham DI, eds. Brain tumours. Scientific basis, clinical investigation and current therapy. London: Butterworth's pp145-167

24. Padma MV, Said S, Jacobs M, Hwang DR, Dunigan K, Satter M, Christian B, Ruppert J, Bernstein T, Kraus G and Mantil JC (2003) Prediction of pathology and survival by FDG PET in gliomas. J Neurooncol. 64: 227-237

25. Gao X, Deeb D, Jiang H, Liu YB, Dulchavsky SA and Gautam SC (2005) Curcumin differentially sensitizes malignant glioma cells to TRAIL/Apo2L-mediated apoptosis through activation of procaspases and release of cytochrome c from mitochondria. J Exp Ther Oncol. 5: 39-48

26. Nagai S, Kurimoto M, Washiyama K, Hirashima Y, Kumanishi T and Endo S (2005) Inhibition of cellular proliferation and induction of apoptosis by curcumin in human malignant astrocytoma cell lines. J Neurooncol. 74: 105-111

27. Kuroda M, Mimaki Y, Nishiyama T, Mae T, Kishida H, Tsukagawa M, Takahashi K, Kawada T, Nakagawa K and Kitahara M (2005) Hypoglycemic effects of turmeric (Curcuma longa L. rhizomes) on genetically diabetic KK-Ay mice. Biol Pharm Bull. 28: 937-939

28. Pari L and Murugan P (2005) Effect of tetrahydrocurcumin on blood glucose, plasma insulin and hepatic key enzymes in streptozotocin induced diabetic rats. J Basic Clin Physiol Pharmacol. 16: 257-274

29. Rao CV, Rivenson A, Simi B and Reddy BS (1995) Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res. 55: 259-266

30. Huang MT, Newmark HL and Frenkel K (1997) Inhibitory effects of curcumin on tumorigenesis in mice. J Cell Biochem. 27: 26-34

31. Annabi B, Thibeault S, Moumdjian R and Beliveau R (2004) Hyaluronan cell surface binding is induced by type I collagen and regulated by caveolae in glioma cells. J Biol Chem. 279: 21888-21896

32. An J, Li Y, van De Werve G and Newgard CB (2001) Overexpression of the P46 (T1) translocase component of the glucose-6-phosphatase complex in hepatocytes impairs glycogen accumulation via hydrolysis of glucose 1-phosphate. J Biol Chem. 276: 10722-10729

33. Shieh JJ, Pan CJ, Mansfield BC and Chou JY (2003) A glucose-6-phosphate hydrolase, widely expressed outside the liver, can explain age-dependent resolution of hypoglycemia in glycogen storage disease type Ia. J Biol Chem. 278: 47098-47103

34. Martin CC, Oeser JK, Svitek CA, Hunter SI, Hutton JC and O'Brien RM (2002) Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. J Mol Endocrinol. 29: 205-222

35. Nilsson OS, Arion WJ, Depierre JW, Dallner G and Ernster L (1978) Evidence for the involvement of a glucose-6-phosphate carrier in microsomal glucose-6-phosphatase activity. Eur J Biochem. 82: 627-634

36. Arion WJ, Lange AJ and Walls HE (1980) Microsomal membrane integrity and the interactions of phlorizin with the glucose-6-phosphatase system. J Biol Chem. 255: 10387-10395

37. Arion WJ, Burchell B, Burchell A. Specific inactivation of the phosphohydrolase component of the hepatic microsomal glucose-6-phosphatase system by diethyl pyrocarbonate. Biochem J. 220: 835-842

38. Zoccoli MA and Karnovsky ML (1980) Effect of two inhibitors of anion transport on the hydrolysis of glucose 6-phosphate by rat liver microsomes. Covalent modification of the glucose 6-P transport component. J Biol Chem. 255: 1113-1119

39. Mithieux G and Zitoun C (1996) Mechanisms by which fatty-acyl-CoA esters inhibit or activate glucose-6-phosphatase in intact and detergent-treated rat liver microsomes. Eur J Biochem. 235: 799-803

40. Brauer S, Almstetter M, Antuch W, Behnke D, Taube R, Furer P and Hess S (2005) Evolutionary chemistry approach toward finding novel inhibitors of the

type 2 diabetes target glucose-6-phosphate translocase. J Comb Chem. 7: 218-226

41. Woodward GE and Hudson MT (1954) The effect of 2-desoxy-D-glucose on glycolysis and respiration of tumor and normal tissues. Cancer Res. 14: 599-605

42. Seyfried TN and Mukherjee P (2005) Targeting energy metabolism in brain cancer: review and hypothesis. Nutr Metab (Lond). 2:30

43. Varshney R, Dwarakanath B and Jain V (2005) Radiosensitization by 6-aminonicotinamide and 2-deoxy-D-glucose in human cancer cells. Int J Radiat Biol. 81: 397-408

44. Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL, Ravindranath T and Jain V (2005) Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol. 181: 507-514

45. Chou JY, Matern D, Mansfield BC and Chen YT (2002) Type I glycogen storage diseases: disorders of the glucose-6-phosphatase complex. Curr Mol Med. 2: 121-143

46. Chen LY, Pan CJ, Shieh JJ and Chou JY (2002) Structure-function analysis of the glucose-6-phosphate transporter deficient in glycogen storage disease type Ib. Hum Mol Genet. 11: 3199-3207

47. Chen LY, Shieh JJ, Lin B, Pan CJ, Gao JL, Murphy PM, Roe TF, Moses S, Ward JM, Lee EJ, Westphal H, Mansfield BC and Chou JY (2003) Impaired glucose homeostasis, neutrophil trafficking and function in mice lacking the glucose-6-phosphate transporter. Hum Mol Genet. 12: 2547-2558

Fig.1

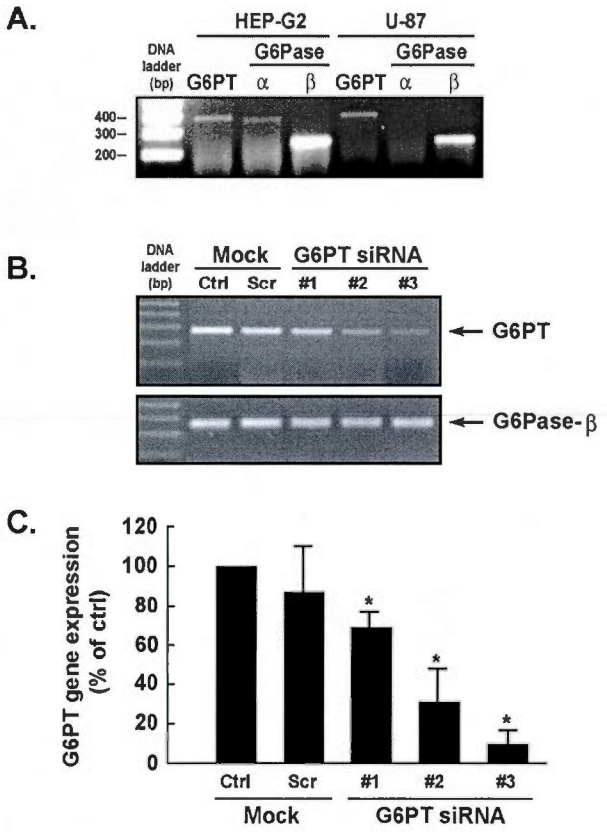

Figure 1. *Specific G6PT gene silencing in U-87 glioma cells.* (A) Brain tumor-derived U-87 glioma cells were cultured until they reached approximately 90% confluency. Total RNA was extracted and RT-PCR performed in order to generate the cDNA reflecting gene expression levels of G6PT (380 bp), G6Pase-α (360 bp), and G6Pase-β (236 bp) as described in the Methods section. HepG2 hepatoma cells were used as positive controls for the presence of all three genes. (B) Three siRNA constructs were generated to knock-down the human G6PT gene in U-87 glioma cells. Cells were either transfected with the siRNA constructs, mock-transfected (lipofectamine) or transfected with a scrambled negative control construct (Neg. Ctrl) as described in the Methods section. Total RNA was isolated 24 hrs post-transfection and the cDNA of G6PT or G6Pase-β amplified as described in the Methods section. A representative ethidium bromide-stained agarose gel of the levels of the respective amplicons is shown. (C) Scanning densitometry of the cDNA products was performed on each gel and the average values ±SEM of four independent experiments is shown.

Fig.2

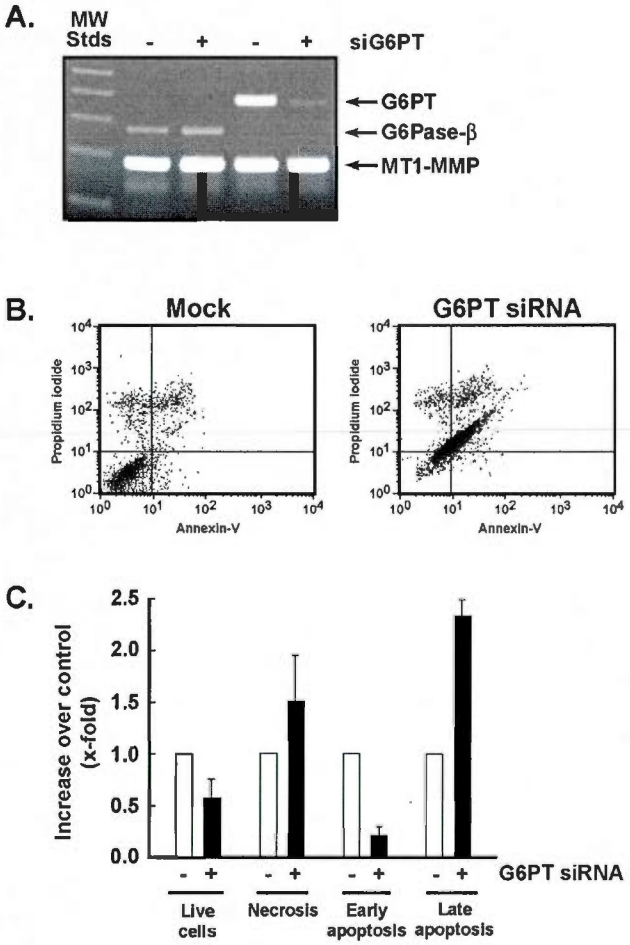

Figure 2. *G6PT gene silencing triggers U-87 glioma cell death.* (A) SiRNA construct #3 was used to transfect U-87 glioma cells in order to decrease G6PT gene expression. Total RNA was isolated and the gene expression of G6PT, G6Pase-β, and MT1-MMP evaluated by RT-PCR as described in the Methods section. (B) Cell apoptosis/necrosis was evaluated by flow cytometry in cells that were stained with propidium iodide (FL2-H) and annexin-V (FL1-H) as described in the Methods section. (C) A representative quantification is shown for each of the quadrants in (B). The results are presented as followes: lower left quadrant, live cells from untransfected (mock) or cells transfected with the G6PT siRNA; upper left quadrant, cells undergoing necrosis; lower right quadrant, cells in the early phase of apoptosis; and upper right quadrant, cells in late phase of apoptosis.

Fig.3

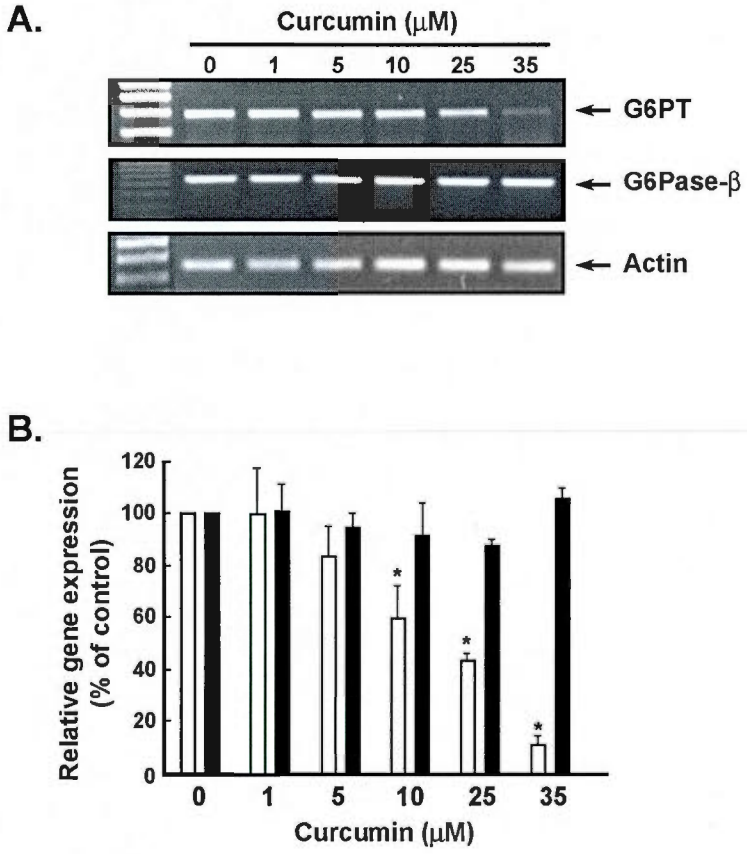

Figure 3. *The anti-cancer molecule curcumin inhibits G6PT gene expression in U-87 glioma cells.* U-87 glioma cells were serum-starved and treated with different concentrations of curcumin for 18 hrs. (A) Total RNA was isolated and G6PT, G6Pase-β, and actin mRNA levels were evaluated by RT-PCR as described in the Methods section. A representative ethidium bromide-stained agarose gel of the observed amplicons is shown. (B) Scanning densitometry was used to evaluate the extent of curcumin effect on G6PT (white bars) and G6Pase-β (black bars) gene expression. Values were normalized with reference to the values for β-actin gene. The results represent the mean values ±SEM of three experiments.

Fig.4

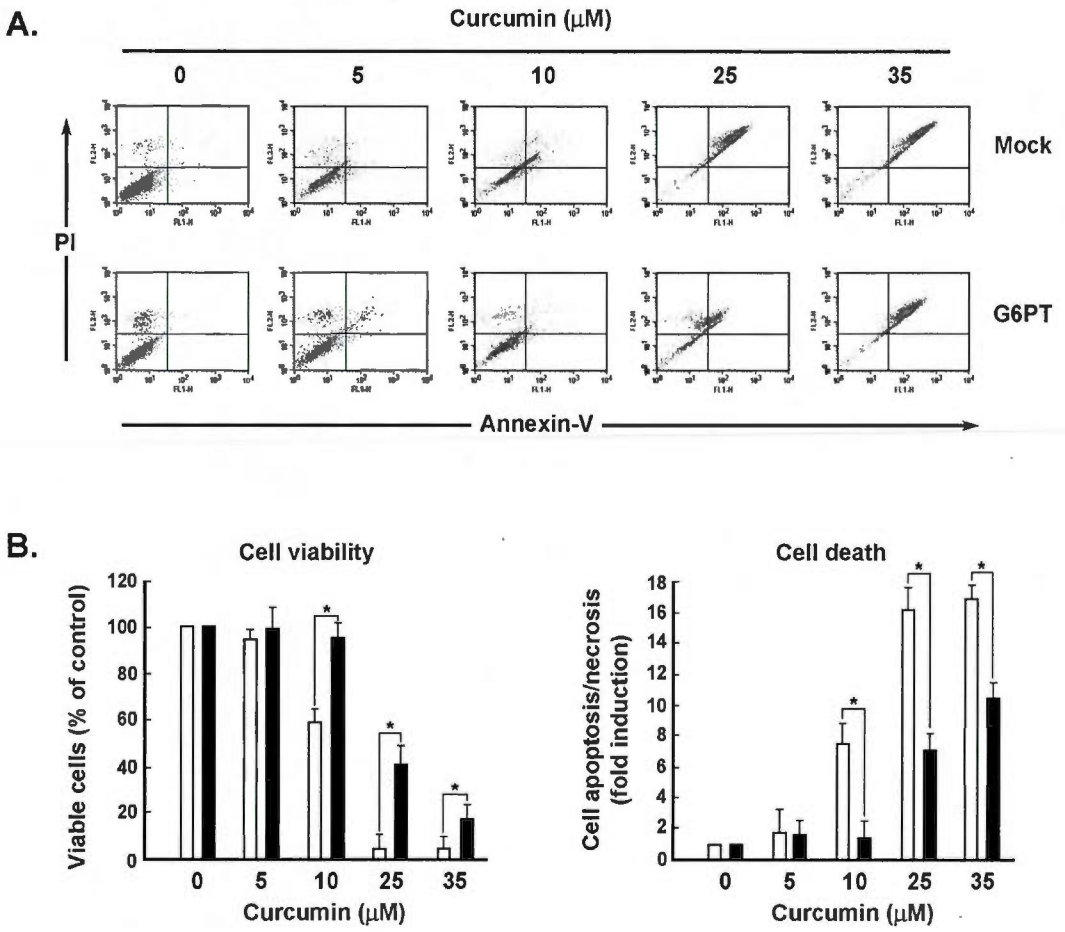

Figure 4. *The overexpression of recombinant G6PT rescues U-87 glioma cells from curcumin-induced cell death.* (A) Untransfected (mock) U-87 glioma cells or cells transfected with the G6PT expression vector were serum-starved and treated with different concentrations of curcumin for 18 hrs. To evaluate cell death, we used flow cytometry of propidium iodide and annexin-V-stained cells as described in the Methods section. (B) Quantification was performed as in the legend to Fig.2. Cell viability values come from the lower, left quadrant, while cell death represents the combined values of necrosis, early, and late apoptosis. White bars : mock cells; black bars : G6PT-transfected cells.

ARTICLE # 3

Necrosis induction in glioblastoma cells reveals a new *"bio-switch"* function for the MT1-MMP/G6PT signalling axis in proMMP-2 activation versus death cell decision

Necrosis induction in glioblastoma cells reveals a new *"bio-switch"* function for the MT1-MMP/G6PT signalling axis in proMMP-2 activation versus death cell decision

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Abstract: Cytoskeleton disorganization is an early step in the activation process of matrix metalloproteinase-2 (MMP-2) by membrane type-1 (MT-1)-MMP, but is also associated to endoplasmic reticulum (ER) dysfunction and subsequent cell death. Given evidence that the ER-embedded glucose-6-phosphate transporter (G6PT) regulates glioblastoma cell survival and that MT1-MMP is a key enzyme in the cancer cells invasive phenotype, we explored the molecular link between G6PT and MT1-MMP. Cytoskeleton disrupting agents such as Concanavalin-A (ConA) and Cytochalasin-D triggered proMMP-2 activation and cell death in U87 glioma cells. ConA decreased G6PT gene expression, an event that was also observed in cells overexpressing the full-length recombinant MT1-MMP protein. Overexpression of a membrane-bound catalytically active but cytoplasmic domain-deleted MT1-MMP was unable to downregulate G6PT gene expression or to trigger necrosis. Gene silencing of MT1-MMP with siRNA prevented proMMP-2 activation and induced G6PT gene expression. ConA inhibited Akt phosphorylation, while overexpression of recombinant G6PT rescued the cells from ConA-induced proMMP-2 activation and increased Akt phosphorylation. Altogether, new functions of MT1-MMP in cell death signalling may be linked to those of G6PT. Our study indicates a molecular signalling axis regulating the invasive phenotype of brain tumor cells and highlights a new *"bio-switch"* function for G6PT in cell survival.

INTRODUCTION

The endoplasmic reticulum (ER) is a membrane-bound organelle present in all eukaryotic cells. Recently, ER stress signaling has been linked to disease states involving insulin resistance, disordered lipid metabolism, and to hypoxia tolerance in tumor progression (1, 2). In addition, the ER is a multifunctional metabolic compartment that controls entry and release of calcium, sterol biosynthesis, apoptosis and the release of arachidonic acid (3, 4). Despite its complex organization, the ER is a continuous membrane compartment whose architecture depends upon microtubule dynamics (5). The ER is primarily known as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. Recent evidence suggests that the microtubulin cytoskeleton and the centrosomes (the microtubulin cytoskeleton-organizing centers) are essential for the trafficking and the internalization of the membrane-bound matrix metalloproteinase MT1-MMP (6), involved in brain tumor cell invasion, extracellular matrix (ECM) degradation and cell-ECM interaction (7). Interestingly, altered expression, maturation and trafficking of MT1-MMP to the plasma membrane were observed in diabetic states (8, 9), a condition known to upregulate the expression of an ER-embedded protein, the glucose-6-phosphate transporter (G6PT) (10). G6PT expression was shown to be downregulated by MT1-MMP in bone marrow-derived stromal cells, where it was suggested to provide a molecular link between proMMP-2 activation and chemotaxis processes in cell mobilization (11).

Several factors are required for optimum protein folding, including ATP, Ca^{2+} and an oxidizing environment which will allow disulphide-bond formation (12). As a consequence of requiring this specialized environment, the stresses which perturb cellular energy levels, redox state or Ca^{2+} concentration can often result in the intracellular accumulation of unfolded protein, which is called ER stress response.

Recently, we have provided evidence that G6PT regulated U87 glioma cell chemotaxis (13) and survival (14). Tumor cells often show evidence of constitutive ER stress, possibly due to hypoxia and glucose depletion (15). In fact, the ATP depleting agents and ER stress inducers 2-Deoxyglucose and 5-Thioglucose have been shown to inhibit MMP-2 secretion from U87 glioma cells (13) a process known to contribute to tumor development (16). G6PT is thought to have a role in sequestering intracellular Ca^{2+} within the ER through an ATP-mediated process (17). Since manipulating the ER stress response of tumor cells is a promising therapeutic strategy (15) and because various anticancer drugs have been shown to induce ER stress and to affect the invasive or metabolic control of cancer cells (18, 19), we explored the potential molecular link between MT1-MMP and G6PT functions within the ER that could potentially regulate the brain tumor cell invasive phenotype.

The abbreviations used are : Concanavalin-A, ConA; Cytochalasin-D, CytoD; ECM, extracellular matrix; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P transporter; GSD, glycogen storage disease; MMP, matrix metalloproteinase; PI, propidium iodide; siRNA, small interfering ribonucleic acid

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). The polyclonal antibodies against Akt and phospho-Akt were purchased from Cell Signaling (Danvers, MA). All other reagents were from Sigma-Aldrich Canada.

Cell culture and transfection method : The U87 glioblastoma cell line was purchased from American Type Culture Collection (Manassasa, VA) and cultured in Eagle's Minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 2 mM glutamine, at 37°C under a humidified atmosphere containing 5% CO_2. U87 glioblastoma cells were transiently 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.

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 and 0.02% NaN_3, 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 death by flow cytometry: Cell death was assessed by flow cytometry as described previously (13). Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Then, 10^5 cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin-V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Mississauga, ON). 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 indicate 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 well known to translocate 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 dye 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 probability values of less than 0.05 were considered significant; an asterisk (*) identifies such significance in each figure.

RESULTS

Differential induction of cell necrosis and cell apoptosis by cytoskeleton disrupting agents. Concanavalin-A (ConA) and cytochalasin-D (CytoD) have been shown to disrupt the cytoskeleton architecture (21). Following cell staining with Annexin-V/Propidium iodide (PI), flow cytometry was used to assess the extent of cell death induced by both agents. From the SSC/FSC plots, the changes in cell morphology that each agent induced are clearly visible (Fig.1a). When Annexin-V/PI cell staining was performed, ConA treatment resulted in a marked increase in necrosis (Fig.1a, lower panels, upper left quadrant), while Cyto-D triggered late apotosis (Fig.1a, lower panels, upper right quadrant). Cell viability and total cell death were quantified (Fig.1b). Furthermore, cell necrosis and cell apoptosis (early and late) (Fig.1c) were also separately quantified in order to show the differential induction of necrosis by ConA, and induction of apoptosis by CytoD.

Recombinant MT1-MMP and cytoskeleton disrupting agents induce proMMP-2 activation. Latent proMMP-2 activation into its active MMP-2 form is an MT1-MMP-mediated event that has also been correlated to cell death (22, 23). Accordingly, we have previously shown that the cytoskeleton disrupting agents ConA and CytoD triggered proMMP-2 activation in U87 glioma cells (24). In particular, overexpression of full-length wild-type (Wt) recombinant MT1-MMP or of cytoplasmic-deleted recombinant MT1-MMP (Fig.2a) also triggered proMMP-2 activation as demonstrated by gelatine zymography (Fig.2b). Transfections of cells with cDNA encoding GFP alone did not affect ConA or CytoD ability to induce proMMP-2 activation (data not shown). These results suggest that, similar to cytoskeleton disrupting agents ConA and CytoD, MT1-MMP-mediated proMMP-2 activation is also potentially linked to the control of cell survival in U87 glioma cells.

Concanavalin-A-mediated activation of proMMP-2 requires MT1-MMP. In order to assess the involvement of MT1-MMP in both the proMMP-2 activation process and the necrotic effects of ConA, we specifically downregulated MT1-MMP gene expression using a specific MT1-MMP gene silencing strategy (20). Cells were transfected with mismatched (Mock) or MT1-MMP targeted siRNA duplexes as described in the Methods section, and then treated with increasing concentrations of ConA. Total RNA was isolated and RT-PCR confirmed that the MT1-MMP gene downregulation was successfully achieved (Fig.3a). Conditioned media from these conditions were also isolated in order to assess the MT1-MMP-mediated proMMP-2 activation by ConA. Gelatin zymography clearly showed significantly decreased proMMP-2 activation in the cells in which MT1-MMP gene expression had been knocked-down (Fig.3b). This was quantified by scanning densitometry, showing close to 90% inhibition (data not shown). This clearly suggests that the ConA-mediated events that we previously observed involve MT1-MMP in either proMMP-2 activation or cell survival.

MT1-MMP cytoplasmic domain is responsible for cell death signalling. Whether MT1-MMP also controls some cell death processes was next evaluated. Transient transfection using cDNA plasmids encoding either the full-length or the cytoplasmic-deleted recombinant forms of MT1-MMP was performed in U87 glioma cells. Annexin-V/PI staining was then performed and cell death (necrosis and apoptosis) assessed by flow cytometry (Fig.4a). Overexpression of native MT1-MMP significantly triggered cell necrosis by more than 10-times, while cell apoptosis was also induced approximately 2-fold (Fig.4b, grey bars). Although still catalytically active at the cell surface (Fig.2b), the deletion of the MT1-MMP cytoplasmic domain significantly abrogated the induction of both cell necrosis and apoptosis (Fig.4b, black bars). These observations suggest that active MMP-2 is not responsible for cell death and that some MT1-MMP-mediated intracellular signalling is a pre-requisite for the control of cell survival.

MT1-MMP overexpression and concanavalin-A treatment downregulate G6PT gene expression. To investigate the intracellular events involved in MT1-MMP- and ConA-mediated cell death, we examined the pro-survival microsomal glucose-6-phosphate translocase (G6PT) as a potential link. Cytoskeleton disruption is often linked to ER stress (25, 26), and silencing of G6PT, a microsomal resident protein, has recently been shown to induce cell death in U87 glioma cells (13). We thus isolated total RNA from ConA-treated cells and from MT1-MMP-transfected cells since cell necrosis was a common event in both conditions. RT-PCR was performed as described in the Methods section and we found that G6PT gene expression was significantly reduced in ConA-treated and in the MT1-MMP-transfected cells (Fig.5a). Interestingly, in agreement with its inability to trigger cell death, deletion of MT1-MMP's cytoplasmic domain was also ineffective in reducing G6PT gene expression. Gene expression of G6Pase-β, the only other component of the gluscose-6-phosphatase system that was expressed in U87 cells (12) and of GAPDH remained unaffected and can be considered as unaffected internal controls (Fig.5a). Con-A treatment and MT1-MMP overexpression resulted, as expected, in an increase in MT1-MMP transcript levels. Altogether, this demonstrates that necrosis-inducing conditions, such as those triggered by ConA or overexpression of recombinant MT1-MMP, are molecularly linked to the pro-survival functions of G6PT. Interestingly, when MT1-MMP gene expression was silenced, the expression of G6PT increased significantly in comparison to the mismatched siRNA-transfected cells (Mock), suggesting that MT1-MMP exerted a repressive effect on G6PT gene regulation (Fig.5b). G6PT gene expression modulation was further confirmed at the protein level. We showed that ConA treatment or Wt-MT1-MMP overexpression downregulated G6PT protein expression (Fig.5c). Thus, our results show that G6PT gene regulation is signalled by the intracellular MT1-MMP cytoplasmic domain.

G6PT overexpression antagonizes the ConA-mediated lethal effect and rescues cells from MT1-MMP-mediated cell death. In order to characterize the molecular mechanism linking MT1-MMP to G6PT, we next assessed whether constitutively expressed recombinant G6PT could overcome the lethal effect of ConA. MT1-MMP synthesis by a mechanism that involves Phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR was recently highlighted (27) and Akt phosphorylation state explored. U87 cells were transiently transfected with cDNA encoding G6PT and then treated with the cell death-inducer ConA. Immunodetection of total and phosphorylated Akt was performed on Mock and G6PT-transfected cell lysates. We observed that ConA decreased the basal levels of phosphorylated Akt by up to 50%, though not of total Akt protein expression, in Mock-transfected cells (Fig.6a). Transient overexpression of recombinant G6PT completely reversed the effects of ConA on Akt phosphorylation (Fig.6b). These observations confirm the G6PT prosurvival activity (13) and show that targeting PI3K/Akt signaling by ConA induces apoptosis (28). Finally, we showed by zymography that G6PT overexpression significantly antagonized ConA-mediated proMMP-2 activation (Fig.6c), an effect that may involve inhibiting MT1-MMP functions.

DISCUSSION

Gliomas remain a great challenge in oncology today as they account for more than 50% of all brain tumors and are by far the most common primary brain tumors in adults (29). More importantly, the mechanisms involved in the resistance of migrating glioblastoma cells to chemotherapy or to radiation-induced cell death have long been recognized (30), and still receive much attention in order to optimize future cellular targets for the treatment of glioblastomas (31). A relationship between cell migration and apoptosis was highlighted by the observations that resistance to apoptosis is closely linked to tumorigenesis, but that paradoxically migrating tumor cells can also still be induced to die by non-apoptotic mechanisms such as necrosis (32). 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 oxygenated by the pre-existing vasculature (33). Because tumor cells respond to hypoxic stress by upregulating a variety of genes involved in glucose uptake, glycolysis and angiogenesis, all essential to maintaining nutrient availability and intracellular ATP levels (34), the intracellular metabolic compartments regulating cell survival and invasiveness are of particular interest. Besides mitochondria and lysosomes, the ER is very important in this respect as it is now recognized as an important sensor of cellular stress and it plays a key role in the release and activation of death factors such as cathepsins, calpains, and other proteases through intracellular calcium flux (35). For instance, migrating glioblastoma cells have recently been shown to overexpress death-associated protein-3 (36). Therefore new routes should be investigated as possible issues to combat apoptotic-resistant migrating glioblastoma cells.

Given the ER localization of G6PT and the crucial role that the ER plays as a metabolic compartment, we suggest that G6PT is a key mediator in the regulation of cancer cell survival and ECM degradation signalling. In fact, regulation of G6PT

expression may function as a *"bio-switch"* (Fig.7a) enabling cells to promote either migration or cell death processes. Switching from one state to another may occur in response to external stimuli, such as hypoxia, or as a result of intracellular metabolic changes (37). Intracellular regulation of Ca^{2+} flux, cytosolic ATP and G6P levels are among the parameters that G6PT may modulate in the transformed proliferating cells. 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 (38, 39). Among the several brain tumor-derived cell lines tested, G6PT expression was found the highest in the highly infiltrating and angiogenic U87 glioma cells (13). This potentially suggests that metabolic adaptative capacity, in part through G6PT, may regulate the invasive phenotype of aggressive cancer cells. Documenting the pleiotropic roles of G6PT in cancer cells will thus help optimize or design new anti-tumor therapies.

We previously showed that inhibiting G6PT function by chlorogenic acid or by ATP-depleting agents such as 2-Deoxyglucose in brain-tumor-derived cells did not directly affect MT1-MMP catalytic function but still resulted in decreased invasiveness (13). Moreover, cancer cells frequently display high rates of aerobic glycolysis, in comparison to their nontransformed counterparts, and the possible applications of 2-Deoxyglucose in anticancer therapies further supports the theory that inhibiting G6PT function in cancer cells could decrease tumor progression. Some evidence also suggests that ER stress-inducing agents are useful as cancer agents, and that excessive ER stress leads to apoptosis. These agents include glycosylation inhibitors (e.g. tunicamycin and 2-Deoxyglucose), agents that deplete ER Ca^{2+} (e.g. the sarcoendoplasmic-reticulum Ca^{2+}-ATPase inhibitor thapsigargin and various ionophores) and agents that induce reductive stress (dithiotreitol and β-mercaptoethanol) (40).

We also found that cytoskeleton remodeling is among the first events in a cascade of activation that leads to MT1-MMP-mediated downregulation of brain cancer cell survival, in part through PI3K/AKT-mediated plasma membrane to nucleus signaling. Interestingly, part of the switch between cell migration and cell death comes under the control of the PTEN/Akt/PI3K/mTOR pathway (30). Survival through PI3K/Akt signaling is complex (41) and the activity of the PI3K/Akt pathway is, in fact, often upregulated in brain tumors as a result of excessive stimulation by growth factor receptors and Ras (42). Moreover, glioblastomas frequently carry mutations in the *PTEN* tumor suppressor gene, whose tumor suppressor properties are closely related to its inhibitory effect on the PI3K-dependent activation of Akt signaling (43). The activation of the PI3K pathway is significantly associated with increasing tumor grade, lower levels of apoptosis and an adverse clinical outcome in the case of human gliomas (44). All these factors indicate that aberrant PI3K/Akt signaling means that both cell proliferation in glioma cells and cell migration have become abnormal. A number of publications have already reported that an aberrantly activated PI3K/Akt pathway renders tumor cells resistant to cytotoxic insults, including those related to anticancer drugs (45, 46). In light of these results and because G6PT was able to reverse the cytotoxic effects of ConA, it is tempting to suggest that functional targeting of G6PT such as by the use of chlorogenic acid or by its analogs, the most potent G6PT inhibitors, could augment the effectiveness of chemotherapy on glioma cells.

Maintenance of cytoarchitecture is required for cell survival, since its perturbation by Cytochalasin-D- or ConA-mediated MT1-MMP mechanisms diminished cell survival and were correlated to proMMP-2 activation (21, 22, this study). In fact, silencing of the MT1-MMP gene prevented ConA from activating proMMP-2. Moreover, we showed that the intracellular domain of MT1-MMP is an absolute requirement for transducing the intracellular signaling that leads to cell death. Although the exact identity of the amino acid residues from the MT1-MMP

intracellular domain remains to be addressed, speculations about the Tyr-573, Cys-574, and Val-582 have been put forward as important for MT1-MMP signaling (47, 48). Similarly, a caspase-dependent mechanism has recently been associated with MT1-MMP function in endothelial cell morphogenic differentiation (49). This suggests that MT1-MMP acts as a potential cell death sensor/effector that signals ECM degradation processes to be activated. Interestingly, hypoxia increased MT1-MMP and the MT1-MMP transcription factor regulator Egr-1 levels in bone marrow-derived stromal cells (50), a condition that led to cell death (51). Moreover, ConA was found ineffective in activating proMMP-2 or inhibiting G6PT gene expression in bone marrow stromal cells isolated from Egr-$1^{-/-}$ mouse (11).

The fact that G6PT overexpression inhibited ConA-induced proMMP-2 activation, but not cell death, further suggests that complex differential regulation takes place and highlights the pleiotropic intracellular functions of G6PT. Moreover, this observation also provides insight into the cellular event chronology, confirming that MT1-MMP-mediated activity and signaling are among the first steps that inhibit G6PT expression, ultimately leading to cell death. Interestingly, our data are consistent with some of the abnormal polymorphonuclear neutrophil (PMN) phenotypes observed in GSD type 1b, a clinical condition where the G6PT gene and/or protein activity is defective (52, 53). In fact, it has been hypothesized that G6PT might function as a G6P receptor/sensor (53) or that it could favor calcium sequestration in the endoplasmic reticulum lumen (17). Finally, although no effects in response to MT1-MMP or cytoskeleton disruption was observed on the ER-embedded G6Pase-β, recent evidence regarding G6Pase-β involvement in cell survival was demonstrated in neutrophils as disruption of the G6Pase–β gene expression also lead to cell death, an event suggestive of a vital interaction between G6PT and G6Pase-β (54).

In summary, we highlight new functions of MT1-MMP in cell death signalling which may potentially be linked to those of the ER-embedded functions of G6PT. In fact, we believe that this signaling axis may not be exclusive to one cell line, but that it may rather regulate cell mobilization processes through metabolic and/or cell survival control such as similarly demonstrated for bone marrow-derived stromal cells (11). Our study further shows a molecular axis linking the invasive phenotype of brain tumor cells to their potential metabolic control by G6PT and supports the notion of an MT1-MMP/G6PT *"bio-switch"* (Fig.7b) that could regulate glucose homeostasis and thus restrain cancer cell proliferation, inhibit ECM degradation, or induce cell death. By revealing tumor-specific metabolic shifts in tumor cells, metabolic profiling studies will further enable drug developers to identify the metabolic steps that control cell proliferation, thus aiding the identification of new anti-cancer targets and screening of lead compounds for anti-proliferative metabolic effects.

REFERENCES

1. Marciniak, S.J., and Ron, D. (2006) *Physiol. Rev.* **86**, 1133-1149
2. Koumenis, C. (2006) *Curr. Mol. Med.* **6**, 55-69
3. Csala, M., Banhegyi, G., and Benedetti, A. (2006) *FEBS Lett.* **580**, 2160-2165
4. Szegezdi, E., Logue, S.E., Gorman, A.M., and Samali, A. (2006) *EMBO Rep.* **7**, 880-885
5. Vedrenne, C., and Hauri, H.P. (2006) *Traffic.* **7**, 639-646
6. Remacle, A.G., Rozanov, D.V., Baciu, P.C., Chekanov, A.V., Golubkov, V.S., and Strongin, A.Y. (2005) *J. Cell Sci.* **118**, 4975-4984
7. Sato, H., Takino, T., and Miyamori, H. (2005) *Cancer Sci.* **96**, 212-217
8. Boucher, E., Mayer, G., Londono, I., and Bendayan, M. (2006) *Kidney Int.* **69**, 1570-1577
9. McLennan, S.V., Fisher, E., Martell, S.Y., Death, A.K., Williams, P.F., Lyons, J.G., Yue, D.K. (2000) *Kidney Int. Suppl.* **77**, S81-87
10. Van de Werve, G., Lange, A., Newgard, C., Mechin, M.C., Li, Y., and Berteloot A. (2000) *Eur. J. Biochem.* **267**, 1533-1549
11. Gaut, J.R., and Hendershot, L.M. (1993) *Curr. Opin. Cell. Biol.* **5**, 589-595
12. Belkaid, A., Currie, J.C., Desgagnes, J., and Annabi B. (2006) *Cancer Cell Int.* **6**, 7
13. Belkaid, A., Copland, I.B., Massillon, D., and Annabi B. (2006) *FEBS Lett.* **580**, 3746-3752
14. Linder, S., and Shoshan, M.C. (2005) *Drug Resist. Updat.* **8**, 199-204
15. Chintala, S.K., Tonn, J.C., and Rao, J.S. (1999) *Int. J. Dev. Neurosci.* **17**, 495-502
16. Chen, P.Y., Csutora, P., Veyna-Burke, N.A., and Marchase, R.B. (1998) *Diabetes.* **47**, 874-881
17. Carew, J.S., Nawrocki, S.T., Krupnik, Y.V., Dunner, K. Jr., McConkey, D.J., Keating, M.J., and Huang, P. (2006) *Blood.* **107**, 222-231

18. Nawrocki, S.T., Carew, J.S., Pino, M.S., Highshaw, R.A., Dunner, K. Jr., Huang, P., Abbruzzese, J.L., and McConkey, D.J. (2005) *Cancer Res.* **65**, 11658-11666
19. Cao, J., Chiarelli, C., Kozarekar, P., and Adler, H.L. (2005) *Thromb Haemost.* **93**, 770-778
20. Neth, P., Ciccarella, M., Egea, V., Hoelters, J., Jochum, M., and Ries, C. (2006) *Stem Cells.* **24**, 1892-1903
21. Allenberg, M., Weinstein, T., Li, I., and Silverman, M. (1994) *J. Am. Soc. Nephrol.* **4**, 1760-1770
22. Hinoue, A., Takigawa, T., Miura, T., Nishimura, Y., Suzuki, S., and Shiota, K. (2005) *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **282**, 130-137
23. Preaux, A.M., D'ortho, M.P., Bralet, M.P., Laperche, Y., (2002) Mavier, P. *Hepatology.* **36**, 615-622
24. Gingras, D., Page, M., Annabi, B., and Beliveau, R. (2000) *Biochim. Biophys. Acta.* **1497**, 341-350
25. Grzanka, D., Domaniewski, J., Grzanka, A., and Zuryn, A. (2006) Ultraviolet radiation (UV) induces reorganization of actin cytoskeleton in CHOAA8 cells. *Neoplasma.* **53**, 328-332
26. Seyb, K.I., Ansar, S., Bean, J., and Michaelis, M.L. (2006) *J. Mol. Neurosci.* **28**, 111-123
27. Zhang, D., and Brodt, P. (2003) *Oncogene.* **22**, 974-982
28. Kim, D., Cheng, G.Z., Lindsley, C.W., Yang, H., and Cheng, J.Q. (2005) *Curr. Opin. Investig. Drugs.* **6**, 1250-1258
29. Glioma Meta-analysis Trialists (GMT) Group. (2002) *Lancet.* **359**, 1011-1018
30. Lefranc, F., Brotchi, J., and Kiss, R. (2005) *J. Clin. Oncol.* **23**, 2411-2422
31. McLaughlin, N., Annabi, B., Bouzeghrane, M., Temme, A., Bahary, J.P., Moumdjian, R., and Beliveau R. (2006) *Brain Res.* **1071**, 1-9
32. Okada, H., and Mak, T.W. (2004) *Nat. Rev. Cancer.* **4**, 592-603
33. Raza, S.M., Lang, F.F., Aggarwal, B.B., Fuller, G.N., Wildrick, D.M., and Sawaya, R. (2002) *Neurosurgery.* **51**, 2-12

34. Neurath, K.M., Keough, M.P., Mikkelsen, T., and Claffey, K.P. (2006) *Glia.* **53,** 733-743

35. Broker, L.E., Kruyt, F.A., and Giaccone, G. *Clin. Cancer Res.* **11**, 3155-3162

36. Mariani, L., Beaudry, C., McDonough, W.S., Hoelzinger, D.B., Kaczmarek, E., Ponce, F., Coons, S.W., Giese, A., Seiler, R.W., and Berens, M.E. (2001) *Clin. Cancer Res.* **7**, 2480-2489

37. Slepchenko, B.M., and Terasaki, M. (2004) *Curr. Opin. Genet. Dev.* **14**, 428-434

38. Boros, L.G., Cascante, M., and Lee, W.N. (2002) *Drug Discov. Today.* **7**, 364-372

39. Ramos-Montoya, A., Lee, W.N., Bassilian, S., Lim, S., Trebukhina, R.V., Kazhyna, M.V., Ciudad, C.J., Noe, V., Centelles, J.J., and Cascante, M. (2006) *Int. J. Cancer.* **119**, 2733-2741

40. Lee, A.S. (2001) *Trends Biochem. Sci.* **26**, 504-510

41. McCormick, F. (2004) *Nature*. **428**, 267-269

42. Newton, H.B. (2005) *Expert Rev. Anticancer Ther.* **5**, 355-378

43. Knobbe, C.B., and Reifenberger G. (2003) *Brain Pathol.* **13**, 507-518

44. Chakravarti, A., Zhai, G., Suzuki, Y., Sarkesh, S., Black, P.M., Muzikansky, A., and Loeffler, J.S. (2004) *J. Clin. Oncol.* **22**, 1926-1933

45. Shingu, T., Yamada, K., Hara, N., Moritake, K., Osago, H., Terashima, M., Uemura, T., Yamasaki, T., and Tsuchiya, M. (2003) *Cancer Res.* **63**, 4044-4047

46. Joy, A.M., Beaudry, C.E., Tran, N.L., Ponce, F.A., Holz, D.R., Demuth, T., and Berens, M.E. (2003) *J. Cell Sci.* **116**, 4409-4417

47. Langlois, S., Di Tomasso, G., Boivin, D., Roghi, C., Murphy, G., Gingras, D., and Beliveau, R. (2005) *Exp. Cell Res.* **307**, 452-464

48. Annabi, B., Lee, Y.T., Turcotte, S., Naud, E., Desrosiers, R.R., Champagne, M., Eliopoulos, N., Galipeau, J., and Beliveau, R. (2003) *Stem Cells.* **21**, 337-347

49. Zhu, W., Chen, J., Cong, X., Hu, S., and Chen, X. (2006) *Stem Cells.* **24**, 416-425

50. Leuzzi, R., Banhegyi, G., Kardon, T., Marcolongo, P., Capecchi, P.L., Burger, H.J., Benedetti, A., and Fulceri, R. (2003) *Blood.* **101**, 2381-2387

51. Hiraiwa, H., Pan, C.J., Lin, B., Moses, S.W., and Chou JY. (1999) *J. Biol. Chem.* **274**, 5532-5536

Fig.1

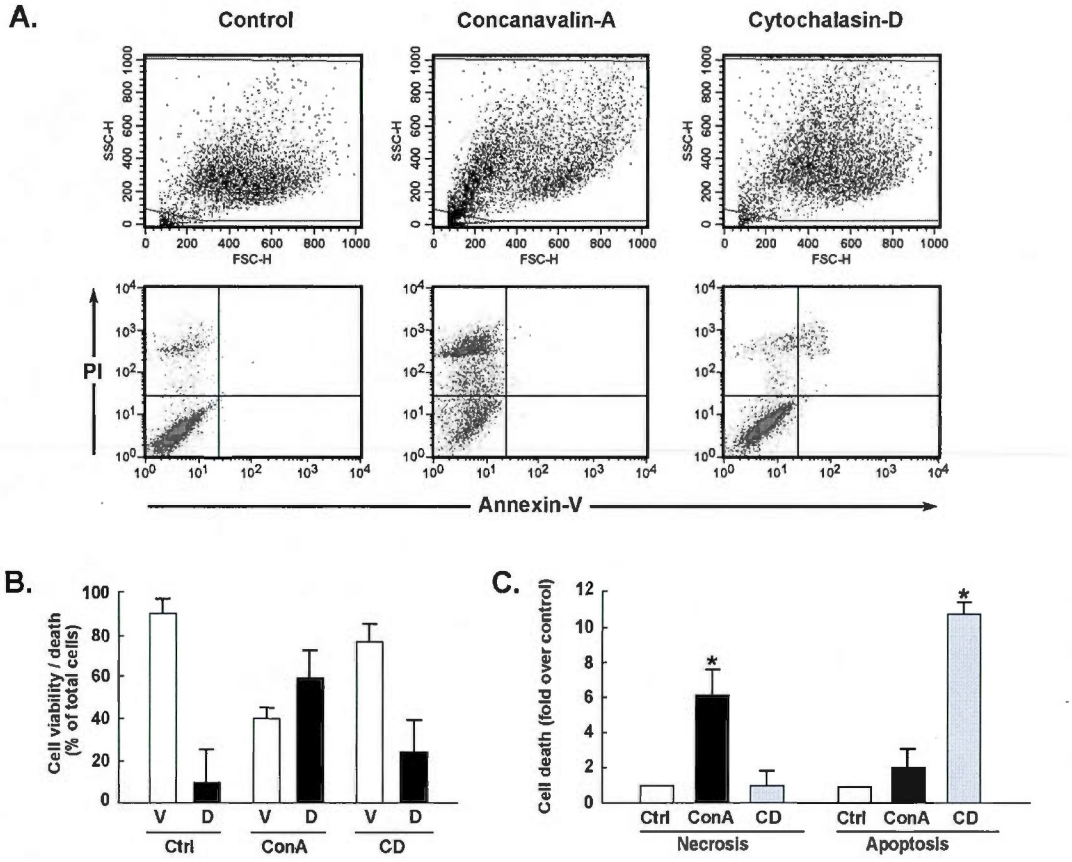


Figure 1. *Differential induction of cell necrosis and cell apoptosis by cytoskeleton disrupting agents.* U87 glioblastoma cells were cultured as described in the Methods section until they reached ~75-90% confluence. They were then serum-starved for 24 hours prior to the addition of 10 μg/ml Concanavalin-A (ConA) or 1 μM Cytochalasin-D (CD). Incubation was continued for another 24 hours. (A) Flow cytometry was then used to either assess cell morphology changes (three upper panels), or cell death from Annexin-V/propidium iodide (PI) stained cells (three lower panels). (B) Cell viability (white boxes) was assessed as the percentage of total cells present in the lower left quadrants. Cell death (black boxes) represents the combined cells present in necrosis, early/late apoptosis (black boxes). (C) The respective cell death proportions attributable to either necrosis or apoptosis (early and late) are shown. Data are the averages ± SEM of 4 independent experiments. Statistical significance is represented by (*).

Fig.2

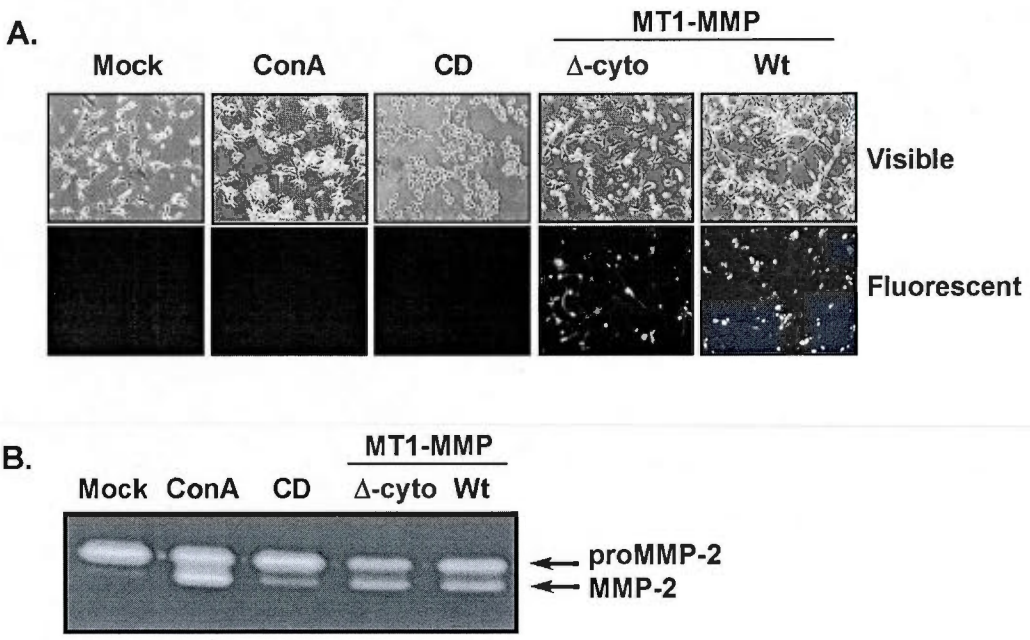


Figure 2. *Recombinant MT1-MMP and cytoskeleton disrupting agents induce proMMP-2 activation.* U87 glioblastoma cells were treated as described in the legend to Fig.1. Transfections of cDNA plasmids encoding full-length Wt-MT1-MMP and cytoplasmic domain-truncated MT1-MMP both fused to GFP were carried out as described in the Methods section. (A) Changes in cell morphology are shown through phase contrast microphotography (visible light), while transfection efficiency was validated using fluorescence microscopy. (B) Gelatin zymography was carried out to assess the extent of proMMP-2 activation levels, as described in the Methods section, using conditioned media isolated from each of the serum-starved cells conditions.

Fig.3

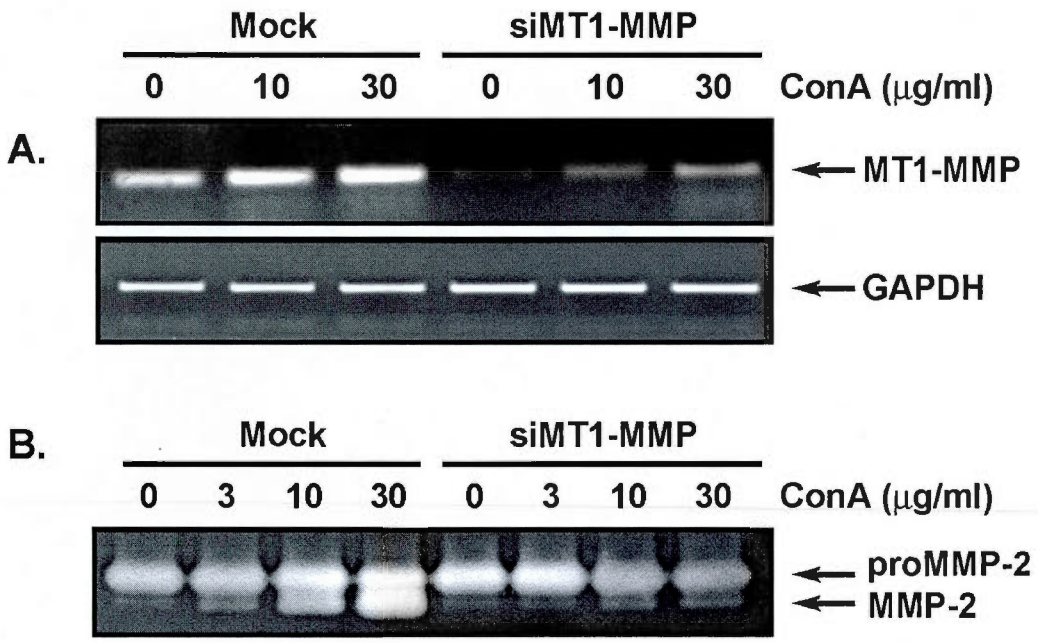


Figure 3. *Concanavalin-A-mediated activation is an MT1-MMP-mediated event.* Since proMMP-2 activation is thought to proceed through an MT1-MMP-mediated event, U87 glioblastoma cells were transfected with siRNA against MT1-MMP or mismatched siRNA (Mock) for 48 hours as described in the Methods section, prior to treatment with increasing concentrations of ConA. (A) Total RNA was isolated and MT1-MMP gene expression was assessed by RT-PCR as described in the Methods section. The gene expression level of GAPDH was used as the internal control. (B) Gelatin zymography was performed to assess the extent of proMMP-2 activation in the conditioned media of serum-starved Mock and siMT1-MMP cells treated with increasing concentrations of ConA.

Fig.4

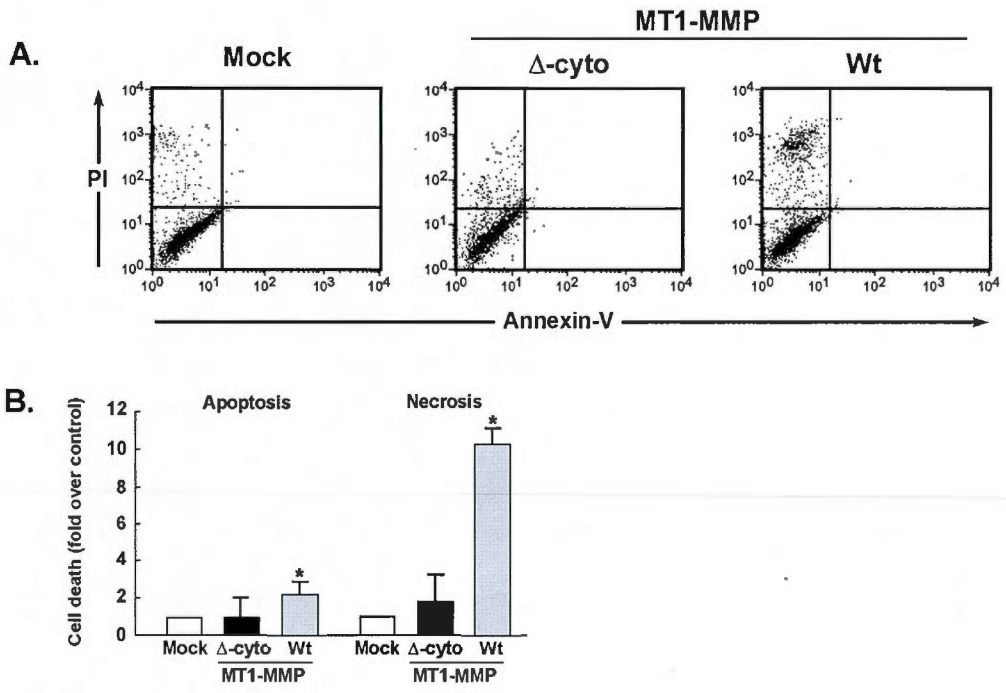


Figure 4. *MT1-MMP overexpression triggers cell death and is signalled through its intracellular cytoplasmic domain.* U87 glioblastoma cells were transfected with cDNA encoding full length wild type (Wt) MT1-MMP or its cytoplasmic domain-truncated form as described in the Methods section. (A) Flow cytometry was used to assess the extent of cell death in Annexin-V/Propidium iodide (PI)-stained cells. (B) Quantification of cell death caused by either combined early and late apoptosis (upper and lower right quadrants) or necrosis (upper left quadrants) was performed for cells transfected with cytoplasmic domain-truncated MT1-MMP (Δ-cyto) or full length (Wt) MT1-MMP.

Fig.5

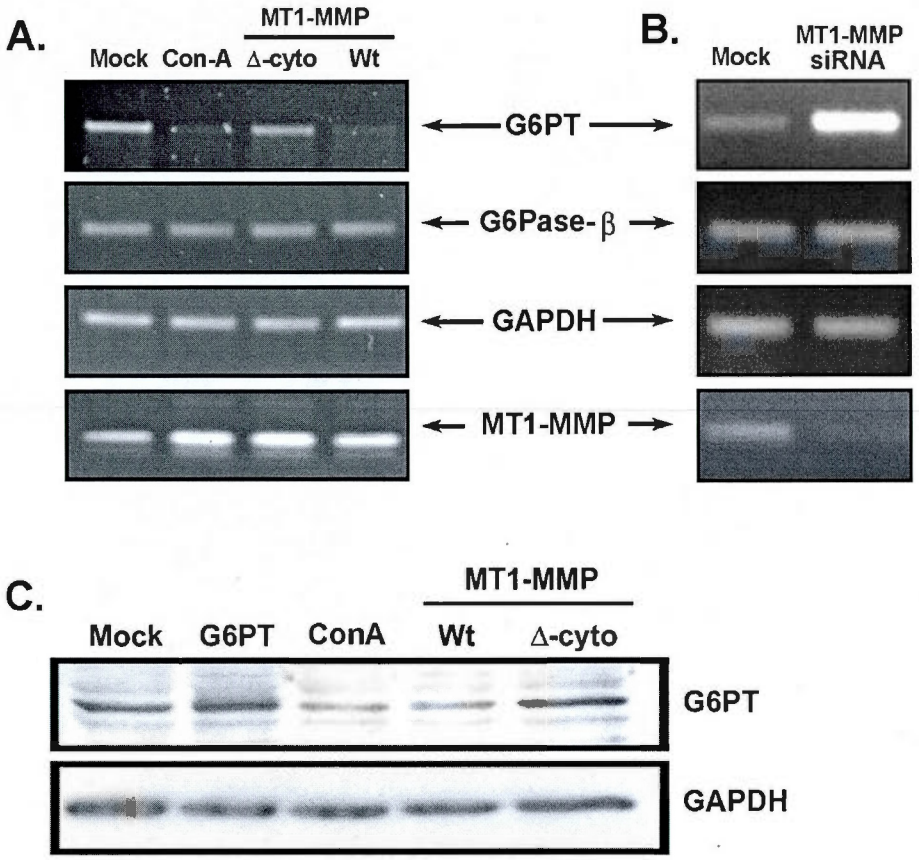


Figure 5. *Both MT1-MMP overexpression and Concanavalin-A treatment downregulate G6PT gene and protein expression.* (A) Total RNA was isolated from untreated (Mock), ConA-treated, or U87 glioblastoma cells transfected with either the cytoplasmic domain-truncated MT1-MMP (Δ-cyto) or full length (Wt) MT1-MMP cDNA. RT-PCR was performed in order to assess the changes in G6PT, G6Pase-β, GAPDH, or MT1-MMP gene expression in each condition. (B) MT1-MMP gene expression was specifically downregulated in U87 glioblastoma cells transfected with siMT1-MMP but not in mismatched siRNA-transfected cells (Mock) as described in the Methods section. Total RNA and RT-PCR were performed as in (A). (C) Cell lysates were isolated from U87 cells transfected with cDNA encoding G6PT, Wt-MT1-MMP, or Δ-cyto MT1-MMP, or treated with ConA. Western blotting and immunodetecetion was performed with anti-G6PT and anti-GAPDH antibodies.

Fig.6

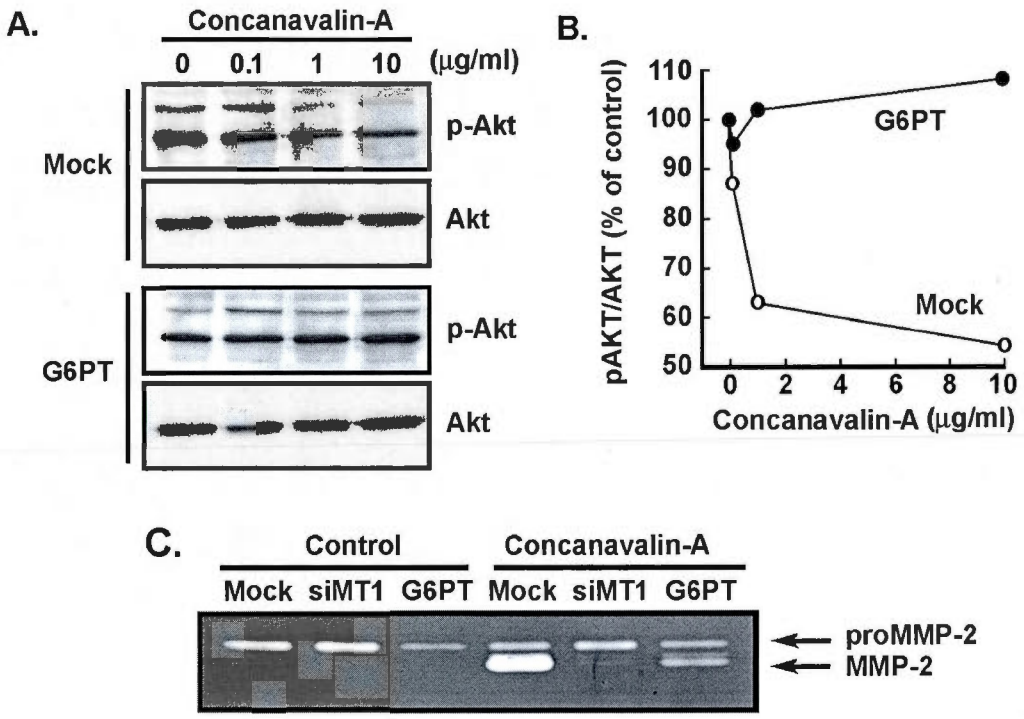


Figure 6 . *G6PT overexpression antagonizes the ConA-mediated lethal effect and rescues cells from MT1-MMP-mediated cell death.* (A) Mock or G6PT-transfected U87 cells were treated with increasing ConA concentrations and cell lysates were used to immunodetect the levels of total or phosphorylated Akt (p-Akt). (B) Scanning densitometry was used to quantify the Akt immunoreactive bands, and results were expressed as the ration of p-Akt over Akt. (C) Conditioned media isolated from serum starved Mock, siMT1-MMP (siMT1), or G6PT-transfected U87 cells were used to perform gelatin zymography.

Fig.7

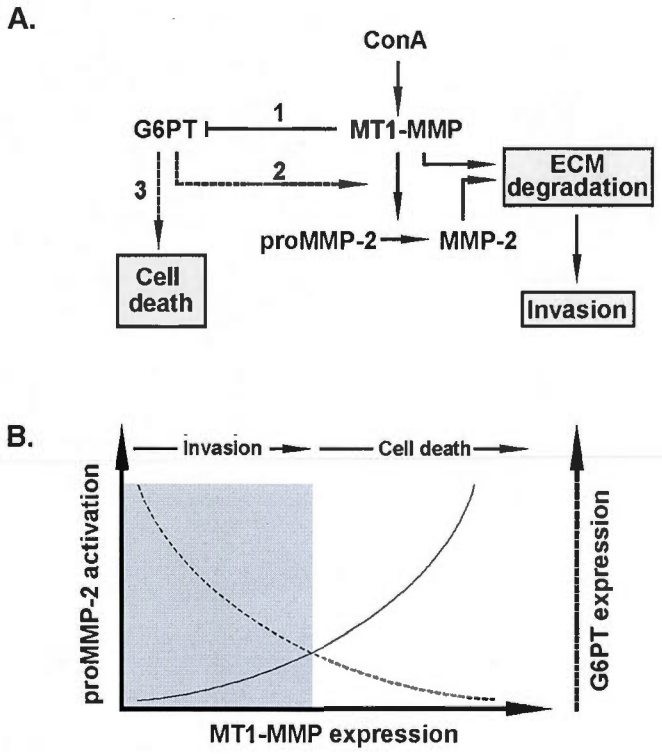


Figure 7 . *G6PT as a "bio-switch" intermediate in the regulation of cell invasion and cell death signalling.* (A) Summarized scheme of the events that lead to MT1-MMP-mediated signalling in cell invasion and cell death. Concanavalin-A (ConA) upregulates MT1-MMP gene and protein expression, which in turn downregulates G6PT expression **(1)**. Low levels of G6PT release the inhibitory effect on MT1-MMP-mediated proMMP-2 activation **(2)** which altogether leads to ECM degradation and cell invasion. When a specific balance is reached between MT1-MMP and G6PT expression (i.e. high MT1-MMP expression, low G6PT expression), cell death signalling is then activated **(3)**. (B) Our data currently show that MT1-MMP expression leads to both proMMP-2 activation (full line) and to concomitant downregulation of G6PT expression (dashed line). Conceptually, we suggest that a balance between the early invasive processes that are initiated by the increased MT1-MMP expression correlate with proMMP-2 activation in order to hydrolyse the ECM and promote cell migration (left shaded area). Concurrently, G6PT expression decreases until it reaches a threshold where its inhibitory effect upon proMMP-2 activation is released, which then leads to cell death (right area). The intersection between the G6PT expression curve and that of the proMMP-2 activation is the *"bio-switch"* that reflects the balance between cell invasion and cell death signalling.

CONCLUSIONS ET PERSPECTIVES

Les travaux présentés dans ce mémoire illustrent bien la complexité des mécanismes pouvant réguler le phénotype invasif et infiltrant des glioblastomes. Le but principal de notre recherche est de trouver le traitement le plus efficace, certains produits naturels étant de bons candidats vu leur grande teneur en molécules anti-angiogéniques et anti-cancéreuses dont les fonctions demeurent néanmoins souvent à être caractérisées. En ce sens, l'acide chlorogénique (CHL), une molécule retrouvée généralement dans le café, la tomate et le thé, semble avoir des propriétés anti-cancérigènes (Mori et al., 2000; Jin et al., 2005). Dans nos travaux, nous donnons pour la première fois une explication moléculaire à ses propriétés anti-cancéreuses.

L'étude publiée dans *Cancer Cell International* démontre une nouvelle fonction de G6PT dans la régulation du phénotype invasif des glioblastomes, tout en postulant que G6PT puisse être une cible pharmacologique potentielle pour le CHL. Il a été maintes fois rapporté que le CHL, un inhibiteur fonctionnel de G6PT, avait des propriétés anti-cancérigènes, mais on n'a jamais associé ces dernières avec les fonctions de G6PT dans la tumorigenèse. En plus de mettre en évidence l'existence de G6PT dans les glioblastomes, nos travaux ont démontré une diminution significative de la migration cellulaire et de la sécrétion des métalloprotéases (MMP-2) lors de traitements avec le CHL, deux pré-requis pour l'invasion tumorale. Ces résultats corrèlent avec l'effet anti-cancérigène attribué au CHL.

Par ailleurs, nous mettons en évidence de nouvelles fonctions insoupçonnées de G6PT dans la régulation du phénotype invasif, en plus de sa fonction primaire de transport microsomal de G6P. Etant donné l'implication secondaire de G6PT dans la régulation de la concentration du calcium intracellulaire, il est possible de présumer son implication dans la signalisation cellulaire médiée à travers un flux calcique. Nos

résultats nous ont permis de déduire que l'inhibition de G6PT par le CHL aurait des répercutions sur des voies clés de signalisation régulant l'invasion des glioblastomes.

Dans un deuxième temps, nous nous sommes penchés sur le rôle de G6PT dans la survie des glioblastomes. Il est intéressant de noter que l'activité de G6PT dans la lumière du RE va de pair avec celle de l'hexose-6-phosphate déshydrogénase, retrouvée aussi dans tous les tissus et qui serait responsable de la protection du RE contre les agents du stress oxydatif (Van schaftingen et al., 2002). Toute perte d'intégrité du RE serait responsable d'une mort cellulaire prématurée par apoptose. Il est déjà connu que toute déficience de G6PT cause la GSD-1b, et toute mutation responsable d'une diminution ou abolition de ces fonctions causeraient une mort cellulaire certaine (Chou et al., 2002; Chen et al., 2002; Chen et al., 2003). Nos travaux démontrent clairement qu'une inhibition ciblée de l'expression de G6PT serait une bonne alternative pour un contrôle efficace de la croissance tumorale.

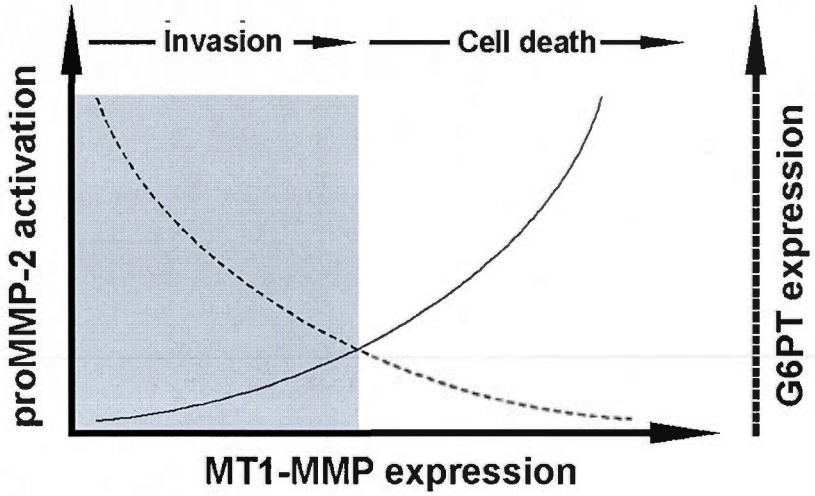
Finalement, on s'est intéressé à l'implication moléculaire de G6PT dans la décision "mort cellulaire" versus "invasion tumorale". Différentes voies de signalisation sont étudiées pour tenter d'expliquer l'action de G6PT dans ces processus. Il semble exister une relation paradoxale entre les cellules cancéreuses et l'apoptose. En effet, dans certains cas, la cellule cancéreuse est une cellule résistante à l'apoptose, mais chaque masse tumorale possède un centre hypoxique avec des cellules nécrotiques autour en réponse à une insuffisance d'oxygénation. Dans ce cas, la cellule réagit en augmentant l'expression de différents gènes impliqués dans la glycolyse et l'angiogenèse, afin d'assurer des nivaux d'ATP convenables et un apport en oxygène suffisant pour sa survie (Neurath et al., 2006). Mis à part la mitochondrie et les lysosomes, le RE est une organelle importante dans la régulation de la survie cellulaire, elle serait responsable de la sécrétion de certains facteurs de mort tels que les cathepsines, les calpaines, et d'autres protéases par son contrôle du flux calcique intracellulaire (Broker et al., 2004). Vu que G6PT est une protéine du RE, et que ce

dernier joue un rôle clé dans la signalisation cellulaire, il serait opportun de revoir les fonctions de G6PT dans un cadre autre que celui du contrôle du métabolisme glucidique.

La ligne est mince entre le passage d'une cellule en mode survie avec une mort cellulaire programmé à un mode invasif (angiogenèse et migration cellulaire). Ce contrôle serait attribué à des changements dans le métabolisme intracellulaire en réponse à des stimuli extérieurs (Slepchenko et al., 2004). Par ailleurs, l'élément déclencheur pourrait commencer par la perte de l'organisation structurale cellulaire, responsable d'une cascade d'évènements aboutissant à l'activation de la pro-MMP2 via un mécanisme MT1-MMP. D'autres études ont avancé que l'hypoxie induisait l'expression de MT1-MMP en augmentant son facteur de transcription Egr-1 et par le fait même la mort cellulaire (Zhu et al., 2006). Nos travaux, démontrent qu'une surexpression de MT1-MMP pourrait contribuer de par sa signalisation, à inhiber l'expression de G6PT ce qui induirait une mort cellulaire.

D'un point de vue signalisation, une activation soutenue de MT1-MMP diminuerait, probablement, les chances de survie des cellules cancéreuses en partie via la voie PI3K/AKT. Il est intéressant de souligner l'importance de cette dernière voie dans le passage d'une cellule d'un fonctionnement normal à un état invasif (Lefranc et al., 2005). L'activation de la voie PI3K est souvent associée à un niveau élevé de malignité, des nivaux bas d'apoptose, et une résistance au traitement. Toutefois, les glioblastomes présentent des mutations pour le gène suppresseur de tumeur PTEN, un inhibiteur de la voie PI3K/AKT (Knobbe et al., 2003). Il a d'ailleurs été rapporté que MT1-MMP serait impliqué dans la mort cellulaire (Langlois et al., 2005), ce qui pourrait confirmer l'existence d'un lien moléculaire entre ce dernier et G6PT. Une description de ce lien et l'étude de son spectre d'action est nécessaire pour identifier l'étape clé du métabolisme responsable du contrôle de la

prolifération cellulaire (voir le schéma tiré de l'article 3 figure 7 du journal *NEOPLASIA*).



Des études effectuées ultérieurement dans notre laboratoire ont permis d'établir une certaine similarité des propriétés anti-cancérigène, en plus de la ressemblance dans la structure chimique, entre l'épigallocatéchine-gallate (EGCg) et CHL (Annabi et al., 2005; Baatout et al., 2004; McLaughlin et al., 2006). L'échec de la radiothérapie face aux glioblastomes est dû essentiellement à leur caractère infiltrant et à la présence d'un centre hypoxique. Des études ont prouvé que toute augmentation du métabolisme glucidique chez les glioblastomes est aussi associée à un haut niveau de malignité et à un mauvais pronostic (Timperley, 1980). De récentes études ont par ailleurs révélé que le CHL serait un hypoglycémiant (Nicasio et al., 2005). L'usage du 2-D-Deoxyglucose (2DG) et du 5-Thioglucose (5TG), deux agents déplétant l'ATP cellulaire, en association avec la radiothérapie donnerait des résultats significatifs en augmentant les dommages causés à la cellule cancéreuse (Singh et al., 2005; Xu et al., 2005). Ceci laisse supposer qu'une combinaison entre le CHL et/ou le 2DG lors des traitements de radiothérapie serait efficace, car cela pourrait causer un déséquilibre dans l'apport énergétique de la cellule cancéreuse.

Un autre volet d'étude pourrait aborder l'effet du 2DG et du 5TG sur le RE. Ces derniers semblent exercer un stress au niveau du RE. Ce qui est particulièrement intéressant, c'est leur capacité à diminuer les niveaux extracellulaires de la MMP-2. Les résultats de récentes études associent le stress du RE avec des désordres métaboliques, des maladies insulinodépendantes et avec la progression tumorale (Marciniak et al., 2006; Koumenis, 2006). L'avantage de cette approche thérapeutique via le RE serait non seulement d'affecter G6PT mais aussi le RE, une organelle impliquée dans la synthèse protéique, et le contrôle du flux calcique intracellulaire et par ce fait avoir des effets sur la signalisation cellulaire.

Durant nos travaux, on a démontré une implication de G6PT dans divers processus d'invasion tumorale (Belkaid et al., 2006). D'autres maladies sont aussi causées par des déficiences de G6PT notamment la GSD Ib. Cette dernière est provoquée par des mutations causant la diminution ou l'abolition totale de l'activité de G6PT. Il serait pertinent pour des travaux futurs de déterminer quelles mutations sur le gène régulent l'invasion tumorale, et lesquelles induisent une mort cellulaire. Il nous semble intéressant d'amorcer une étude génétique chez des patients atteints de GSD Ib afin d'évaluer leurs risques de cancer, et de comparer ces résultats avec ceux obtenus chez des patients dont la fonction de G6PT n'est pas affectée.

D'autres travaux futurs pourront tenter d'expliquer la présence de G6PT particulièrement dans les glioblastomes (cancer de l'adulte), car durant nos recherches nous avons constaté l'absence de celui-ci dans les médulloblastomes qui sont des tumeurs pédiatriques (Belkaid et al., 2006). Ceci semble corréler avec l'étude du groupe de Benedetti sur des fœtus de rat (Puskas et al., 1999). Il semblerait que l'ARNm de G6PT est déjà exprimé dans les tissus du fœtus aux environs du 19^{éme} jours de vie intra-utérine, et que sa concentration s'accroît dans les premiers jours de la vie extra-utérine. Une autre étude supporte la théorie selon laquelle G6PT aurait

des niveaux d'expression intra-utérine assez élevés pour rester stable jusqu'après la naissance (Mechin et al., 2000). L'expression de G6PT dans les glioblastomes et son absence dans les médulloblastomes aurait-elle un lien avec son expression et son activité durant la vie intra et extra-utérine?

La compréhension des fonctions de G6PT dans l'invasion tumorale pourrait donc représenter une percée dans le traitement des glioblastomes, puisque c'est une protéine avec diverses fonctions dont seules les implications dans le métabolisme glucidique ont été à ce jour documentées. Nos recherches ont permis d'attribuer à G6PT des rôles clés dans l'invasion tumorale. Le fait de le placer comme nouvelle cible thérapeutique pourrait contribuer au développement de médicaments contre ce dernier afin d'améliorer le traitement des glioblastomes.

CONCLUSION FINALE

L'identification d'autres protéines que G6PT, susceptibles d'être affectées par le CHL, pourrait permettre le développement éventuel de nouvelles cibles thérapeutiques. De plus, l'étude de l'impact du CHL sur d'autres phénomènes associés à l'invasion tumorale tel que la tubulogenèse, qui n'a pas été abordée au cours de nos travaux, pourrait être une bonne piste pour une meilleure compréhension des mécanismes d'action de ce produit anti-cancérigène.

Un autre volet d'étude pourrait aborder l'effet du CHL sur des glioblastomes ayant subit des irradiations représentatives du traitement de radiothérapie. L'étude de la protéine G6PT et l'impact du CHL sur les propriétés radiorésistantes de ces cellules, ajouterait de nouvelles données qui mettraient en valeur le potentiel de l'usage du CHL et l'importance de cibler G6PT dans les nouvelles stratégies thérapeutiques.

Nos travaux ont permis de démontrer que G6PT a des implications plus complexes dans l'invasion tumorale, au delà du transport du G6P vers le RE, et ceci en partie par le contrôle du calcium intracellulaire. Ces projets, qui seront poursuivis au cours des prochaines années, permettront sans aucun doute d'améliorer notre connaissance de l'angiogenèse et de l'invasion tumorale, afin de développer de nouvelles cibles thérapeutiques dans la lutte contre le cancer.

BIBLIOGRAPHIE

Aldape, K.D., M. F Okcu, et al., (2003). Molecular epidemiology of glioblastoma. Cancer 9, 99-106.

Annabi, B., M. Bouzeghrane., et al. (2005). Probing the infiltrating character of brain tumors : inhibition of rhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. J Neurochem 94, 906-916.

Arion, W.J., W. K. Canfield., et al. (1997). Chlorogenic acid and hydroxynitrobenzaldehyde: new inhibitors of hepatic glucose-6-phosphatase. Arch Biochem Biophysi 339, 315-322.

Arion, W.J., W. K. Canfield., et al. (1998). Chlorogenic acid analogue S3483: a potent competitive inhibitor of hepatic and renal glucose-6-phosphatase systems, Arch Biochem Biophysi 351, 279-285.

Arion, W.J., J. A. Lange, et al. (1980) Evidence for the participation of independant translocation for phosphate and glucose-6-phosphate in the microsomal glucose-6-phosphatase system. Interaction of the system with orthophosphate, inorganic pyrophosphate, and carbamyl phosphate. J Biol Chem 255, 10396-10406.

Arion, W. J., B.K. Wallin , et al. (1975). On the involvment of a glucose-6-phosphate transport system in the function of microsomal glucose-6-phosphatase. Mol Cell Biochem. 6, 75-83.

Atkinson, S. J., T. Crabbe., et al. (1995). Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membrane. J Biol Chem 270, 30479-30485.

Baatout, S., P. Jacquet., et al. (2004). Study of the combined effect of X-irradiation and epigallo-catechin-galate (a tea component) on the growth inhibition and induction of apoptosis in human cancer cell lines. Ocolo Rep 12, 159-167.

Baldwin, R. T., S. Preston-Martin. (2004). Epidemiology of brain in childhood. Toxi and Appl Pharmacol 199, 118-131.

Ballas, L. M., W. J. Arion. (1977). Measurment of glucose-6-phosphate penetration into liver microsomes. Confirmation of substrate transport in the glucose-6-phosphatase system. J Biol Chem 252, 8512-8518.

Baochuan, L., B. Annabi., et al. (1998). Cloning characterization of cDNA encoding a candidate glycogen storage disease typeIb protein rodents. J Biol Chem 273, 31656-31660.

Barthel, A., D. Schmoll., et al.. (2001). Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinse gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells. Biochem Biophys Res 285, 897-902.

Beck, K., et B. Brodsky. (1998). Supercoiled protein motifs: the collagen triple-helix and the alphahelical coiled coil. J. Strucl Biol 122, 157-162.

Birkedal-Hansen, H., W. G. Moore., et al. (1993). Matrix metalloproteinase: a review. Crit Rev Oral Biol Med 4, 197-250.

Brat, D. J., A. Castellano-Sanchez., et al (2002). Genetic and biologic progression in astrocytomas and their relation to angiogenic dysregulation. Adv Anat Pathol 9, 24-36.

Chatelain, F., J. P. Pegorier., et al. (1998). Development and regulation of glucose-6-phosphatase gene expression in rat liver, intestine, and kidney : *in vivo* an *in vitro* studies in cultured fetal hepatocytes. Diabetes 47, 882-889.

Chen, L. Y., B. Lin., et al . (2000). Structural requirements for the stability and microsomal transport activity of the humain glucose-6-phosphate transporter. J Biol Chem 275, 34280-34286.

Chen, L. Y., J. J. Sheih., et al, (2003). Impaired glucose homeostasis, neutrophil trafficking anf function in mice lacking the glucose-6-phosphate transporter. Hum Mol Genet 12, 2547-2558.

Chou, J. Y., D. Matern., et al. (2002). Type I glycogen storage diseases: disorders of the glucose-6-phosphatase complex. Curr Mol. Med 2, 121-123.

Cuvelier, A., C. Kuntz.., et al. (1997) Les métalloprotéases de la matrice extracellulaire (MMP): structure activité. Rev mal Res 14, 1-10.

Dickens, M., C. A. Svitek., et al. (1998). Central role for phosphatidylinositide-3-kinase in the repression of glucose-6-phosphatase gene transcription by insulin. J Biol Chem 273, 20144-20149.

Fam, N. P., S. Verma., et al. (2003). Clinician guide to angiogenesis. Circulation 108, 2613-2618.

Forget, M-A., R. Desrosiers., et al. (1999). Physiological roles of matrix metalloproteinases :implication for timor growth and metastasis. Canc J Physiol Pharmacol 77, 465-480.

Foster, J. D., A. Bode., et al. (1994). Time-dependent inhibition of glucose-6-phosphatase by 3-mercaptopicolinic acid. Biochem Biophys Acta 1208, 222-228.

Gerin, I., G. Noel., et al. (2001). Novel arguments in favor of the substrate-transport model of glucose-6-phosphatase. Diabetes 50, 1531-1538.

Ghosh, A., Y. Y. Cheung., et al. (2005). Brain contain a functionnal glucose-6-phosphatase complex capable of endogenous glucose production. J Biol Chem 280, 11114-11119.

Gibb, G., G. P. Reid., et al. (1986). Purification and characterisation of the phosphate/hydroxyl ion antiport protein from rat liver mitochandria. Biochem J. 238, 543-551.

Hanahan, D., R. Weinberg. (2000). The halmarks of cancer. Cell 100, 57-70.

Hemmerle, H., H. J. Burger., et al. (1997). Chlorogenic acid and synthetic chlorogenic derivates: novel inhibitors of hepatic glucose-6-phophate translocase. J Med Chem 40, 137-145.

Hers, H. G., C. Duve. (1950). Le système hexose-phosphatasique II- répartition de l'activité glucose-6-phosphatasique dans les tissus. Bull Soc Chim Biol 32, 20-29.

Hers, H. G. (1976). The control of glycogen metabolism in the liver. Annu Rev Biochem 45, 167-189.

Hla, T., M. J. Lee., et al. (2000). Sphingosine-1-phosphate signaling via the EDG-1 family of G-protein-coupled receptors. Ann NY Acad 905, 16-24.

Horwitz, J. (1999). Adenovirus-mediated p53 gene therapy: overiview of preclinical studies and potential clinical applications. Curr Opin Mol Ther 1, 500-509.

Ichai, C., L. Guignoy., et al (2001). Glucose 6-phosphate hydrolysis is activated by glucagon in a low temperature manner. J Biol Chem 276, 28126-28133.

Janecke, A. R., N. U. Bosshard., et al. (1999). Molecular diagnosis of type 1c glycogen storage disease. Hum Genet 104, 275-277.

Jin, U. H., Y. J. Lee., et al. (2005). Aphenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor:isolation and identification from methanol extract of euonymus alatus. Life 77, 2760-2769.

Khan, A., Z. C. Ling., et al. (1998). Effects of 3-mercaptopicolinic acid and a derivate of chlorogenic acid (S-3483) on hepatic and islet glucose-6-phosphatase activity. Eur J Pharmacol 349, 325-331.

Kishnani, P. S., Y. Bao., et al. (1997). Isolation and nucleotide sequence of canine glucose-6-phosphatase mRNA: identification of mutation in poppies with glycogen storage disease type Ia. Biochem Mol Med 61, 168-177.

Kramer, W., H. J. Burger., et al. (1999). Identification of protein components of the microsomal glucose-6-phosphate transporter by photoafinity labeling. Biochem J 339, 629-638.

Kristiansen, K., S. Hagen., et al. (1981). Combined modality therapy of operated astrocytomas grades III and IV. Confirmation of the value of postoperative irradiation and lack of potentiation of bleomycin on survival time: a prospective multicancer trial of scandinavian glioblastoma study group. Cancer 47. 649-652.

Langlois, S., G. Di Tomasso., et al. (2005) Membrane type 1-matrix metalloproteinase induces endothelial cell morphogenic differentiation by a caspase-dependent mechanism. *Exp. Cell Res.* **307**, 452-464.

Lehti, K., L. Lohi., et al. (2002). Oligomerization throught hemopexin and cytoplasmic domainsregulates the activity and turnover of MT1-MMp. J Biol Chem 277, 8440-8448.

Lei, K. J., Y. T. Chen., et al. (1995). Genetic basis of glycogen storage disease type 1a: prevalent mutations at the glucose-6-phosphatase locus. Am J Hum Genet 57, 766-771.

Lei, K. J., C. J. Pan. (1994). Identification of mutations in the gene for glucose-6-phosphatase, the enzyme deficient in glycogen storage disaese type Ia. J Clin Invest 93, 1994-1999.

Lei, K. J., C. J. Shelly, et al. (1993). Mutation in the glucose-6-phosphatase gene that cause glycogen disease type Ia. Science 262, 580-583.

Lei, K. J., L. L. Shelly., et al.(1995). Mutations in glucose-6-phosphatase gene are associated with glycogen storage disease type 1a and 1aSP but not 1b and 1c. J Clin Invest 95, 234-240.

Lemeke, D. M. (2004). Epidemiology, diagnosis, and treatment of patient with metastatic cancer and high-grade gliomas of central nervous syste. J of Infu Nurs 27, 263-269.

Lelievre, S., V. Weaver., et al. (1996). Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation. Recent Prog Horm 51, 417-432.

Leuzzi, R., G. Banhegyi., et al. (2003). Inhibition of microsomal glucose-6-phosphate transport in humain neutrophilis results in apopotosis : apotential explanation for neutrophyldisfonction in glycoge storage disease type ib. Blood 101, 2381-2387.

Lin, B., H. Hiraiwa., et al. (1999). Type 1c glycogen storage disease is not caused by mutations in the glucose-6phosphate transporter gene. Hum Genet 105, 515-517.

Marieb, E. N. (1993). Anatomie et physiologie humaine. Deuxième édition . Canada : Édition du renouveau pédagogique.

Martin, C. C., J. K. Oeser., et al. (2002). Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. J Mol Endoc 29, 205-222.

Massillon, D. (2001). Regulation of the glucose-6-phosphatase gene by glucose occurs by transcriptional and post-transcriptional mechanisms. Differential effect of glucose annd xylitol. J Biol Chem 276, 4055-4062.

Mclaughlin, N., B. Annabi., et al. (2006). The survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate. Brain Reserch 1071, 1-9.

Mori, H., K. Kawabata., et al (2000). Chemopreventive effects of coffee ben and rice constituents on colorectal carcinogenesis. Biofact 12, 101-105.

Moreno-Sanchez, R., S. Rodriguez-enriquez., et al. (2007). Energy metabolism in tumor cells. The FEBS J. 274, 1393-1418.

Murphy, G., H. Stanton., et al. (1999). Mechanism for pro matrix metalloproteinase activation. APMIS 107, 38-44.

Nicasio, P., L. Aguilar-santamaria., et al. (2005). Hypoglycemic effect and chlorogenic acid content in two cecropia apecies. Phyto Res 19, 661-664.

Nordlie, R. C., K. A. Sukalski., et al. (1983). Type ic, a novel glycogenosis underlying mechanism. J Biol Chem 258, 9739-9744.

Nordlie, R. C., H. M. Scott., et al. (1992). Analysis of humain hepatic microsomal glucose-6-phosphatase in clinical conditions where the T2 pyrophosphate/phosphate transport protein is absent. Biochem J 281, 859-863.

O'Brien, R. M., R. S. Streeper., et al. (2001). Insulin-regulated gene expresion. Biochem Soc Trans29, 552-558.

Parker, J. C., M. A. Vanvolkenberg., et al. (1998). Plasma glucose levels are reduced in rats and mice microsomes treated with an inhibitor of glucose-6-phosphate translocase. Diabetes 47, 1630-1636.

Polette, M., P. Birembaut. (1998). Membrane-type metalloproteinase in tumor invasion. Int J Biochem Cell Biol 30, 1195-1202.

Rake, J. P., A. M. Ten Berge., et al. (2000). Glycogen storage disease type a: recent experience with mutation analysis, a summary of mutations reported in the literature and newly developped diagnostic flow chart. Eur J Pediat 159, 322-330.

Sato, H., Y. Okada., et al. (1996). TNF-alpha and IL-1 beta selectivity induce expression of 39-Kda gelatinase by human macrophaes. J Immunol 157, 159-164.

Schulze, H. U., B. Nolte., et al. (1986). Evidence for changes in the conformational status of rat microsomal glucose-6-phosphate: phosphohydrolase during detergent-dependent membrane. Effect of p-mercuribenzoate and organomercurial agarose gel on glucose-6-phosphatase of native and detergent-modified microsomes. J Biol Chem 261, 16571-16578

Shcmoll, D., K. S. Walker., et al. (2000). Regulation of glucose-6-phosphatase gene expression by protein kinase balpha anf the forkhead transcription factor FKHR. Evidence for indulin response unit-dependent and-independent effects of insulin on promotor activity. J Biol Chem 275, 36324-36333.

Sheih, J. J., C. J. Pan., et al. (2003). A glucose-6-phosphate hydrolase, widly expressed outside the liver can explain age0dependent resolution of hypoglycemia in glycogen storage disease type Ia. J Biol Chem 278, 47098-47103.

Singht, D., A. K. Banerji., et al. (2005). Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalatio studies in patients with glioblastoma multiforme. Stran Onco 181, 507-514.

Speth, M., H. U. Schulze. (1992). The purification of detergent-soluble glucose-6-phosphatase from rat liver. Eur J Biochem 208, 643-650.

Speth, M., H. U. Schulze. (1992). Protease inhibitors but not proteases inhibit the glucose-6-phosphatase of native rat liver microsomes. Biochem Biophys Res Commu 183, 590-597.

Streeper, R. S., C. A. Svitek., et al. (1997). A multicomponent insulin response sequence mediates a strong repression of mouse glucose-6-phosphatase gene transcription by insulin. J Biol Chem 272, 11698-11701.

Strongin, A. Y., I. Collier., et al. (1995). Mechanism of cell surface activation of 72-Kda type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. J Biol Chem 270, 5331-5338.

Timperly, W. R. (1980). Glycolysis in neuroectodermal tumors. In :Thomas DGT, Graham DI, eds brain tumors, Scientific basis, clinical investigation and current therapy. Lond: But Terwort's, 145-167.

Van Schaftingen, E., I. Gerin. (2002). The glucose-6-phophatase system. J Biochem 362, 513-532.

Vertesy, L., M. Kurz., et al. (2001). The chemical structure of mumbaistain, a novel glucose-6-phopsphate translocase inhibitor producer by Streptomyces sp. DSM 11641. J Antibiot 54, 354-363.

Walker, M. D., Jr. Alexander., et al. (1978). Evaluation of BCNU and/or radiotherapy in treatment of anaplastic gliomas. A cooperative clinical trial. J Neurosur 49, 333-343.

Wossner, J. F. Jr. 1998. The matrix metalloproteinases family. Matr Metallop academic press. 1-13.

Xu, R. H., H. Palicano., et al. (2005). Inhibition of glycolysis in cancer cells : a novel strategy to overcome drug resistance associated with mitochondria respiratory defect and hypoxia. Cancer Res. 65, 613-621.

Yancopoulos, G. D., M. Klagdbrun., et al. (1998). Vasculogenesis angiogenesis, and growth factors: ephrins enter the fray at the border. Cell 93, 661-664.

Yu, A., E. Murphy., et al. (1998). 62-KDa gelatinase :structure regulation, and substrate specificity. Mat Met Acad Pres, 83-113.

Zingone, A., H. Hiraiwa., et al. (2000). Correction of glycogen storge disease type 1a in a mouse model by gene therapy. J Biol Chem 275, 828-832.

Zoccoli, M. A., M. L. Karnovsky. (1980). Effect of two inhibitors of anion transport on the hydrolysis of glucose-6phosphate by rat liver microsomes. Covalent modification of the glucose-6-phosphate transport component. J Biol Chem 225, 1113-1119.

Zoccoli, M. A., R. R. Hoopes., et al. (1982). Identification of a rat liver microsomal polypeptide involved in transport of glucose-6-phosphate. Labeling with 4. 4'-diidothiocyano-1-2-diphenyl [3H]ethane-2-2'-disulfonic acid. J Biol Chem 257, 3919-3924.