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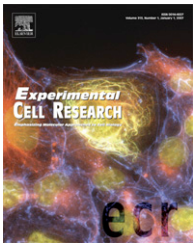

Research Article

Inhibition of tumor growth by a truncated and soluble form of melanotransferrin

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ABSTRACT

Melanotransferrin is a glycoprotein expressed at the cell membrane and secreted in the extracellular environment. Recombinant truncated form of membrane-bound melanotransferrin (sMTf) was reported to exert *in vitro* anti-angiogenic properties. Here we show that sMTf treatment leads to a 50% inhibition of neovascularization in Matrigel™ implants when stimulated by growth factors. Using a glioblastoma xenograft model, we demonstrate that sMTf delivery at 2.5 and 10 mg/kg/day by micro-osmotic pump inhibits tumor growth by 73% and 91%, respectively. In a lung carcinoma xenograft model, sMTf treatment at 2.5 and 10 mg/kg/day impeded tumor growth by 87% and 97%. Furthermore, subcutaneous glioblastoma and lung carcinoma tumors from mice treated with 10 mg/kg/day of sMTf present insignificant growth toward the study. In association with a reduction in endoglin mRNA expression, the hemoglobin content decreased by half in sMTf-treated glioblastoma tumors. *In vitro* experiments revealed that NCI-H460 cells treated with sMTf display an inhibition in their invasive capabilities with a concomitant reduction in the expression of the low-density lipoprotein receptor protein and urokinase plasminogen activator receptor. Altogether, our results demonstrate that sMTf exerts anti-cancer and anti-angiogenic activities, suggesting that its administration may provide novel therapeutic strategies for the treatment of cancer.

Introduction

First identified in human melanoma, melanotransferrin (MTf) is a glycosylated protein that was found to play an important role in numerous processes associated with tumor development and angiogenesis [1–4]. Currently, two distinct forms of MTf have been identified, either associated to the cell membrane by a glycosyl phosphatidylinositol anchor (mMTf) or secreted into the extracellular environment. While the secreted form of MTf is present at relatively low concentrations in human plasma (33 pM) [5], mMTf is reported to be expressed in many tissues and cell lines [6–8]. In a previous study, mMTf was reported to bind plasminogen and facilitate its conversion into plasmin at the cell surface leading to an increase in the motility, migration and invasion of Chinese hamster ovary cells overexpressing MTf [4]. Moreover, it was recently demonstrated that down-regulation of MTf mRNA by post-transcriptional gene silencing in SK-Mel-28 melanoma cells resulted in the inhibition of proliferation, migration, tumor

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growth and metastasis [9,10]. A recombinant truncated form of mMTf (sMTf) was proposed to exert anti-angiogenic properties since it reduces endothelial cell (EC) migration and capillary-like structures formation by interacting with the plasminogen activation system [1,2,11]. Altogether, these results suggest the involvement of an important balance between mMTf and sMTf in various mechanisms related to plasminogen activation.

Of all malignancies, lung cancer is the leading cause of cancer-related mortality [12]. Of newly diagnosed patients with lung cancer, 75–80% are defined as non-small cell lung carcinoma (NSCLC) and roughly 20% as small-cell lung cancer. According to the National Cancer Institute, NSCLC can be divided in three subtypes: squamous cell carcinoma, adenocarcinoma and large lung carcinoma. Recent clinical trials have demonstrated that the benefit of combined chemotherapy among the fittest patients with advanced NSCLC is marginal and that only patients who undergo curative surgery have a significant potential for cure [13]. Another type of cancer much less frequent but reaching over 18,000 new cases per year in the U.S. is multiform glioblastoma [14]. Although these tumors are resistant to both radiotherapy and chemotherapy, the established treatment for patients diagnosed with malignant gliomas currently involves a combination of surgery, radiation and chemotherapy [15]. Thus, novel treatment strategies are needed to improve the clinical management of these diseases.

Tumor growth over 1–3 mm^3 requires the development of newly formed vessels from pre-existing vasculature [16,17]. The angiogenic process is regulated by a balance between stimulatory and inhibitory factors, which may be released by the tumor itself or by the surrounding tissues. The urokinase-type plasminogen activator receptor (u-PAR) is considered a key element in EC proliferation and migration since the activation of plasminogen at the cell surface is tightly controlled by the internalization and degradation of activator/inhibitor complexes by the u-PAR/LRP system. The members of the low-density lipoprotein (LDL) receptor family are a group of cell surface receptors that transport a large number of macromolecules into cells. This receptor includes binding sites for numerous ligands, including complexes of plasminogen activator inhibitor-1 (PAI-1) with urokinase- (u-PA) or tissue-type plasminogen activator (t-PA) [18] and receptor-associated protein (RAP) [19]. Due to the broad spectrum of ligands for LRP, it has been suggested that this receptor functions in many processes including lipoprotein metabolism, hemostasis, cellular growth and tissue remodeling.

We previously reported that sMTf could inhibit migration of cancer and endothelial cells expressing MTf as well as differentiation of endothelial cells into capillary-like structures *in vitro* [2]. These results led us to further investigate the effects of sMTf on tumor growth and related angiogenesis *in vivo*. Results presented here clearly demonstrate that sMTf inhibits the growth of subcutaneous tumors derived from U-87 MG glioblastoma cells and NCI-H460 large lung carcinoma cells. In addition, sMTf treatment reduced the angiogenesis associated with the development of subcutaneous glioblastoma. Our data suggest that sMTf treatment might exert its anti-cancer and anti-angiogenic properties in MTf-expressing cells by affecting key players involved in plasmin activity such as u-PAR and LRP.

Materials and methods

Materials

Recombinant human melanotransferrin (sMTf) (produced by introducing a stop codon following the glycine residue at position 711) and L235 monoclonal antibody (mAb) were kindly provided by Biomarin Pharmaceutical (Novato, CA).

Cell culture

Human multiform glioblastoma cells (U-87 MG) were grown in minimal essential medium (MEM) containing 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). Human large lung carcinoma cells (NCI-H460) were cultured in RPMI 1640 medium supplemented with 2.5 g/L D-glucose, 1 mM pyruvate, 10 mM HEPES and 10% calf serum (CS). Human dermal microvascular endothelial cells (HMEC-1) (Center for Disease Control and Prevention; Atlanta, GA) were cultured as previously described [2]. Chinese hamster ovary (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, BC). CHO cells were maintained in F12 medium containing 1 mM HEPES and 10% CS. Cell lines were cultured at 37 °C under 5% CO_2/95% air atmosphere.

In vivo Matrigel™ plug angiogenesis assay

The Matrigel™ implantation assay was based on the method of Passaniti et al. [20]. Angiogenesis is considered to be indicated by the growth of blood vessels from subcutaneous tissue into a solid piece of Matrigel™. Specific pathogen-free, female Crl:CD-1®-nuBR nude mice were obtained from Charles River Laboratories (Lasalle, QC). All of the mice used were 5–10 weeks of age. All animal studies were conducted in accordance with recommendations from the Canadian Council on Animal Care (CCAC) for care and use of experimental animals. Prior to injection, heparin was incubated with or without bFGF (250 ng/mL) or $VEGF_{165}$ (200 ng/mL) for 5 min then diluted into phenol red-free Matrigel™ on ice for a final concentration of 0.0025 units/mL heparin. Then, mice were subcutaneously injected under anesthesia in the ventral midline region of the right flank with 0.5 mL of Matrigel™ alone or with Matrigel™ containing bFGF or VEGF. After 7 days, mice were sacrificed and Matrigel™ implants were harvested, washed with PBS and photographed with a Nikon Coolpix™ 5000 digital camera. The remaining implants were immediately frozen and lyophilized overnight in order to evaluate the hemoglobin (Hb) concentrations.

Testing of anti-angiogenic substances was initiated at the time of Matrigel™ implantation. Systemic treatment with repeated injection of sMTf (5.0 mg/kg) was performed by subcutaneous injection at days 0, 2, 4 and 6 post-implantation. A Ringer/HEPES solution was used for control treatment in all experiments.

Subcutaneous xenograft model

For tumor cell inoculation in nude mice, U-87 MG or NCI-H460 cells were harvested by trypsinization using a trypsin/EDTA solution. Cells were washed three times with phosphate-buffered saline Ca^{2+} – Mg^{2+} free (PBS-CMF) and centrifuged. The resulting pellet was resuspended in 1% methylcellulose in serum-free MEM at a concentration of 2.5×10^6 cells per 100 μL. Animals were anesthetized by O_2/isoflurane inhalation and tumors were established by subcutaneous injection of 100 μL from cell suspension into the right flank of female Crl:CD-1®-nuBR nude mice. At 3 days after cell inoculation, animals were randomly separated in three groups. Alzet micro-osmotic pumps (model 1007D-1002; Durect Corporation) filled with Ringer/HEPES (R/H) or sMTf were implanted in the left flanks of O_2/isoflurane anesthetized mice. Prior to the surgery, pumps were incubated in a NaCl solution (0.9%) at 37 °C for 16 h and filled following the supplier's directions.

Tumors were measured in O_2/isoflurane anesthetized mice every 3 days using a digital caliper and tumor volume was calculated as: $\pi/6 \times \text{length} \times \text{width}^2$. Tumor volume measurements less than initial values define partial tumor regression in sMTf-treated mice. When the first mouse from control group held a tumor reaching 1000 mm^3, tumors were photographed and mice from every group were sacrificed. Experiments were performed on 8 animals for each group. Tumors were then cut in equal parts for the quantification of hemoglobin and RT–PCR. Recombinant sMTf treatment toxicity was evaluated by monitoring the body weight of control and treated mice, considering that a weight loss > 20% results in a toxic effect. A brief necropsy was also performed to assess the toxicity to other organs.

Hemoglobin quantification

Matrigel™ implants or tumor sections were washed twice with PBS–CMF and freeze-dried for 24 h. Samples were then crushed and resuspended in 0.1% Triton X-100. Samples were mixed frequently for 1 h and centrifuged at 14,000 × *g* for 15 min to remove particles. The concentration of hemoglobin (Hb) in the supernatant was then determined directly by measuring the absorbance at 405 nm using a Spectramax Plus spectrophotometer (Molecular Devices) and compared with a standard curve of purified Hb (Sigma-Aldrich, Oakville, ON). Hemoglobin content was expressed in μg Hb/mg of dried sample.

In vivo metastasis assay

The metastasis assay was based on the method of Muto [21] with minor modifications. Briefly, CHO Mock and MTf-transfected cells were incubated with [^{3}H]-thymidine for 48 h. After the metabolic labeling, 5×10^5 cells were injected into the tail vein of 5- to 10-week-old male Crl:CD-1 mice. After 24 h, whole body perfusion was performed with PBS–CMF for 15 min. Then, organs were removed and solubilized with Solvable™ for 48 h at 37 °C. The amount of [^{3}H]-thymidine labeled CHO cells was measured by liquid-scintillation spectrometry. Results are expressed as the amount of labeled cells by mg of tissue.

Cell migration assay

Cell migration was performed using Transwell filters (Costar, 8-μm pore size) precoated (top and bottom) with 0.15% gelatin as previously described [1]. Briefly, 1×10^4 cells were resuspended in 100 μL of fresh medium with or without sMTf (100 nM) and added to the upper chamber of each Transwell. Medium in the lower chamber contained 10% serum with or without sMTf. After 18 h of migration, cells were fixed, stained and visualized at a 100 × magnification using a Nikon Coolpix™ 5000 digital camera (Nikon Canada, Mississauga, ON) fixed to a Nikon TMS-F microscope. Migrated cells were quantified by counting at least 5 random fields per filter using Northern Eclipse software (Empix Imaging, Mississauga, ON).

Cell invasion assay

Tumor cell invasion was assessed using Transwell filters (Costar; 8-μm pore size) precoated with 50 μg air-dried Matrigel™ matrix (BD Bioscience, Mississauga, ON) as described in [4]. Cells were resuspended in serum-free medium with or without recombinant sMTf (100 nM) and added to the upper chambers of the Transwells. The lower chambers contained 10% serum as well as recombinant sMTf. After 48 h, invading cells were fixed, stained and quantified as described through the cell migration assay.

Western blot analysis

Cells (HMEC-1, NCI-H460 and U-87 MG) were incubated with sMTf (100 nM) during 18 h at 37 °C under a 5% CO_2/95% air atmosphere. Treated cells were washed twice with PBS–CMF and solubilized on ice in lysis buffer [2]. Proteins were separated by SDS–PAGE and blotted onto a polyvinylidene difluoride membrane. Following transfer, the immunodetection of LRP-α (clone 8G1; Research Diagnostics Inc.), u-PAR (3937; American Diagnostica Inc.), MTf (mAb L235) and GAPDH (RGM2; Advanced Immunochemical Inc.) were performed using enhanced chemiluminescent reagents (Perkin Elmer).

Reverse transcriptase–polymerase chain reaction

HMEC-1 cells were exposed to fresh medium containing 100 nM sMTf for 18 h at 37 °C under a 5% CO_2/95% air atmosphere. Total RNA was extracted using TRIzol™ reagent from Invitrogen (Burlington, ON). RT–PCR reactions were performed using SuperSript™ One-Step RT–PCR from Invitrogen. Specific primers used in this study were designed by MacVector™ software using human cDNA sequences deposited at the NCBI data bank. The upstream and downstream primers used are described in [2] and as follows: for MTf: 5′-CCTAAGTGGCGAGGACATTTACAC-3′ and 5′-TAGTTCTTGGGGTTGTTCACGGGC-3′, CD105: 5′-CCTTTGGTG-CCTTCCTGATTG-3′ and 5′-TGTTTGGTTCCTGG-GACAAGTTC-3′, for CD31: 5′-TATCCAAGGTCAGCAGCATCGTGG-3′ and 5′-GGGTTGTCTTTGAAT ACCGCAG-3′ and for β-tubulin: 5′-CTGGCACCACACCTTCTACAATG-3′ and 5′-AATGTC ACGCAC-GATTTCCCGC-3′. RT–PCR conditions were optimized so that gene products were obtained during the exponential phase of amplification. Gene product amplifications were performed for 35 cycles of PCR [94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min]. RT–PCR was also performed using RNA extracted from subcutaneous U-87

MG-derived tumors previously excised from mice. In this case, gene product amplifications were performed for 35 cycles of PCR [94 °C for 30 s, 60 °C for 30 s (55 °C for CD31), 72 °C for 1 min]. Amplification products were electrophoresed on 2% (w/v) agarose gels and visualized under UV light following staining with ethidium bromide.

Statistical analysis

Experimental data were evaluated by Student's *t*-test using GraphPad Prism (San Diego, USA). The resulting *P*-values designate the level of significance. Tumor growth inhibition is determined from median tumor volumes in mm^3 from the treated and control groups. Time points used to determine the median tumor volumes were designated as the first day of treatment and the day when the first mouse from the control group held a tumor of 1000 mm^3.

Results

sMTf treatment inhibits bFGF- and VEGF-induced neovascularization

The recombinant truncated and soluble form of mMTf (sMTf) was previously reported to reduce endothelial cell (EC) migration and differentiation into capillary-like structure without affecting EC proliferation [1,2,11]. Hence, we first confirmed the effects of sMTf on EC migration. Fig. 1A shows that 100 nM sMTf decreased by half the migration of HMEC-1 cells on gelatin. We then evaluated the capacity of sMTf to interfere with angiogenesis stimulation by various growth factors. Malignant tumors develop their own vascular networks by secreting growth factors which stimulate endothelial migration and proliferation [22,23]. The Matrigel™ plug neovascularization assay allows evaluation of the angiogenesis levels by measuring the hemoglobin content in the implant. The factor-free Matrigel™ implants showed barely apparent pink hue coloration. However, the addition of bFGF and VEGF to Matrigel™ induced strong neovascularization (Fig. 1B) and an increase in hemoglobin content of about three- to four-fold higher than in the control Matrigel™ samples (Fig. 1C). In mice treated with sMTf, the stimulation of neovascularization by bFGF and VEGF was diminished by 50% as judged by the corresponding hemoglobin content (Fig. 1C). These data suggest that sMTf interferes with bFGF- and VEGF-induced angiogenesis.

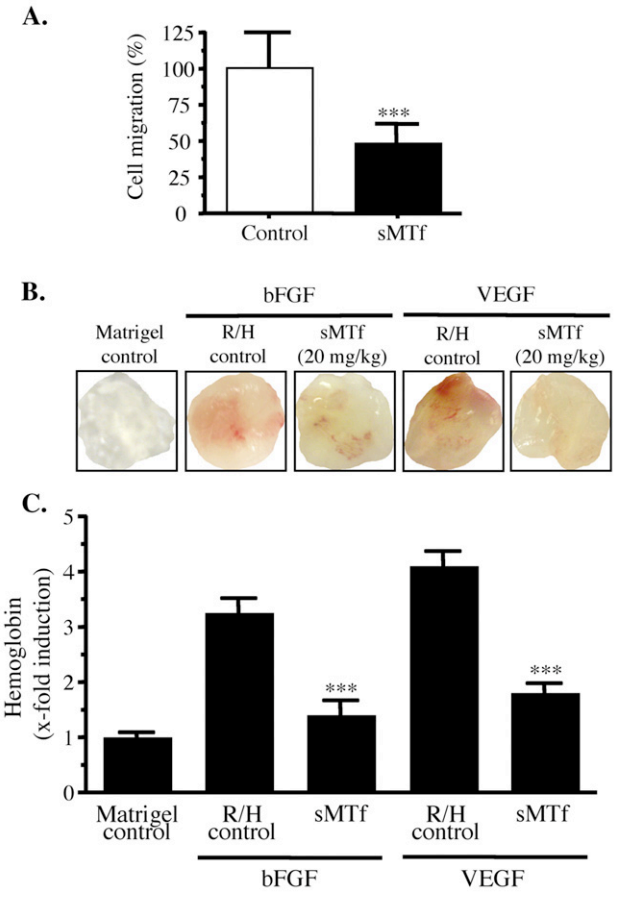

Fig. 1 – Inhibition of bFGF- and VEGF-induced neovascularization by sMTf treatment. (A) The migration of HMEC-1 endothelial cell was performed in presence and in absence of sMTf (100 nM) during 18 h as described in Materials and methods. (B) bFGF- and VEGF-induced Matrigel™ plug *in vivo* angiogenesis assay in nude mice treated for 7 days with repeated subcutaneous injection of either Ringer/HEPES (R/H) control solution or sMTf (20 mg/kg/week) as described in Materials and methods. Photos obtained from representative samples are shown. (C) Relative Hb content from Matrigel™ implants containing or lacking bFGF and VEGF in nude mice treated for 7 days with subcutaneous administration of recombinant sMTf. Results are expressed as *x*-fold induction of angiogenesis as compared to Matrigel™ control conditions. $n = 7$ for all conditions and the means ± S.E. are shown. Statistically significant differences compared to R/H control conditions are indicated by * for $p < 0.001$ (Student's *t*-test).**

sMTf reduces the growth of glioblastoma and lung carcinoma subcutaneous tumors

Considering the results obtained in the neovascularization of Matrigel™ implants, the impact of sMTf administration on xenografts tumor growth was further investigated. U-87 MG and NCI-H460 cells were inoculated into the right flank of nude mice whereas Alzet osmotic micro-pumps containing sMTf or Ringer/HEPES control solution were implanted in their left flank. In the glioblastoma model, control mice showed a tumor growth upon the fifth day of treatment, while mice treated with sMTf (2.5 mg/kg/day) display a tumor growth at 18 days of treatment. Interestingly, tumors from mice treated with sMTf 10 mg/kg/day do not present significant growth toward the study (Fig. 2). Indeed, we observed a 41.3% regression in tumor volume for 3 out of 8 mice treated with sMTf 10 mg/kg/day. After 18 days of treatment, i.e. when the first tumor of control mice reached 1000 mm^3, the difference in tumor size between control and sMTf-treated mice was significant. We were able to determine a tumor growth inhibition of 73.8% and 91.8% in sMTf-treated mice at 2.5 and 10 mg/kg/day, respectively (Table 1). In the lung carcinoma model, sMTf treatment (2.5 mg/kg/day) inhibits tumor growth

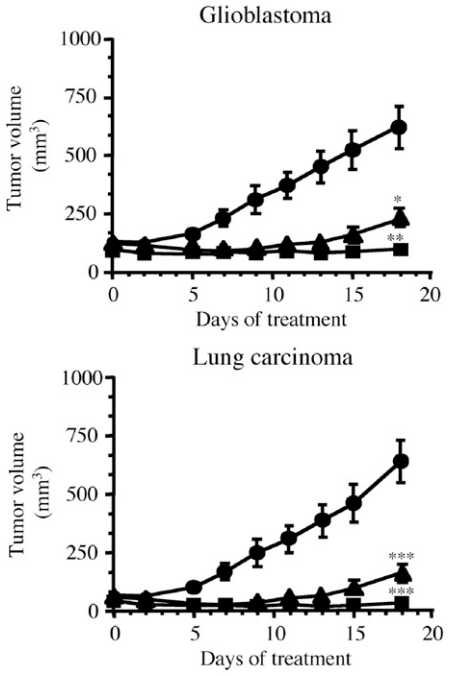

Fig. 2 – Inhibition of tumor growth by sMTf. Nude mice were subcutaneously inoculated with U-87 MG glioblastoma cells and NCI-H460 large lung carcinoma cells. At 3 days after cell inoculation, animals were randomly separated in three groups and Alzet micro-osmotic pumps filled with Ringer/HEPES control solution (●) or sMTf at 2.5 (▲) and 10 mg/kg/day (■) were implanted as described in Materials and methods. Experiments were performed on eight different animals for all conditions and the means ± S.E. are shown. Statistically significant differences in tumor volumes are indicated by ** for $p < 0.005$ and * for $p < 0.001$ (Student's *t*-test) when compared to tumors from the control group.**

for a 15-day period. After 18 days of treatment, mice treated with sMTf at 2.5 and 10 mg/kg/day have tumors with average volumes reaching 140 and 65 mm^3 respectively, compared to 642 mm^3 for tumors in the control group (Table 1). Furthermore, tumor growth inhibition was sustained for 18 days in mice treated with 10 mg/kg/day of sMTf (Fig. 2) and complete remission was observed in one case. Seven out of 9 mice treated with sMTf 10 mg/kg/day showed a regression in tumor size of about 50% when compared to the initial tumor volume. The corresponding tumor growth inhibition values reached 87.6% and 97.2% in mice treated with sMTf at 2.5 and 10 mg/kg/day, respectively (Table 1). Additionally, tumor growth inhibition by sMTf at 10 mg/kg/day is significantly different from that obtained at 2.5 mg/kg/day in both subcutaneous xenograft models. sMTf can thus be considered as an active antitumor protein. In both cases, sMTf treatment had no significant effect on mouse body weight and a gross necropsy revealed no physiological modifications. Results from molecular stability demonstrated that sMTf is stable at 37 °C for 28 days without the identification of any low molecular weight fragments by SDS–PAGE analysis (data not shown).

sMTf inhibits angiogenesis in subcutaneous glioblastoma development

In order to demonstrate that sMTf induced a reduction in tumor growth by interfering throughout the angiogenic process, Hb content was quantified in subcutaneous tumors derived from U-87 MG and NCI-H460 cells. The results demonstrated that Hb content was 2-fold higher in U-87 MG than in NCI-H460 tumors (Fig. 3A), indicating that angiogenesis in subcutaneous NCI-H460-derived tumor is much lower than in U-87 MG-derived ones. This led us to evaluate angiogenic development in U-87 MG-derived tumors, rather than in the lung carcinoma model. As soon as the first tumor from the control group reached 1000 mm^3, tumors were excised and the Hb content was measured. Fig. 3B demonstrates that sMTf treatment resulted in a reduction of Hb levels at both doses by approximately 50%. Additionally, mRNA expression of CD105, an endothelial cell marker from newly formed vessels, was greatly down regulated in sMTf-treated U-87 MG-derived tumors (Fig. 3C). However, the mRNA expression of another endothelial cell marker, CD31 or PECAM, was unaffected by sMTf treatments when compared to the loading control β-tubulin (Fig. 3C). Western blot analysis

Table 1 – Inhibition of tumor growth by sMTf

Tumor origin	sMTf dosage (mg/kg/day)	Volumes (mm^3)		Tumor growth inhibition (%)	Partial tumor regression (9–11 days)
		Initial	Final		
Glioblastoma	0	88 ± 32	591 ± 229	0	0/8
	2.5	92 ± 33	229 ± 120	73.8	1/8
	10	63 ± 7	104 ± 25	91.8[a]	3/8
Lung carcinoma	0	71 ± 29	642 ± 240	0	0/9
	2.5	69 ± 26	140 ± 69	87.6	5/9
	10	49 ± 15	65 ± 32	97.2[a]	7/9

Tumor cells from human glioblastoma (U-87 MG) and lung carcinoma (NCI-H460) were inoculated subcutaneously in Crl:CD-1®-nuBR nude mice. After 3 days, micro-osmotic pumps containing Ringer/HEPES control solution or sMTf were implanted as described in Materials and methods. The activity end points used to determine tumor inhibition is the median tumor volumes in mm^3 of the treated control groups. Median tumor volumes were determined on the first day treatment and at the end of the study – i.e. when the first mouse of the control group held a tumor of 1000 mm^3. Tumor growth inhibition by sMTf is expressed as percent of control. Animals bearing tumor with a final volume below its initial one are defined here as partial tumor regression.

[a] Results are significantly different when compared to sMTf 2.5 mg/kg dose, $p < 0.01$.

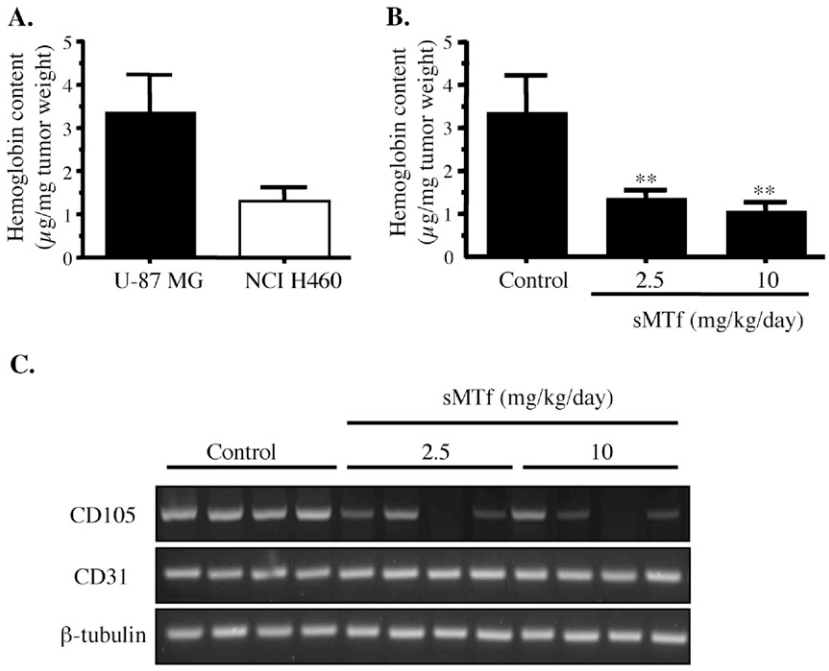

Fig. 3 – sMTf treatment reduces angiogenesis in vivo. Nude mice bearing tumors derived from U-87 MG and NCI-H460 cells were treated using Alzet micro-osmotic pumps containing with Ringer/HEPES control solution or sMTf (2.5 and 10 mg/kg). (A) Control tumors derived from U-87 MG and NCI-H460 cells with a volume of around 1000 mm^3 were excised and the hemoglobin contents were determined as described in Materials and methods. (B) When the first U-87 derived tumor from the control group reached 1000 mm^3, control and sMTf-treated tumors were excised and lyophilized to evaluate their hemoglobin content. (C) mRNA expression of CD105 (endoglin) and CD31 (PECAM) were also assessed on U-87 MG-derived tumors treated or not with sMTf (2.5 and 10 mg/kg). RT–PCR analysis of β-tubulin was used as a loading control. Experiments were performed on eight different animals for all conditions and the means ± S.E. are shown. Statistically significant differences between hemoglobin contents are indicated by ** for $p < 0.005$ (Student's *t*-test) when compared to the hemoglobin content in tumors from the control group.

also revealed that CD31 protein expression in U-87 MG-derived tumors was unchanged after sMTf treatments (data not shown). These results are in agreement with recent studies reporting that CD105 is a better marker than CD31 for newly formed vessels in glioblastoma [24]. In conclusion, the hemoglobin content and CD105 mRNA levels (even though semi-quantitative) in U-87 MG-derived tumors suggest that sMTf exerts an anti-angiogenic activity during the development of subcutaneous glioblastoma.

Since angiogenesis is strongly induced by cytokines from tumor cells, levels of bFGF and VEGF mRNA were studied in tumor tissue derived from U-87 MG cells. The mRNA expression of these growth factors was unchanged in subcutaneous glioblastoma tumors treated with sMTf when compared to tumor tissue from the control group (data not shown). These results indicate that although sMTf inhibits bFGF- and VEGF-induced angiogenesis, the inhibition of angiogenesis by sMTf is not the result of a negative regulation in the expression of these growth factors in U-87 MG-derived tumors.

MTf expression is associated with cell invasion in vivo

MTf was previously demonstrated to be involved in cell migration and invasion *in vitro* [2]. More recently, it was reported that MTf overexpression in melanoma cells contributes to tumor progression as well as cell invasion [9,10]. In order to determine the impact of MTf expression in tissue invasion, an *in vivo* metastasis assay was performed. The transfection of CHO cells with full-length human MTf cDNA led to the detection of a band around 97 kDa by Western blot analysis corresponding to MTf (Fig. 4A). This band is effectively lacking in CHO cells transfected with the control vector, referred as Mock control cells. CHO cells (Mock control and MTf-transfected) were then metabolically stained with [^{3}H]-thymidine and injected into the tail vein of CD-1 mice. After 24 h, the accumulation of labelled cells in several organs was quantified by liquid-scintillation spectrometry (Fig. 4B). It appears that the presence of [^{3}H]-thymidine-labelled CHO MTf-transfected cells is increased by 8-, 10- and 6-fold in the brain, lung and kidney, respectively. The accumulation of CHO cells in the heart was not modulated by the expression of MTf. Meanwhile, the accumulation of CHO cells (Mock control and MTf-transfected) was barely detectable in the liver (Fig. 4B). Results show that MTf overexpression in CHO cells is associated to brain, lung and kidney invasion. These results also indicate that MTf is a key player during cell invasion *in vivo* and that its expression might contribute to metastasis formation.

Effects of sMTf on tumor cell-invasive capabilities

We already established that sMTf could modulate the generation of plasmin through the reduction of the LRP-mediated u-PAR

endocytosis at the surface of ECs [2]. Considering that plasmin is involved in cell migration and invasion, we then investigated the effects of sMTf on tumor cells' migratory abilities. Figs. 5A–B indicates that the presence of sMTf reduced by 30% and 70% the migration and the invasion of NCI-H460 large cell lung carcinoma cells. Nevertheless, the invasive capabilities of U-87 MG glioblastoma cells were unaffected by sMTf (Figs. 5C–D). The proliferation of NCI-H460 and U-87 MG cells measured by [^{3}H]-thymidine incorporation were unaffected by sMTf treatment (data not shown).

sMTf was reported to reduce the protein expression of LRP and u-PAR in endothelial cells [2]. We decided to extend this analysis to U-87 MG and NCI-H460 cells. While LRP and u-PAR protein expression were unchanged in sMTf-treated U-87 MG glioma cells, the same treatment induced a significant reduction in the expression of both receptors in NCI-H460 large lung carcinoma cells. In fact, LRP and u-PAR could not be detected in these cells after sMTf treatment, as shown in Fig. 6A. In agreement with prior results, the treatment of HMEC-1 endothelial cells with sMTf also resulted in a great reduction of both LRP and u-PAR protein expression when compared to the loading control GAPDH (Fig. 6A). It is to note that mMTf expression was unaffected by sMTf in NCI-H460 and HMEC-1 cells (Fig. 6A–B). In addition, endogenous mMTf levels were under the detectable limit in U-87 MG cells.

Several members of the LDLR family, including LRP, are involved in the regulation of u-PA/u-PAR activity. Even though

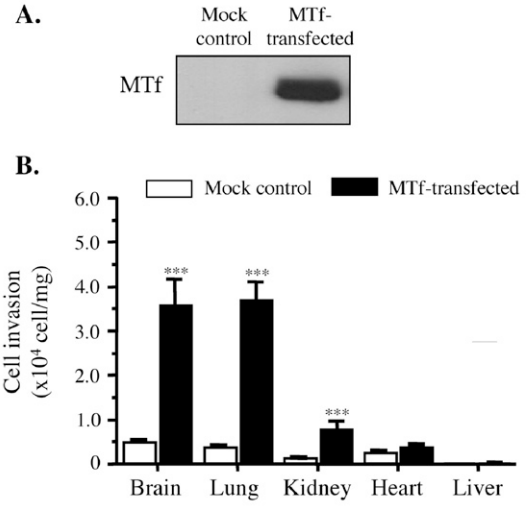

Fig. 4 – MTf expression correlates with cell invasion in vivo. Chinese hamster ovary (CHO) cells were transfected with full-length human MTf cDNA (MTf-transfected) or with control vector (Mock control). (A) The protein expression of MTf was studied by Western blot in CHO Mock control and MTf-transfected cells as detailed in Materials and methods. (B) CHO Mock control and MTf-transfected cells were metabolically labeled with [^{3}H]-thymidine and injected in the tail vein of Crl:CD-1 mice as described in the *in vivo* metastasis assay. After 24 h, cell accumulation was quantified by liquid-scintillation spectrometry. Statistically significant differences compared to the control condition are indicated by * for $p < 0.001$ (Student's *t*-test).**

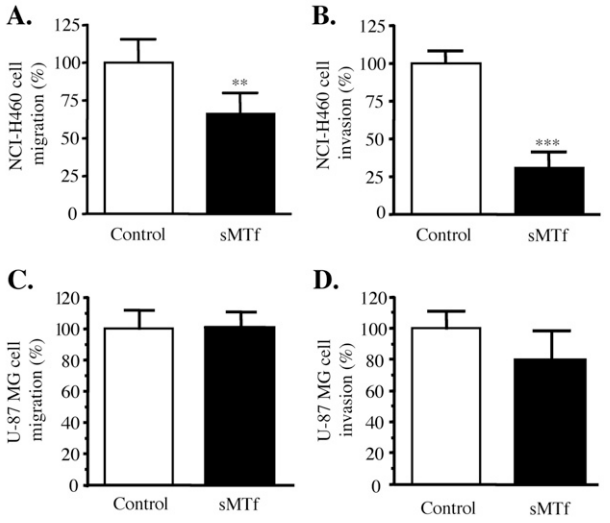

Fig. 5 – Effects of sMTf on tumor cell migratory properties. The migration of NCI-H460 lung carcinoma cells (A) and U-87 MG glioblastoma cells (C) was performed on gelatin-coated Transwell filters in presence and in absence of sMTf (100 nM). The invasion of NCI-H460 (B) and U-87 MG (D) cells was also studied with or without sMTf (100 nM) on dried Matrigel™-coated filters as described in Materials and methods. After 18 h, cells were fixed, stained and the filter tops were cleaned. Cellular migration and invasion were quantified from 5 random fields using Northern Eclipse software. Statistically significant differences compared to the control condition are indicated by ** for $p < 0.005$ and * for $p < 0.001$ (Student's *t*-test).**

previous studies report modulation of these receptors during the regulation of the u-PA/u-PAR system [25–27], LRP mRNA levels were unaffected in NCI-H460 and HMEC-1 cells treated with sMTf under our experimental conditions (Fig. 6B). The mRNA expression of other LDLR family members such as LRP1b, LRP8, LR11, LRP2, LDL-R and VLDL-R were not modulated by sMTf (data not shown). u-PAR mRNA levels were also similar in HMEC-1 and NCI-H460 cells in presence and in absence of exogenous sMTf, suggesting that sMTf treatment seems to act downstream of u-PAR and LRP gene regulation (Fig. 5B). Results presented here further propose an association between endogenous MTf expression and the *in vitro* u-PAR/LRP system targeting by sMTf treatment.

Discussion

Our previous results demonstrate that sMTf inhibits ECs movement as well as tubulogenesis [2]. The purpose of the current study is to evaluate the anti-cancer and anti-angiogenic properties of sMTf during tumor development. We show here that sMTf treatment clearly decreases the angiogenesis stimulated by growth factors and leads to efficient growth inhibition of subcutaneous U-87 MG and NCI-H460 cells.

Tumor growth and metastasis require persistent angiogenesis and in the absence of new vasculature, tumor cells become necrotic or apoptotic [28]. New blood vessel growth occurs in multiple stages and is under complex controls by angiogenic

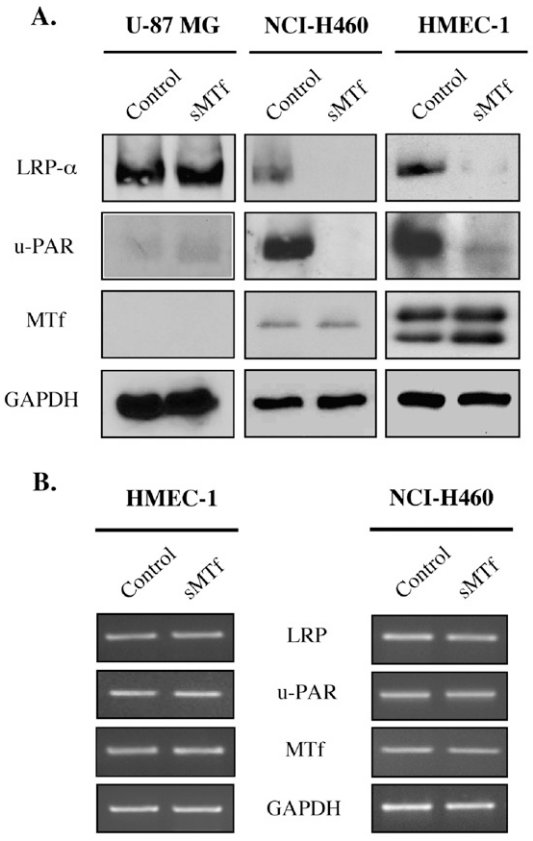

Fig. 6 – sMTf treatment affects the u-PAR/LRP system in endothelial and large lung carcinoma cells. (A) Protein expression of u-PAR and LRP were studied by Western blot in cells from human glioblastoma (U-87 MG), large lung carcinoma (NCI-H460) and in microvascular endothelial cells (HMEC-1) treated or not with 100 nM sMTf for 18 h. (B) mRNA expression of u-PAR and LRP was also studied by semi-quantitative PCR in U-87 MG, NCI-H460 and HMEC-1 cells treated or not with sMTf. GAPDH expression was used as a loading control. Results are representative of three independent experiments.

inducers such as bFGF and VEGF. ECs entering tumor tissue in response to chemo-attractants and growth factors, such as VEGF, proliferate to initiate neovascularization. In this study we demonstrate that sMTf interferes with angiogenesis induced by growth factors. Tumor cells in very aggressive glioblastomas, especially those surrounding micro vessels, express high levels of VEGF, suggesting that tumor and ECs vigorously interact [29]. Thus, while cancer cells may actively recruit ECs to support their proliferation and promote the formation of new vasculature, sMTf strongly interferes with these processes. Hence, further studies are required to determine whether sMTf affects the expression of the receptors involved in VEGF signaling pathway, such as VEGFR-1 and VEGFR-2.

We recently reported that mMTf-induced angiogenesis was inhibited by systemic treatment with sMTf [11]. Here, we show that constant delivery of sMTf into nude mice with Alzet micro-osmotic pumps contributed to a significant reduction in the growth of subcutaneous U-87 MG-derived tumor. This potent inhibition of tumor growth was also associated with a decrease of the hemoglobin content in the glioma tumors. In fact, the evaluation of angiogenesis in tumors, as determined by the hemoglobin content, revealed that U-87 MG-derived tumor development appears to be strongly dependent on angiogenesis. Analysis of microvessel density in astrocytic tumors in association with prognosis has therefore produced conflicting results [30,31]. Although pan-endothelial markers, such as anti-CD31, -CD34 and -factor-VIII antibodies, are generally used in the evaluation of angiogenesis, they not only react with newly formed vessels but also with normal vessels, which are entrapped within tumor tissues. Endoglin, or CD105, is a transforming growth factor-β (TGF-β) binding protein expressed on the surface of endothelial cells which is essential for angiogenesis [32]. It was reported that anti-CD105 antibodies preferentially react with activated endothelial cells in angiogenic tissues, but only weakly or not at all with those of most normal tissues [33,34]. Although Gomez-Esquer et al. showed that endoglin mRNA did not correlate with breast tumor aggressiveness [35], several reports have identified CD105 as a superior marker for the evaluation of angiogenesis in various types of cancer [24], notably in glioblastomas [36]. Overall, our results strengthen the fact that sMTf interferes with pathological angiogenesis by inhibiting endothelial cell migration *in vitro*, reducing the hemoglobin content and the CD105 mRNA expression in the U-87 MG-derived tumors, and by leading to the inhibition of VEGF- and bFGF-induced neovascularization of Matrigel™ plug. Therefore, sMTf appears to be an effective anti-angiogenic agent in subcutaneous glioblastoma tumors by targeting the endothelial environment.

In this study, we show that sMTf treatment inhibits the growth of subcutaneous NCI-H460-derived tumors. These results are consistent with the inhibition of NCI-H460 cell migration and invasion *in vitro* by sMTf. Considering that mMTf binds to plasminogen and facilitates its activation [4], the truncated form sMTf may interfere with cell motility via the modulation of plasmin formation. Hence, our results demonstrate that the addition of sMTf to endothelial and lung carcinoma cells reduced their invasive capacities *in vitro* in association with a decrease in their u-PAR and LRP protein expression. A disruption in the equilibrium of plasminogen activation system, as it occurs in the pathogenesis of cancer, may drive malignant cells to invade surrounding tissue and eventually to metastasize to distant body regions [37]. In addition to the binding of plasminogen to sMTf, down-regulation of u-PAR expression in ECs may act as a strong modulator of plasmin formation. Considering that sMTf reduces the free and active u-PAR regeneration at the HMEC-1 cell surface by both increasing the internalization of his scavenger receptor LRP and reducing its protein expression [2], we believe that sMTf acts similarly in NCI-H460 cells. Thus, modulation of these receptors – such as decrease in their synthesis or/and increase in their turnover – by sMTf could greatly participate to the inhibition of cell migration in these cells. Indeed, modulation of u-PAR/LRP expression by sMTf appears to be an important feature in cellular invasion and migration *in vitro*. Nevertheless, LRP has a protective role in maintaining the vascular wall structure and its deficiency or overexpression can cause vascular disorders (reviewed in Llorente-Cortes and Badimon [38]). Therefore, further studies

are needed to determine the effects of sMTf on healthy tissue. Our results suggest that sMTf treatment may affect the growth of tumor cells such as NCI-H460 by targeting the u-PAR/LRP system.

Inhibition of tumor growth by sMTf may result from different molecular events. Here we suggest that sMTf treatment modulate the equilibrium between mMTf and its soluble secreted form. Considering that the average concentration of circulating MTf in human plasma reaches 33 pM [5], the addition of exogenous sMTf can disturb the MTf equilibrium. Disruption in this balance could greatly modulate plasmin formation at the cell surface in response to the binding of plasminogen to sMTf rather than endogenous mMTf. We already reported that the binding of plasminogen to mMTf could favour its conversion into plasmin, thereby promoting cell migration and invasion [4,9]. The accumulation of MTf transfected cells in mice brain, lung and liver reported in this study confirm that mMTf expression triggers cell invasion *in vivo*. Downregulation of MTf expression also provides further evidence that endogenous mMTf plays a key role during SK-Mel-28 melanoma cell proliferation, migration, tumor growth and metastasis [9,10]. Altogether, our findings and that from others indicate that mMTf is a key player in tumor growth and invasiveness. In this regard, sMTf treatment seems to exert anti-angiogenic and anti-tumor activities in a MTf-expressing environment, while the equivalent treatment performed in a situation lacking MTf expression triggers no specific response. Although additional analyses are required to support this hypothesis, the association between endogenous MTf expression and sMTf treatment efficacy could partly explain the results obtained in both subcutaneous models. This study has produced further evidence that the anti-angiogenic activity of sMTf may be considered as a significant potential clinical treatment. In addition, the duality in sMTf treatment distinguishes it from single-target, conventional anti-angiogenic and anti-cancer therapies. Likewise, mMTf would be an interesting therapeutic target since its expression in tumors could increase their invasion to adjacent tissues. We could also consider mMTf as a potential biomarker for the selection of cancer patients in the use of sMTf treatment.

These are the first *in vivo* results showing that sMTf could reduce the growth of U-87 MG- and NCI-H460-derived tumors. This present study demonstrates that sMTf contributes to the reduction of angiogenesis and tumor growth. Consequently, sMTf would provide a novel approach to tumor treatment by targeting both tumoral endothelial and cancer cells.

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