Inhibition of melanoma brain metastasis by targeting melanotransferrin at the cell surface

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Summary

Brain metastases are a common feature of malignant melanoma and are associated with poor prognosis. Melanotransferrin (MTf), one of several antigens associated with the surface of melanoma cells, has been demonstrated to promote cell invasion. In this study, we investigated the role of membrane-bound MTf in several of the steps leading to the development of melanoma brain metastasis. Our results indicated that MTf-positive cells were detected in the brains of nude mice injected intravenously with human melanoma SK-Mel 28 cells. Moreover, administration of a single dose of a monoclonal antibody (L235) directed against human MTf significantly reduced the development of human melanoma brain metastases in nude mice. The ability of melanoma cells to cross the blood–brain barrier (BBB) in vitro is correlated with their MTf expression levels at the cell surface. Overall, our results indicated that membrane-bound MTf is a key element in melanoma cell transmigration across the BBB and subsequent brain metastasis. Thus, these data suggest MTf as an attractive target and demonstrate the therapeutic potential of an anti-MTf mAb for preventing metastatic melanoma.

Introduction

Brain metastases represent an important cause of morbidity and are the most common intracranial tumours in adults. Recent population-based data suggest that 10–30% of cancer patients exhibit clinical signs of metastasis to the brain whereas 80% are shown to have metastatic brain tumours at autopsy (Barnholtz-Sloan et al., 2004). The majority of brain metastases originate from one of the three primary malignancies: lung cancer (40–50%), breast cancer (15–25%), and melanoma (5–20%). Amongst these, melanoma has the highest propensity to metastasize to the brain (Wen and Loeffler, 2001). Limited therapeutic success in the treatment of central nervous system (CNS) neoplasia with chemotherapy is generally attributed to natural or acquired resistance to

Significance

This study evaluated the contribution of melanotransferrin (MTf) to the formation of melanoma brain metastasis. Melanotransferrin was first identified as an antigen of melanoma cells and has more recently been reported to stimulate plasmin formation and thus cell invasion. Results presented here show that MTf over-expression is closely related to the capacity of melanoma cells to transmigrate across the blood–brain barrier in vitro in order to produce brain metastases. We also demonstrate that the administration of a monoclonal antibody directed against human MTf significantly reduced the development of melanoma brain metastases in nude mice. Overall, our results suggest that MTf participates in the establishment of melanoma brain metastases, and that membrane-bound MTf is a promising target for preventing tumour progression.

chemotherapy expressed by tumour cells, and to delivery impediment related to the blood–brain barrier (BBB).

Interposed between the circulatory system and the CNS, the BBB is composed of a continuous, non-fenestrated endothelium lining the cerebral microvasculature and is an important mechanism for maintaining homeostasis in the brain microenvironment (Bart et al., 2000). The development of the BBB is influenced by the astrocytic glia and pericytes, and leads to endothelial cells (EC) with unique permeability characteristics both due to high electrical resistance and to the expression of specific transporters (Dermietzel and Krause, 1991; Risau and Wolburg, 1990). Once tumour cells have invaded the BBB to establish a brain metastasis, the ECs now comprise a blood–tumour barrier. Little is known concerning this barrier in either human or animal models. Although a number of alterations in the brain capillary ultrastructure have been described in primary and metastatic brain tumours (Stewart, 1994), a normal BBB can co-exist with brain metastases (Bertossi et al., 1997). Hence, one hallmark of brain metastases is the oedema that surrounds the tumour, an effect possibly caused by altered permeability of tumour-associated ECs permitting greater leakage of fluid into the tumour (Lesniak and Brem, 2004).

To produce brain metastases, tumours progress through several steps including: reaching the brain vasculature through a process named intravasation, attaching to the endothelial wall, extravasating into the brain parenchyma, proliferating and inducing neoangiogenesis (Fidler et al., 2002). The plasminogen activation system plays a crucial role not only in blood clot lysis (Pawse and Tarachand, 1997) but also in various physiological and pathological events where localized proteolysis is required, such as inflammation (Del Rosso et al., 2008), tissue remodelling and tumour metastasis (Andreasen et al., 2000). Plasmin is a key component of the fibrinolytic system that is capable of degrading, directly or through the activation of matrix metalloproteinases (MMPs), most of the major protein components of the extracellular matrix. Matrix degradation attributed to MMP-2 and MMP-9 (also known as gelatinase-A and -B, respectively) has been shown to play an important role in the progression of diseases such as atherosclerosis (Galis et al., 1995), inflammation (Romanic and Madri, 1994), tumour growth and metastasis (Hofmann et al., 2003).

Melanotransferrin (MTf), first identified in human melanoma, has been found to play an important role in numerous processes associated with tumour development and angiogenesis (Demeule et al., 2003; Michaud-Levesque et al., 2005a,b, 2007a; Rolland et al., 2007). MTf can either be associated with the cell membrane by a glycosyl phosphatidylinositol anchor or secreted into the extracellular environment in trace amounts (Brown et al., 1981). Studies have shown that the most pronounced MTf expression in normal tissues was within the epidermis of the skin, the brain endothelium, the kidney tubules and the ducts of sweat and salivary glands (Rothenberger et al., 1996; Sekyere et al., 2005). Expressed only slightly in normal tissues, MTf is present in much larger amounts in neoplastic cells and foetal tissues (Brown et al., 1981). We previously reported that MTf interaction with plasminogen modulates plasmin formation and stimulates cell motility (Demeule et al., 2003; Michaud-Levesque et al., 2005a). In addition, MTf is involved in melanoma cell proliferation and subcutaneous tumour growth (Bertrand et al., 2007; Dunn et al., 2006).

In this study, we evaluated the contribution of MTf in the development of brain metastases from melanoma. We show that MTf targeting by a monoclonal antibody (mAb L235) reduced brain metastasis from SK-Mel 28 cells in nude mice. The results presented here also indicate that transendothelial migration of cancer cells across an in vitro BBB model is correlated with MTf expression levels at their cell surface. Altogether, our results strongly suggest that membrane-bound MTf targeting may represent a promising therapeutic approach in preventing development of brain metastases.

Results

Expression of MTf at the cell surface increases cell invasion in vivo

The expression of membrane-bound MTf in CHO cells was previously reported to stimulate cell migration and invasion in vitro (Michaud-Levesque et al., 2005a). Hence, an in vivo invasion assay was used to determine the ability of MTf-transfected cells to penetrate distant organs from the blood circulation. CHO Mock and MTf-transfected cells were radiolabelled with [^{3}H]-thymidine and injected into Crl:CD-1 mice tail veins. After 24 h, the distribution of [^{3}H]-thymidine in mice tissues was determined using Ultima Gold^{TM} liquid scintillation cocktail and Packard liquid scintillation analyzer from Perkin Elmer (Woodbridge, Canada). Figure 1 shows that the amount of radioactivity in brain and lungs was significantly increased when mice were injected intravenously (i.v.) with MTf-transfected CHO cells. More precisely, MTf expression at the surface of CHO cells increased brain and lung invasion by 7- and 9-fold, respectively. The radioactivity levels detected in kidney, heart and liver were similar whether mice were injected with Mock or with MTf-transfected CHO cells (Figure 1). These results suggest that invasion of MTf-expressing cells to the brain over a short-term period could promote the development of brain metastases over time.

Intravenous injection of human melanoma cells leads to the development of brain metastasis in nude mice

To investigate whether MTf is involved in brain invasion of melanoma cells, we developed a quantitative assay

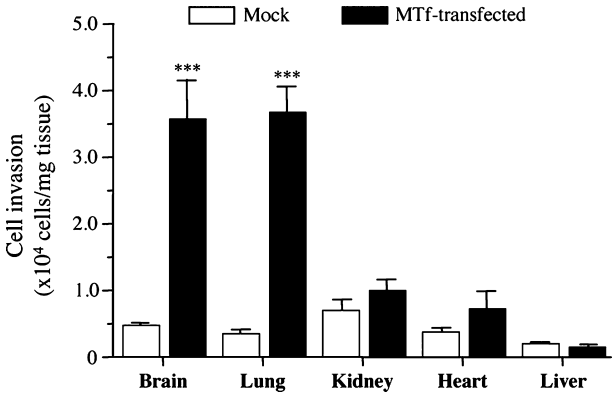

Figure 1. Melanotransferrin (MTf) expression at the surface of CHO cells promotes cell invasion in vivo. CHO Mock and MTf-transfected cells were labelled with [^{3}H]-thymidine over two days and injected into Crl:CD-1 mice tail veins. The amount of radiotracer in whole tissues was determined after 24 h by liquid scintillation spectrometry as described in the Materials and methods section. In vivo invasion is expressed as number of cells per mg of tissue. Experiments were performed on eight different animals for both conditions. Means ± SE are shown.

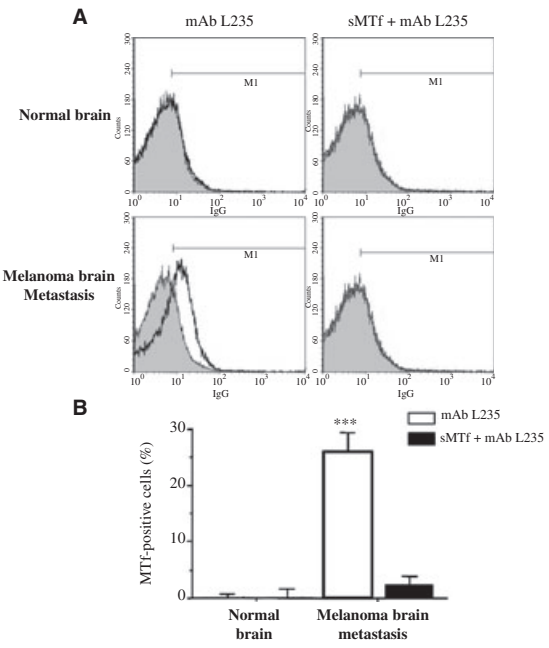

Figure 2. Intravenous (i.v.) human melanoma cells produce brain metastasis in nude mice. (A) SK-Mel 28 cells were injected i.v. in female athymic nude mice. Brain homogenates were analyzed for their content of human melanotransferrin (MTf)-expressing cells using flow cytometric analysis using mAb L235 (black line) or IgG_1 control (grey area) and Alexa Fluor 488® conjugated antibodies, as described in the Materials and methods section. The specificity of the signal was also evaluated by the presence of recombinant truncated MTf (sMTf). All images are representative of five independent experiments. (B) The portion of mice brain cells expressing human membrane-bound MTf was quantified within the M1-gated cell population. Statistically significant differences compared to the mAb L235 signal from normal mice brain are marked as described. Experiments were performed on three normal mice and five melanoma injected mice. Data represent the means ± SE.

to measure the presence of human MTf-expressing cells. Human melanoma SK-Mel 28 cells were injected i.v. into athymic nude mice and the content of human MTf-expressing cells was quantified in total brain after 8, 16 and 24 weeks. MTf-positive cells were only detected in brains of mice, which received the injection of tumour cells 24 weeks earlier. During this period of time, mice did not show any symptom or indication of brain metastasis, such as weight loss, dehydration or arched position. Flow cytometric analysis using mAb L235 demonstrated that human MTf could be detected in brain homogenates of mice injected with SK-Mel 28 cells (Figure 2A). As this mAb specifically recognizes human MTf, this signal can be attributed to human MTf expressed at the surface of melanoma cells. Results obtained by fluorescence-activated cell-sorting (FACS) analysis indicated that the mean fluorescence intensity associated with the detection of MTf-positive cells was increased by 25% in brain homogenates from mice injected i.v. with melanoma cells (Figure 2B). Other organs (i.e. lungs, liver and kidneys) from mice injected with SK-Mel 28 cells were also analysed using FACS. However, in contrast to the brain, MTf-expressing cells were undetectable (data not shown). To confirm the MTf-detection specificity, excess soluble MTf (sMTf) was added during mAb staining. The added sMTf completely blocked staining by the mAb L235, indicating that it is specific to MTf present in the brain homogenates (Figure 2).

Single dose of mAb L235 reduces melanoma brain metastasis in nude mice

To confirm and validate that membrane-bound MTf is associated with SK-Mel 28 cell invasion in vivo, we evaluated the effect of mAb L235 on the establishment of melanoma brain metastasis in mice. Human melanoma SK-Mel 28 cells were injected i.v. into female athymic nude mice treated (or not) with either mAb L235 or an IgG_1 control isotype. The content of melanoma cells, defined as human MTf-expressing cells, was determined using flow cytometry (Figure 3A). The administration of a single dose of mAb L235 intraperitoneally (i.p.) at 20 mg/kg was sufficient to reduce by half the level of human melanoma MTf-expressing cells in mouse brain when compared with brain from untreated or IgG_1-treated mice (Figure 3B). Overall, these results show that a single dose of mAb L235 can reduce the development of brain metastases from SK-Mel 28 human melanoma cells.

Pharmacokinetic characteristics of mAb L235

Plasma levels of mAb L235 were measured following a single i.p. injection of [^{125}I]-L235 (20 mg/kg) into female athymic nude mice. The mean plasma concentrations of

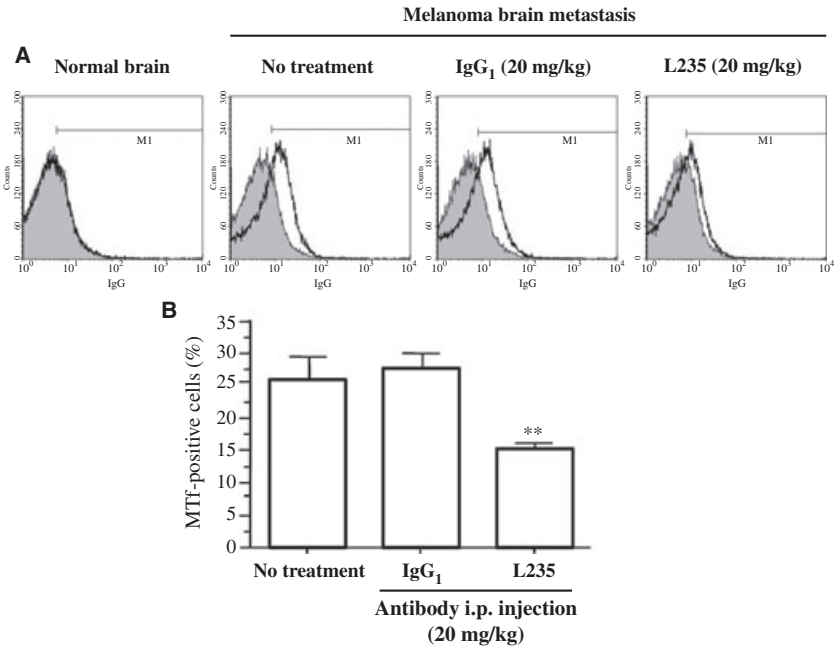

Figure 3. Monoclonal antibody L235 reduces the establishment of melanoma brain metastasis. Female athymic nude mice received a single dose of 20 mg/kg mAb L235 or IgG_1 in saline solution by i.p. administration. Six hours later, SK-Mel 28 melanoma cells were injected into mice tail veins. After 24 weeks, mice brain were collected and their content of cells expressing human MTf was determined by flow cytometry as described in the Materials and methods section. (A) Brain homogenates from treated and untreated mice were incubated with mAb L235 (black line) or IgG_1 control isotype (grey area) and Alexa Fluor 488® conjugated antibodies. All images are representative of four different experiments. (B) Portion of MTf-positive cells in brain homogenates was quantified within the M1-gated cell population. Statistically significant differences are shown for comparisons with the mAb L235 signal in normal mouse brain. Experiments were performed on three normal mice and five mice with melanoma brain metastasis (treated or not). Data represent the means ± SE.

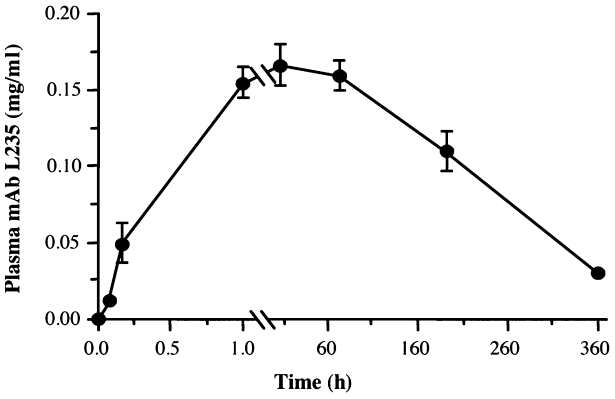

Figure 4. Plasma concentration of mAb L235 in nude mice. Female athymic nude mice received a single dose of 20mg/kg [^{125}I]-L235 in saline solution by intraperitoneal injection. At the allotted times, blood was collected and the quantity of the radiolabel was quantified using a gamma counter. Results are expressed as plasma concentration of mAb L235 (μM). The pharmacokinetic parameters derived from these data are summarized in Table 1. For each condition, n = 3 and the means ± SE are shown.

mAb L235 were plotted as a function of time (Figure 4). The pharmacokinetic parameters derived from these data are summarized in Table 1. Monoclonal antibody L235 was detected in the circulation 10 min after its i.p. injection. The maximum concentration (C_{max}) of 0.17 μg/ml (1.1 μM) was reached 24 h later and the terminal half-life ($t_{1/2}$) was 115 h (~5 days). The high area under curve (AUC_{0-360}) of 45.5 μg*h/ml for the mAb L235 also indicate that its plasma concentration remains stable for a relatively long period of time (Table 1). In addition, the ability of mAb L235 in mouse plasma to bind to MTf was confirmed by Western blot analysis (data not shown). Similar levels of recombinant MTf were detected with original mAb L235 and with mouse plasma collected at 6, 24, 48 and 72 h after i.p. mAb administration.

MTf expression correlates with increased cell transmigration across the blood-brain barrier in vitro

We next evaluated the migration of MTf-expressing cells across the BBB using an in vitro model consisting of brain capillary EC (BCEC) co-cultured with glial cells. This model presents ultrastructural features characteristic of brain endothelium, including tight junctions, lack of fenestration and transendothelial channels, low permeability for hydrophilic molecules and a high electrical resistance (Dehouck et al., 1990). Cell transmigration across this BBB in vitro model was evaluated for several

Dose (mg/kg)	C_{max} (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	$AUC_{(0-360)}$ (μg*h/ml)	Vd (ml/kg)	CL (ml/h/kg)
20	0.17 ± 0.01	24	115 ± 4	45.5 ± 1.1	73 ± 2	0.44 ± 0.01

Pharmacokinetic parameters were determined by inspection of the data using PK Solutions 2.0 software (Summit Research Services; Montrose, CO, USA). Data are expressed as means ± SD (n = 3).

Table 1. Pharmacokinetic parameters of [^{125}I]-L235 mAb in plasma of female athymic nude mice

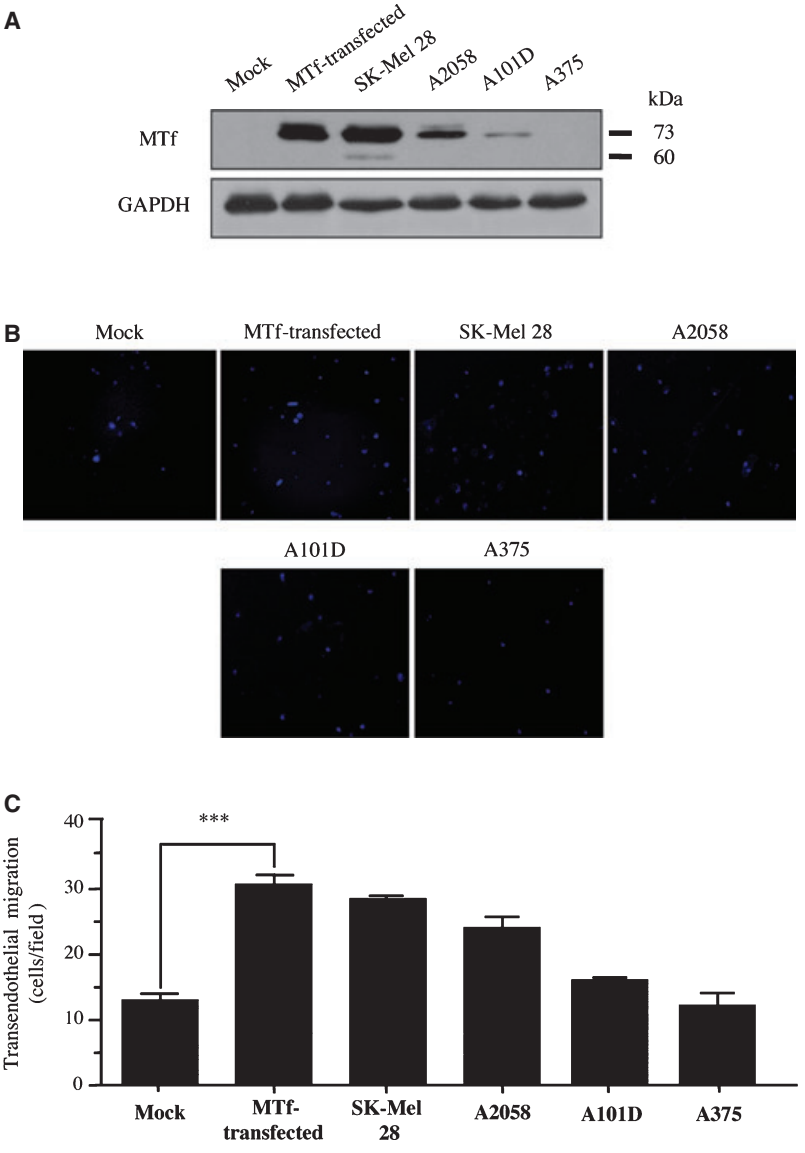

Figure 5. Membrane-bound melanotransferrin facilitates blood–brain barrier invasion in vitro. (A) Protein expression of membrane-bound melanotransferrin (MTf) was studied by Western blot analysis in CHO cells transfected with the full-length human MTf cDNA (MTf-transfected) or with the control vector (Mock), as well as in SK-Mel 28, A2058, A101D and A375 human melanoma cells. The immunodetection of GAPDH was used as a loading control. (B) Cells were incubated with Hoechst 33342 and loaded onto the BBB in vitro model as described in the Materials and methods sections. Fluorescent cells that migrated across the BCEC monolayer were visualized by fluorescent microscopy. Photos obtained from representative experiments are shown at an original magnification of 40×. (C) The transendothelial migration assay was quantified using Northern Eclipse software and expressed as the number of transmigrated cells per field. Data represent the means ± SE of results obtained from four different experiments performed in triplicate (10 random fields were considered for each filter).

cell lines expressing different levels of MTf. Western blot analysis revealed that SK-Mel 28 and A2058 cells express higher levels of MTf among melanoma cell lines, when compared with A101D cells (Figure 5A). Meanwhile, MTf protein expression was undetected in A375 melanomas and CHO Mock cells. As previously reported (Demeule et al., 2003), Western blot analysis allowed detection of two bands at 73 and 60 kDa in human melanoma SK-Mel 28 cells.

Cell transmigration across the in vitro BBB model was monitored by fluorescent cell staining with bisbenzimide (Hoechst 33342; Sigma-Aldrich, Oakville, ON, USA), a molecule that becomes fluorescent after binding to DNA. Cell staining first revealed that the four cell types used were labelled equally with bisbenzimide in a very short period of time, and that the fluorescence remained stable over 72 h (data not shown). Fluorescent cells were then loaded onto the BCEC monolayer and cells that had

transmigrated to the basolateral side of the filter were visualized using fluorescence microscopy after 48 h (Figure 5B). The quantification of transmigrated fluorescent cells shows that SK-Mel 28 and MTf-transfected CHO cells had migrated through the BBB to a greater extent than had other cell lines (Figure 5C). More specifically, the transendothelial migration of MTf-transfected CHO cells was significantly increased by threefold when compared with Mock cells. Results also showed that SK-Mel 28 and MTf-transfected CHO cells had an equivalent ability to cross the BCEC monolayer. Figure 5C indicates that melanoma cells expressing higher levels of MTf, such as SK-Mel 28 and A2058, are more likely to cross the BBB, with nearly twice as much transmigrated cells than non-expressing cells. To that end, A375 melanoma cells and CHO Mock cells have similar levels of transmigration (Figure 5C).

mAb L235 reduces transendothelial migration of MTf-expressing cells across the BBB

We selected three cell lines with the most abundant MTf expression (SK-Mel 28, A2058 and MTf-transfected CHO cells) in order to fully evaluate the contribution of membrane-bound MTf in cell transmigration across the BBB in vitro model. Cells were incubated with a monoclonal antibody (mAb) and loaded onto BCEC monolayer. Figure 6A shows that the transmigration of fluorescent MTf-transfected CHO cells is significantly reduced by mAb L235, while the transmigration of Mock CHO cells was unaffected. Quantification of cell transmigration indicate that mAb L235 diminished the migration of MTf-transfected CHO cells by 50%, nearly to the basal level seen with Mock CHO cells (Figure 6B). Therefore, the presence of the mAb L235 inhibited the transendothelial migration of MTf-expressing melanoma cells, SK-Mel 28 and A2058 cells, by nearly 50% when compared to an IgG_1 control isotype (Figure 6C).

Down-regulation of MTf expression decreases melanoma cell transmigration across the BBB

To verify that MTf is a key player in melanoma cell transmigration across the BBB, the expression of MTf was downregulated by post-transcriptional gene silencing (siRNA). The efficacy of MTf-specific siRNA was first determined by Western blot analysis. SK-Mel 28 and A2058 cells transfected with siRNA targeting MTf

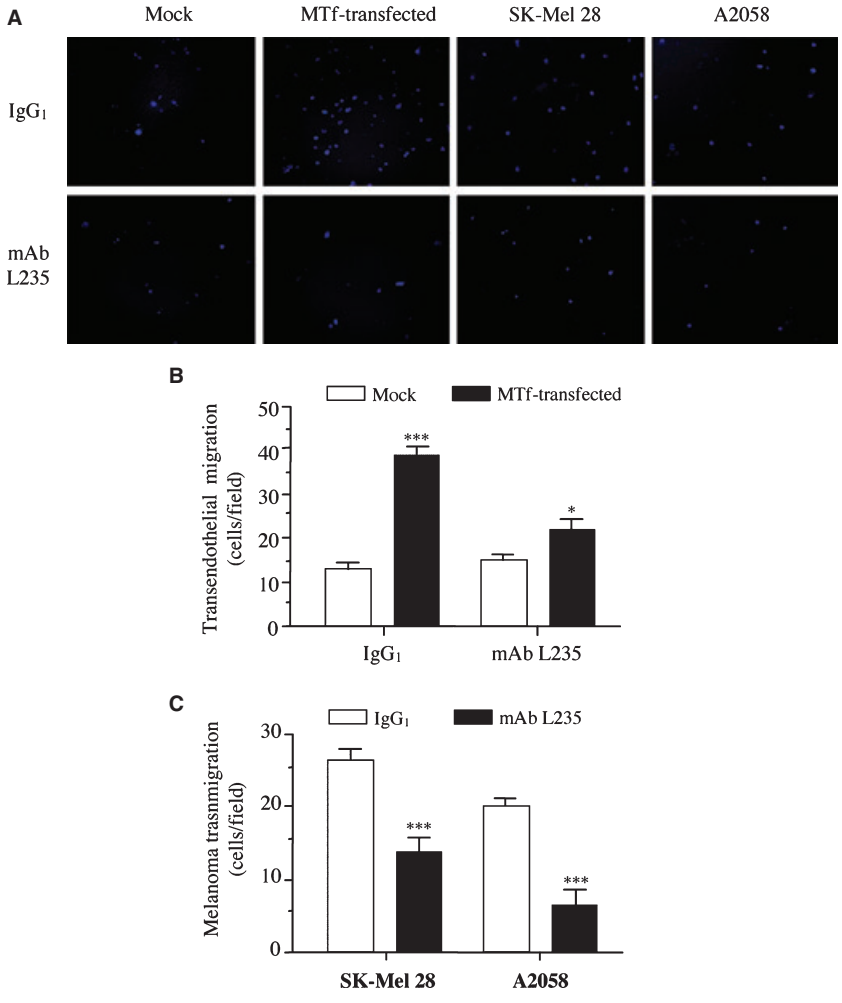

Figure 6. Monoclonal antibody L235 inhibits transmigration of MTf-expressing cells across the blood–brain barrier in vitro. (A) Human melanoma cells and CHO cells (Mock and MTf-transfected) were incubated with an immunoglobulin control (IgG_1) or with mAb L235 directed against MTf. Afterwards, cells were stained with Hoechst 33342 and loaded onto the BBB in vitro model as described in the Materials and methods section. Representative photographs of transmigrated fluorescent cells are shown at the original magnification of 40× for all panels. Transendothelial migration of Mock and MTf-transfected CHO cells (B) as well as of SK-Mel 28 and A2058 human melanoma cells (C) was quantified using Northern Eclipse software in 10 different fields from four independent experiments performed in triplicate. Statistically significant differences are compared to IgG_1 control condition. Means ± SE are shown.

expression show a significant reduction (respectively by 70 and 60%) in MTf protein expression when compared to cells transfected with non-specific siRNA (control siRNA) or to non-transfected cells (Figure 7A). In addition, real-time PCR analysis indicates that mRNA expression of MTf was reduced by 80 and 60% respectively in SK-Mel 28 and A2058 melanoma cells transfected with MTf siRNA (Figure 7B).

Following this, the ability of MTf-silenced melanoma cells to transmigrate across the BBB in vitro was determined. We found that transendothelial migration of SK-Mel 28 cells with low levels of MTf expression was decreased by nearly 80% when compared with non-transfected cells or to melanoma cells transfected with control siRNA (Figure 7C). Similar results were obtained with A2058 melanoma cells, where siRNAs targeting MTf expression succeed to reduce by 70% the transmigration of A2058 cells across the BBB in vitro model (Figure 7C). The integrity of the BBB was studied by monitoring the diffusion of sucrose during the transmigration of SK-Mel 28 melanoma cells (data not shown). While the permeability of sucrose was increased during the transmigration of melanoma cells, it was unaffected and maintained at basal levels during the transmigration of melanoma cells transfected with MTf-siRNA.

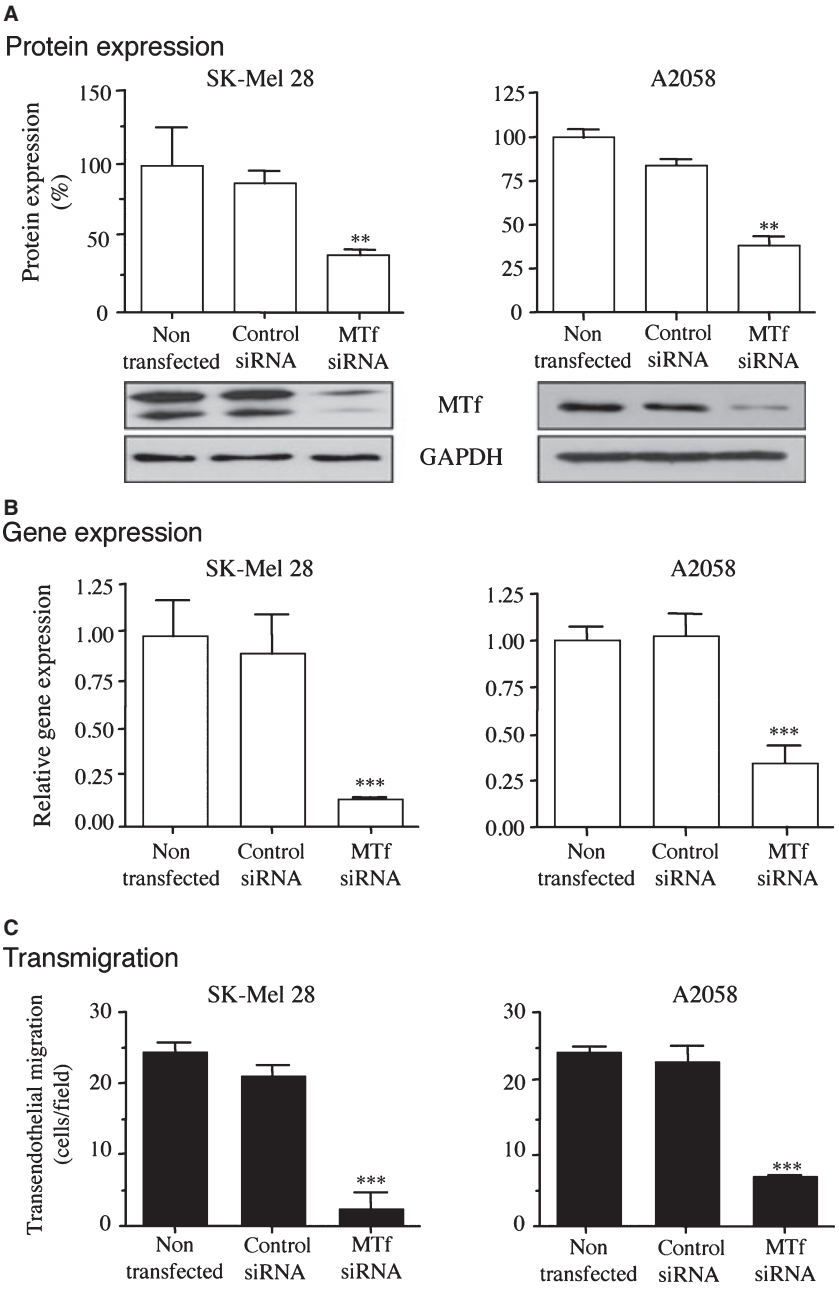

Figure 7. Down-regulation of MTf expression in melanoma cells reduces cell transmigration ability. SK-Mel 28 and A2058 melanoma cells were transfected with non-specific (control siRNA) or MTf-specific (MTf siRNA) siRNA during 48 h. (A) Protein expression of MTf was studied in melanoma cells transfected or not with siRNAs by Western blot analysis. (B) MTf gene expression was quantified in melanoma cells by quantitative PCR and corrected by the measurement of the 18S ribosomal subunit reference gene, as described in the Materials and methods section. (C) Transmigration of fluorescent melanoma cells across the BBB in vitro model was quantified using Northern Eclipse software in 10 different fields from three independent experiments performed in triplicate. Statistically significant differences are compared to cells transfected with the control siRNA. Data represent the means ± SE.

Participation of plasmin and MMPs during the MTf-dependent transmigration process

The activation of plasminogen into plasmin is a key feature in tumour cell invasion and metastasis (Skrzydlewska et al., 2005). As MTf can stimulate the activation of plasminogen (Demeule et al., 2003; Michaud-Levesque et al., 2005a), we evaluated the importance of this system during the transmigration of MTf-expressing cells across the BBB in vitro. In the presence of α_2-antiplasmin (α_2-AP), the transendothelial migration of SK-Mel 28 human melanoma cells was impeded by more than 75% when compared to control conditions (Figure 8A, left panel). While α_2-antiplasmin is known to prevent plasminogen activation and to inhibit the proteolytic activity of plasmin, epsilon aminocaproic acid (EACA) mimics the side chain of lysine and interacts with plasminogen lysine-binding sites (Castellino et al., 1981). When added to the assay, EACA reduced the transendothelial migration of SK-Mel 28 cells by 50%. A similar degree of inhibition was also obtained in the presence of sMTf (Figure 8A, left panel), which is thought to compete with the binding of plasminogen to endogenous membrane bound MTf.

Once formed, plasmin stimulates extracellular matrix degradation by activating pro-MMPs (Skrzydlewska et al., 2005). Treatment of SK-Mel 28 melanoma cells with GM6001, a broad range MMP inhibitor, reduced the transendothelial migration of melanoma cells by 90% (Figure 8A, right panel). MMP-2 and MMP-9 are two major MMPs involved in cancer cells' invasion through the BBB. The presence of monoclonal antibodies directed against human MMP-2 and MMP-9 reduced the invasion of SK-Mel 28 cells by 40 and 75%, respectively, compared to the IgG_1 control condition (Figure 8A, right panel).

Transendothelial migration of MTf-transfected CHO cells was also significantly reduced (more than 50% reduction) by α_2-AP, EACA and sMTf (Figure 8B, left panel). In addition, MTf-expressing CHO cells responded similarly to SK-Mel 28 cells in the presence of MMPs inhibitors and specific mAbs. Figure 8B (right panel) shows that anti-MMP-2 and anti-MMP-9 mAbs inhibit the transmigration of MTf-transfected CHO cells by 50 and 80% respectively, when compared to the isotype control condition. As observed with melanoma cells, the broadrange MMP inhibitor potently reduces the migration of CHO cells across the BBB in vitro model. These results indicate that plasmin as well as MMP-2 and MMP-9 are involved in the MTf-dependent transendothelial migration.

Discussion

Brain is a common site of metastasis from malignant melanoma (Posner, 1996). Among cell surface antigens of human malignant melanoma, there is a growing interest towards MTf. We demonstrate that MTf-expressing cells present a greater ability to cross the endothelium of an in vitro BBB model, when compared with non-expressing cell lines. Previous studies reported that SK-Mel 28 melanoma cells had between 3 and 3.8×10^5 MTf sites/cell, while this number could reach 1.2×10^6 MTf sites/cell in MTf-transfected CHO cells (Brown et al., 1981; Kennard et al., 1995). Among various

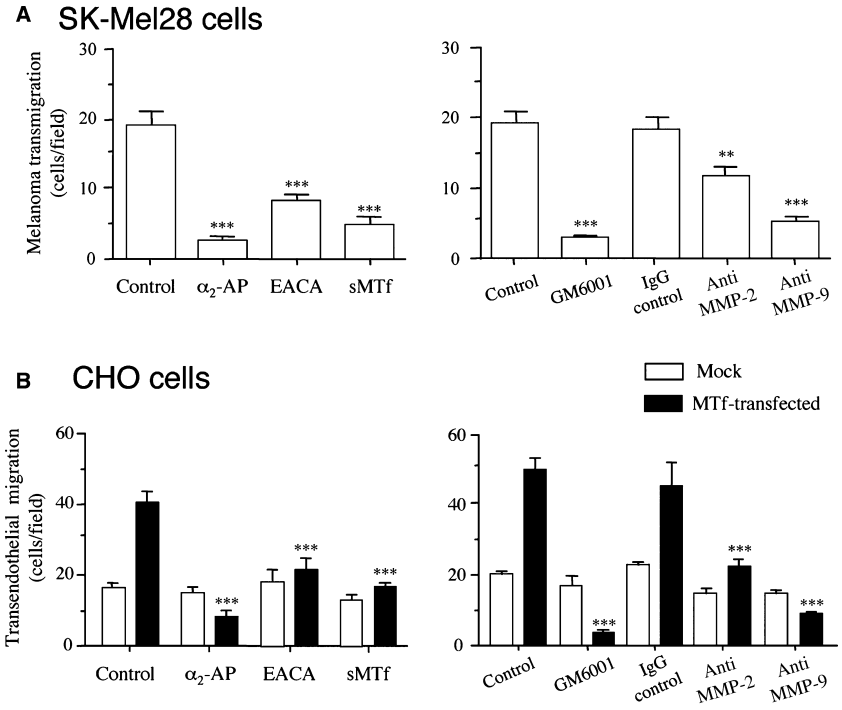

Figure 8. Transmigration of MTf-expressing cells across the BBB in vitro involves the participation of plasminogen and MMP activation systems. Transendothelial migration of melanoma SK-Mel 28 cells (A), as well as of Mock and MTf-transfected CHO cells (B) across the BBB in vitro model was performed in presence of various protease inhibitors. The plasminogen activation system was studied through the addition of α_2-antiplasmin (α_2AP), epsilon aminocaproic acid and a truncated, soluble form of MTf (sMTf). The involvement of MMPs was determined by using a broad range MMP inhibitor (GM6001) and mAbs directed against human MMP-2 and MMP-9. Transmigration was quantified from 10 different fields per conditions (n = 6) using Northern Eclipse software. Statistically significant differences are compared to the transmigration of CHO MTf-transfected cells in the absence of inhibitors. Data represent the means ± SE.

human melanoma cell lines analyzed for cell-surface expression of MTf by quantitative FACS with mAb L235, SK-Mel 28 and A2058 cells expressed abundantly MTf with 1.5×10^5 and 1.45×10^5 L235 sites/cells (Smith et al., 2006). We already established a correlation between MTf expression and cell invasive potential (Demeule et al., 2003; Michaud-Levesque et al., 2005a). Indeed, results obtained by post-transcriptional gene silencing clearly indicate a relationship among MTf over-expression and melanoma cell transmigration across the BBB in vitro. Furthermore, results from in vivo invasion assay revealed that MTf-transfected cells are more likely to invade mice brains and lungs than Mock cells, suggesting that MTf expression could also facilitate cell invasion in vivo. The participation of MTf in melanoma cells' extravasation to the brain was next confirmed using a metastasis model. After reaching the blood vasculature, tumour cells need to accomplish several steps to successfully invade different tissue from a distant site. The identification of MTf-positive cells within nude mice brains after i.v. injection of SK-Mel 28 cells indicate that melanoma cells not only survived in the bloodstream, but also efficiently penetrated into the brain. Our findings are the first to demonstrate the involvement of MTf in melanoma cell invasion leading to the development of brain metastasis. It has already been suggested that MTf expression could modulate cell proliferation. Although MTf expression in CHO cells did not increase their proliferation (Kennard et al., 1995), in vivo studies using MTf down-regulated melanoma cells resulted in significantly reduced initiation and tumour growth in nude mice (Dunn et al., 2006). Hence, MTf over-expression at the surface of melanoma cells could thus facilitate their transmigration across the BBB and stimulate their proliferation after their invasion in the CNS.

The potent inhibition of MTf-expressing cells transmigration across the BCEC monolayer observed with several inhibitors of the plasminogen activation indicates that plasmin is a key player in MTf-dependent processes. As a plausible plasminogen receptor, membrane-bound MTf may stimulate the conversion of plasminogen into plasmin by recruiting plasminogen for its activation by cell-associated plasminogen activator (Michaud-Levesque et al., 2005a). Recently, the involvement of the fibrinolytic system in promoting melanoma cells to pass through the BBB has been demonstrated (Perides et al., 2006). We also previously reported that MTf could equally stimulate the activation of plasminogen by its tissular (Rolland et al., 2006) and urokinase type activators (uPA) (Demeule et al., 2003). As plasminogen activation acts as a trigger in matrix degradation and cell invasion, the stimulation of plasminogen activation by MTf at the surface of melanoma cells could certainly facilitate their extravasation to the brain. Moreover, interfering with MTf at the surface of tumour cells could regulate crucial steps leading to brain metastasis. Plasmin is an important protease that mediates vessel wall extracellular proteolysis (Stalboerger et al., 2001). In this study, results indicate that the permeability of the BBB to sucrose was temporarily increased during SK-Mel 28 cells transmigration, suggesting that plasmin generated at the surface of melanoma cells could locally degrade the BCEC monolayer and affect the BBB integrity. It has been shown that BBB integrity around small lesions, such as metastases, recovers after the passage of metastatic cells into the parenchyma (Fidler et al., 1999). Considering that BBB permeability to sucrose is restored after the transmigration of melanoma cells, we propose that this process is temporary and reversible.

The results presented in this study showed that sMTf affects the transmigration of MTf-expressing cells across the endothelial cell monolayer in vitro. We previously demonstrated that sMTf inhibits plasminogen binding at the cell surface by interacting with the zymogen in the surrounding media and thus reducing its availability in the vicinity of the cell (Michaud-Levesque et al., 2005a). Indeed, sMTf may regulate the motility of MTf-expressing melanoma cells by interfering with plasminogen activation. The participation of the fibrinolytic system during melanoma cell migration across has already been reported (Perides et al., 2006). Previous studies also reported that tPA and MMP-9 greatly affect the BBB integrity by stimulating perivascular tissue proteolysis (Aoki et al., 2002; Romanic and Madri, 1994; Zhang et al., 2002). Low density lipoprotein receptor-related protein (LRP), a member of the LDL receptor family, was shown to mediate tPA and MMP-9 activity (Boucher et al., 2003; Bu et al., 1992; Hahn-Dantona et al., 2001; Yepes et al., 2003). Thus, in the presence of plasminogen, sMTf binds to the cell surface and undergo LRP-mediated endocytosis into cells (Michaud-Levesque et al., 2007b). In addition, sMTf modulates LRP protein expression in EC (Michaud-Levesque et al., 2005b). Based on these findings, sMTf could therefore target LRP-mediated proteolysis during cell transmigration across the BBB in vitro. Hence, these results raise the interesting possibility that sMTf may associate to LRP at the surface of the brain capillary endothelium and interfere with cancer cell invasion by maintaining the BBB integrity.

Interestingly, a single dose of mAb L235 significantly reduced melanoma brain metastasis in nude mice. Results presented in this study demonstrate that the administration of 20 mg/kg mAb L235 can achieve plasma concentrations that are sufficient to inhibit transmigration of melanoma cell in vitro. mAb L235 was reported to inhibit the binding and subsequent activation of plasminogen at the surface of MTf-expressing cells (Michaud-Levesque et al., 2005a). The binding of mAb L235 to MTf at the surface of melanoma cells could interfere with the activation of plasminogen at the vicinity of the cell and thus reduce their ability to digest the vessel wall matrix and extravasate to the brain. Chemotherapy has a limited role in treating brain metastases

and areas for future research include the prevention of CNS metastasis by targeting circulating cancer cells through mAb-based therapy (Stern and Herrmann, 2005; Waldmann, 2003). Immunohistochemical analysis revealed that 62% of metastatic melanoma tumours had strong staining for MTf (Smith et al., 2006). Our findings suggest that an MTf-specific antibody could be used to prevent or limit the development of brain metastasis from primary melanoma. Overexpression of MTf in melanoma as compared with normal tissue, in conjunction with its implication in cell invasion, supports further evaluation of MTf-specific antibody for targeting MTf-overexpressing tumours. Moreover, MTf targeting by sMTf represents an interesting tool for intervening in tumour cells invasion. Thus, in addition to its inhibitory effects on cell invasion, sMTf treatment was also reported to impede the growth of subcutaneous lung cancer cells (Rolland et al., 2007). Considering that other type of cancer cells over-express MTf, one of which is the human lung carcinoma NCI H460 cell line, the efficacy of MTf-specific mAb and sMTf could also be expanded to other cancers expressing MTf. Overall, the overexpression of MTf at the surface of cancer cells along with its participation in several key steps in tumour progression, make it a promising target in cancer therapy.

In conclusion, we provide clear evidence that MTf over-expression at the surface of melanoma cells facilitates their transmigration across the BBB and promotes their invasion to the CNS in vivo in order to produce brain metastases. In addition, we demonstrate that the transmigration of MTf-expressing cells across the BBB requires the participation of the plasminogen activation system. Overall, our results suggest that MTf expression could be a potential prognostic indicator for the development of brain metastases. Moreover, our findings indicate that membrane-bound MTf may represent an attractive target for the development of new cancer therapeutics.

Materials and methods

Materials

Recombinant truncated sMTf (produced by introducing a stop codon following the glycine residue at position 711 of full-length MTf cDNA) and L235 mAb were kindly provided by Biomarin Pharmaceutical (Novato, CA, USA). Antibodies directed against human MMP-2 and MMP-9 as well as the chemical GM6001 were from Calbiochem (La Jolla, CA, USA). Alexa Fluor 488® conjugated antibodies were purchased from Invitrogen (Burlington, Canada). Chinese hamster ovarian (CHO) cells transfected with full-length human MTf cDNA (p97TRVb CHO cells; referred to as MTf-transfected cells in the text) or with control vector (TRVb CHO cells; referred to as Mock cells in the text) were from Dr Malcom Kennard of the University of British Columbia (Vancouver, Canada). Other biochemical reagents were from Sigma-Aldrich (Oakville, Canada). Specific pathogen-free female athymic Crl:CD-1®-nuBR nude mice and male Crl:CD-1 mice were obtained from Charles River Laboratories (LaSalle, Canada).

Cell culture

Human skin malignant melanoma SK-Mel 28 cells were cultured in minimal essential medium (MEM) supplemented with 1 mM pyruvate and 10% calf serum (CS). Human melanoma cells A101D and A375 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% CS. Human A2058 melanoma cells were cultured in DMEM medium containing 1 mM pyruvate and 10% CS. CHO cells (Mock and MTf-transfected) were maintained in F12 medium containing 1 mM Hepes and 10% CS. Cells were grown at 37°C under 5% CO_2/95% air atmosphere. EC from bovine brain capillaries (BCECs) were cultured in DMEM supplemented with heat-inactivated 10% horse (v/v) and 10% CS, 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor, added every other day. Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck et al., 1992). Briefly, after removing the meninges, the brain tissue was gently forced through an 82-μm nylon sieve. Astrocytes were plated on six-well microplates at a concentration of 1.2×10^5 cells/ml in 2 ml of optimal culture medium (DMEM) supplemented with 10% heat-inactivated foetal CS.

BBB model

The in vitro model of the BBB was established by using a co-culture of BCECs and newborn rat astrocytes as previously described (Dehouck et al., 1992). Briefly, prior to cell co-culture, plate inserts (Millicell-PC 3.0 μm; 30-mm diameter; Millipore, Bedford, MA, USA) were coated on the upper side with rat tail collagen. They were then set in the six-multiwell microplates containing astrocytes prepared as described above, and BCECs were plated on the upper side of the filters in 2 ml of co-culture medium. BCEC medium was changed three times a week. Under these conditions, differentiated BCECs formed a confluent monolayer 7 days later. Experiments were performed 5–7 days after confluence was reached. The number of cells at confluence was 400 000 cells/4.2 cm^2 or 90 μg of protein/4.2 cm^2, as evaluated by a micro-BCA assay (Pierce, Rockford, IL, USA). At this point, the permeability coefficient for sucrose attained 0.3–0.5 cm/min as previously described by Dehouck et al. (1992).

Transendothelial migration assay

Cells from melanoma as well as CHO (Mock and MTf-transfected) cells were trypsinized, incubated for 5 min in 10 μM bisbenzimide (Hoescht 33342) and washed three times in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS) solution. Fluorescent cells (1×10^5 cells) were loaded onto a BCEC monolayer. Transendothelial migration was performed at 37°C under 5% CO_2/95% air atmosphere in fresh medium supplemented with (or lacking) recombinant truncated MTf (sMTf; 100 nM), α_2-antiplasmin (α_2-AP; 150 nM), ε-aminocaproic acid (EACA; 1 μM) and GM6001 (10 μM). The inhibitory effects of monoclonal antibodies directed against MTf (L235; 50 nM) and matrix metalloproteinase-2 or -9 (anti-MMP-2 and anti-MMP-9; 20 μg/ml) were also tested.

After 48 h, cells were washed with Ca^{2+}-Mg^{2+}-free PBS solution and fixed during 30 min in 3.7% paraformaldehyde. Filter tops were cleaned with cotton swabs and cells that had migrated to the other side were visualized and photographed at an original magnification of ×40 using a Retiga 1300 camera attached to a NIKON Eclipse TE2000-U microscope (Nikon; Mississauga, Canada). The quantification of transmigrated cells was performed using Northern Eclipse software (Empix Imaging, Mississauga, Canada) by counting at least 10 random fields for each filter.

Post-transcriptional gene silencing

Transfection of SK-Mel 28 and A2058 cells with siRNA was performed as suggested in the standard procedure from Qiagen

(Mississauga, Canada). Shortly before transfection, SK-Mel 28 cells were seeded onto 60-mm dishes (1×10^5 cells/dish) in complete fresh medium at 37°C under a 5% CO_2/95% air atmosphere. Non-specific (siCTL) or MTf-specific siRNA (siMTf; gene accession number M12154): sense r(GGGCGAAGUGUACGAUCAA)dTdT and antisense r(UUGAUCGU ACACUUCGCCC)dAdC were diluted in serum-free culture medium to obtain a final concentration of 50 nM and mixed with HiPerFect Transfection Reagent (Qiagen). The mixture was mixed and added drop-wise onto the cells. Cells were then incubated with siRNAs during 48 h at 37°C under a 5% CO_2/95% air atmosphere.

Western blot analysis

CHO cells (Mock and MTf-transfected), SK-Mel 28 (transfected or not with siRNA) and A375 melanoma cells were solubilized on ice in a lysis buffer (Michaud-Levesque et al., 2005b). Proteins were separated by SDS–PAGE and blotted onto a polyvinylidene difluoride membrane (PVDF) (Perkin-Elmer Life Sciences, Boston, MA, USA). Following transfer, the immunodetection of MTf was performed using mAb L235 (which recognizes a conformational epitope on human MTf). The immunodetection of GAPDH was used as a loading control. Proteins were quantified by laser densitometry using a Chemilmager™ 5500 from Alpha Innotech Corporation (San Leandro, CA, USA).

RNA isolation and real-time PCR analysis

Total RNA from SK-Mel 28 and A2058 cells was extracted using TRIzol™ reagent from Invitrogen and quantified using a spectrophotometer. Reverse-transcription reactions were performed using 1 μg of total RNA and a High-Capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA). The transcript expression of MTf and 18 S RNA were studied using 1 μg and 25 ng of cDNA, respectively. Real time-PCR reactions were performed using the IQ™ SYBR green Supermix and iQ5™ Real Time Detection System from Bio-Rad Laboratories (Mississauga, Canada). Specific primers used in this study were designed with MacVector™ software (MacVector Inc.; Cary, NC, USA) according to cDNA sequences deposited at the NCBI data bank. Specific human MTf primers used are as follows: 5′-CCCTAAGTGGCGAGGACATTTAC-3′ and 5′-TCATCCAAGGTGAAGGCGTG-3′. The relative quantification was normalized to the 18S ribosomal subunit gene expression level (5′- CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCG GCT-3′).

In vivo invasion assay

The in vivo invasion assay was based on the method of Muto (Muto et al., 2003) with minor modifications (Bertrand et al., 2007). All animal studies were conducted in accordance with recommendations from the Canadian Council on Animal Care (CCAC) for care and use of experimental animals. Briefly, CHO Mock and MTf-transfected cells were incubated with [^{3}H]-thymidine for 48 h and injected (5×10^5 cells) into the tail vein of 5 to 10-week-old male Crl:CD-1 mice. After 24 h, mice were anesthetized with ketamine/xylazine (120/10 mg/kg i.p.) and the heart was exposed by opening the thorax. A 18-gauge cannula with a sharp wedge was inserted into the left ventricle of the heart and mice were perfused for 10 min with Ringer/Hepes buffer. The posterior vena cava was cut, allowing the fluid to exit. The perfusion syringe was driven by a Harvard pump (model PHD 2000; Harvard Apparatus, Saint-Laurent, Canada) at the rate of 2 ml/min. Afterward, the organs were removed, minced and solubilized with Solvable™ (Perkin Elmer; Woodbrige, Canada) for 18 h at 37°C. The amount of [^{3}H] was measured using liquid scintillation spectrometry.

Pharmacokinetic parameters determination

Monoclonal antibody L235 was radio-iodinated by standard procedures using Na-[^{125}I] (Amersham Pharmacia Biotech, Baie D'Urfé, Canada) and an Iodo-beads kit from Pierce Chemical Co. (Rockford, IL, USA). Female athymic Crl:CD-1®-nuBR nude mice (aged 5-10 weeks) received a single dose of 20 mg/kg mAb [^{125}I]-L235 in a saline solution by i.p. administration (100 μl; 33 μM; 3.5×10^5 CPM). At the allotted times, blood samples were withdrawn from the tail vein into a heparinized Microvette CB 300 LH container (Sarstedt, Nümbrecht, Germany). The concentration of mAb L235 was quantified by measuring plasma-associated radioactivity using a gamma counter. Peak plasma concentration (C_{max}), time of peak plasma concentration (t_{max}), elimination half-life ($t_{1/2}$), area under the concentration–time curve (AUC), volume of distribution (Vd) and the clearance (CL) were calculated using the software program PK Solutions 2.0 (Montrose, CO, USA).

Metastasis assay

Human melanoma SK-Mel 28 cells were harvested and resuspended in complete culture medium. Cells were washed three times with Ca^{2+}-Mg^{2+}-free PBS solution and the final pellet was resuspended to obtain 1×10^6 cells/50 μl. Specific pathogen-free, female athymic Crl:CD-1®-nuBR nude mice (aged 5–10 weeks) were injected in the tail vein with 1×10^6 SK-Mel 28 cells. After 24 weeks, mice brains were removed and their content of MTf-expressing cells was analyzed using flow cytometry. To evaluate the effect of mAb L235 in melanoma brain metastasis, IgG_1 or mAb L235 (20 mg/kg) were administered i.p. 6 h before the injection of melanoma tumour cells into nude mice.

FACS analysis

Mice brains from the in vivo metastasis assay were dissected and homogenized on ice in Ringer/Hepes buffer containing 0.5% bovine serum albumin (BSA) with a glass homogenizer. Samples were then incubated with collagenase A (1 mg/ml) for 45 min at 37°C under a 5% CO_2/95% air atmosphere with agitation. Cell suspensions were passed through 180-μm and 30-μm Nitex filters. The pellets were washed three times in a Ca^{2+}-Mg^{2+}-free PBS solution containing 0.5% BSA and 2 mM ethylenediamine-tetraacetic acid (EDTA). The final pellets (1×10^6 cells) were resuspended in a binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated at 4°C for 30 min with 1 μg/ml of either mAb L235 or IgG_1 control isotype. Cells were washed twice and incubated at 4°C for 30 min with 1 μg/ml goat anti-mouse IgG Alexa Fluor 488 conjugated antibody. Cells (50 000 events) were then analyzed using flow cytometry on a Becton Dickinson FACS Calibur flow cytometer equipped with CellQuest Pro software (BD Bioscience; Mississauga, Canada). The fluorescence of cell population expressing human membrane-bound MTf was corrected for the background fluorescence intensity measured in the presence of a non-specific IgG_1 and was expressed as mean fluorescence intensities. The specificity of the signal associated with MTf was verified by adding 10 μg/ml of the truncated sMTf during primary antibody incubation.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test using GraphPad Prism (San Diego, CA, USA). The resulting P-values designate the level of significance and are designated by *for $P < 0.01$, **for $P < 0.005$ and ***for $P < 0.001$. When applicable, results are presented as mean ± SE.

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