ORIGINAL ARTICLE

TIMP-1 Overexpression in Lung Carcinoma Enhances Tumor Kinetics and Angiogenesis in Brain Metastasis

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Abstract

Tissue inhibitors of matrix metalloproteinase (TIMP) orchestrate many biologic activities, including inhibition of matrix metalloproteinase activity, activation of pro–matrix metalloproteinases, and regulation of cell proliferation, angiogenesis, and apoptosis induction. Tissue inhibitors of matrix metalloproteinase can play a protective role during tumor invasion and metastasis, but elevated TIMP messenger RNA levels have also been associated with aggressive cancers and poor clinical outcome. We examined the potential roles of TIMP-1 in H2009 lung adenocarcinoma cells and in cells transfected with a human TIMP-1–overexpressing vector (HB-6 and HB-1). Tumors resulting from the implantation of parental cell lines and transfected HB-1 cells into the brains of nude mice had a typical carcinoma profile, but human TIMP-1–overexpressing tumors showed enhanced tumor kinetics and focially more infiltrative features; vessel density assessed with anti-CD31 immunohistochemistry was also greater within HB-1 tumor implants. Similar effects on HB-6 and HB-1 cells versus parental cell lines and empty vector clones were observed in endothelial cell assays. Anchorage-independent growth and invasion through Matrigel were also increased in TIMP-1–overexpressing cells. Together, these results indicate tumor-promoting functions of TIMP-1 through alterations in angiogenesis, increased tumorigenicity, and invasive behavior. Although matrix metalloproteinase inhibition has been the traditionally identified function of TIMP-1, matrix metalloproteinase–independent interactions may contribute to the growth of metastatic carcinomas in the brain.

Key Words: Adenocarcinoma, Brain, Extracellular matrix, Matrix metalloproteinase, Metastasis, Tissue inhibitors of matrix metalloproteinase.

INTRODUCTION

Central nervous system (CNS) metastases remain a major cause of morbidity and mortality in patients with cancer; approximately 200,000 patients are diagnosed as having brain metastases each year (1), and they occur in 20% to 45% of patients with systemic cancer (2, 3). The origin of the most common of these metastases are neoplasms originating in the lung (40%–50%), breast (15%–20%), skin (5%–10%), or gastrointestinal tract (4%–6%) (3). Understanding the mechanisms involved in the establishment of brain metastases is therefore critical for the development of novel therapeutic interventions that will allow us to increase quality of life and reduce mortality in affected patients (4, 5). Despite current aggressive therapeutic interventions, including surgery, whole-brain radiation therapy or stereotactic radiosurgery, and other chemotherapeutic protocols, overall survival may be extended from 5 weeks (for untreated disease) to 3 to 18 months (6).

The development of metastases is complex and involves several necessary sequential steps (7). For CNS metastases, these steps include separation of malignant cells from the primary neoplasm; invasion through the basement membrane and extracellular matrix (ECM), with subsequent entry into the circulatory system; and entrapment and extravasation into the brain after compromise of the blood-brain barrier. Because the growth and spread of metastasis are dependent on the establishment of adequate blood supply, proliferation and tumor expansion are closely associated with angiogenesis (8–12). These processes, particularly the implantation and growth phases, revolve around tumor interactions with the host microenvironment and are modulated by the ECM, matrix metalloproteinases, and their inhibitors (tissue inhibitors of matrix metalloproteinase [TIMP])—cytokines and molecules—within angiogenesis pathways. Both TIMP-1 and TIMP-2 are capable of inhibiting matrix metalloproteinase activity and are therefore important contributors to these interactions. Indeed, evidence suggests that TIMP-1 and TIMP-2 inhibit tumor invasion and metastasis in various experimental models (13, 14). Besides inhibiting active matrix metalloproteinases, TIMP perform other biologic roles, such as activating pro–matrix metalloproteinases, promoting cell growth, binding to the ECM, repressing angiogenesis, and inducing or reducing apoptosis.

Lung carcinoma remains the most frequent metastatic neoplasm in the brain. Several studies have documented a role (including prognostic value) for TIMP-1 in these neoplasms, and high TIMP-1 levels have been correlated with poor survival (15–17). Levels of TIMP-1 have also been found to be higher in adenocarcinomas than in squamous carcinomas (18, 19). Thus, these molecules likely have important biologic roles in the context of malignancy. In this study, we have explored the role of TIMP-1 in the CNS microenvironment and examined the effects of TIMP-1 overexpression on tumor invasion and angiogenesis.
MATERIALS AND METHODS

Cell Culture and Transfection

H2009, a lung adenocarcinoma carcinoma cell line (ATCC, Manassas, VA), was routinely propagated in RPMI 1640 supplemented with 10% fetal bovine serum and 50 μg/mL of gentamicin. H2009 cells were transfected with vectors pBK-CMV (Stratagene, Agilent Technologies, Santa Clara, CA) and pBK-CMV-hTIMP-1 (full-length human TIMP-1 complementary DNA), using lipofectin (GIBCO BRL, Grand Island, NY), according to the manufacturer’s instructions. Five hours later, transfected cells were placed in a medium with serum plus 600 μg/mL of Geneticin (GIBCO BRL). In 2 weeks, individual clones were isolated using cloning cylinders and propagated with 500 μg/mL of Geneticin.

Western Blot Analysis and Reverse Transcription–Polymerase Chain Reaction

Serum-free conditioned medium (SFCM) was collected for each cell line and concentrated, and protein concentration was determined using bicinchoninic acid (BioRad, Hercules, CA). Four micrograms of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions and transferred to nitrocellulose membrane. After being blocked in 5% milk/Tris-buffered saline and Tween 20, the membrane was incubated with 1 μg/mL of anti–TIMP-1 antibodies (Millipore, Billerica, MA). Secondary antibody was used at 1:10000, and development was carried out using the ECL system (Pierce, Waltham, MA). Total RNA was extracted with the Quiagen RNeasy mini kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). One microgram of RNA was used for reverse transcription, using a first-strand complementary DNA synthesis kit (SuperArray, Frederick, MD). Primers of TIMP-1 were purchased from SuperArray, and their polymerase chain reaction protocol was used. Glyceraldehyde 3-phosphate dehydrogenase primers were used for internal normalization. Empty vector controls (empty vector-3 [EV-3]) and empty vector-1 [EV-1]) were compared with parental cell lines and transfected clones. Multiple transfected clones were examined, and HB-1 and HB-6 were selected for additional experiments.

Cell Proliferation Assay

To compare the growth rates of parental cell lines and empty vector clones with those of transfected TIMP-1-overexpressing clones, we performed the MTS cell proliferation assay (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, cells were seeded in triplicate in 96-well plates at 2,500, 5,000, and 7,500 cells/well. After a 48-hour incubation, MTS reagent was added to the wells and incubated for 2 hours. Absorbance was recorded at 490 nm using an ELx800 microplate reader.

In Vivo Tumor Growth

Mice were maintained in accordance with Institutional Animal Care and Use Committee procedures and guidelines. Two microliters of 1 × 10⁵ cells (either H2009 or the TIMP-1-overexpressing clone HB-1) was implanted, with a stereotactic apparatus, into the right cerebral hemisphere of 6- to 8-week-old nude male mice (athymic Ncr–Nu; NCI, Frederick, MD) (n = 12 for H2009; n = 13 for HB-1). Implantation was based on pilot studies in which most animals showed clinical signs of tumor growth, including lethargy, poor grooming, weight loss, and reduced locomotion or paralysis between 8 and 9 weeks; tumors were identifiable in histologic sections at those times. Therefore, all animals were killed within this time frame; brains were removed and fixed in 10% neutral buffered formalin.

Histology and Immunohistochemistry

After 1 week of fixation, brain specimens were coronally sectioned, processed, and embedded in paraffin. Five-micrometer-thick sections were deparaffinized using xylene and hydrated though graded alcohols. Sections were then stained with hematoxylin and eosin, dehydrated, and coverslipped. Parallel sections applied to “Plus” slides (Fisher Scientific) were deparaffinized and hydrated. Antigen retrieval was performed by microwaving in citrate buffer; endogenous peroxidase was quenched using 3% H₂O₂ in methanol, and sections were immunoreacted with rabbit anti–human CD31 antibody (PharMingen Inc, San Jose, CA), a well-documented antibody that reacts with normal and neoplastic endothelial cells. VI Purple (Vector Laboratories, Burlingame, CA) was used as chromogen. Slides were lightly counterstained with hematoxylin and coverslipped. Negative controls included omission of the primary antibody or addition of nonspecific IgG. Mean vessel density was determined on images derived from H2009 and HB-1 tumors. Because tumors were not present in all animals and some tumors were not large enough to fill most of the field (measurements of which will reflect tumor vessel density), data from 24 images of H2009 tumors were pooled and compared with the same number from the HB-1 group.

Endothelial Cell Network Assay

Human umbilical vessel endothelial cells (HUVECs; Lonza, Walkersville, MD) were propagated at approximately 5,000 cells/cm² in an endothelial growth medium (EGM-2) SingleQuot kit (Lonza). Thawed cells underwent 2 passages before being used for endothelial network assay. Three hundred microliters of ice-cold Matrigel (Becton Dickinson, Franklin Lakes, NJ) was added to individual wells of a 24-well tissue culture plate and allowed to polymerize at 37°C for approximately 30 minutes. Seventy percent confluent HUVECs were harvested, counted, and resuspended in EGM-2 (Clonetics; Lonza) at 50,000 cells/mL. One milliliter of cell suspension was mixed with previously concentrated SFCM and overlaid on polymerized Matrigel. The cell suspension with EGM-2 alone, without SFCM, served as control. Endothelial cell sprouting and network formation were best visualized by day 3 in pilot studies. Specific treatments were added to HUVECs as follows: EGM-2 (control) (a), SFCM from H2009 cells (b), EV-3 cells (c), HB-6 cells (d), or HB-1 cells (e). Images were captured on day 3 and morphometrically analyzed using Image Pro Plus software (Media Cybernetics, Rockville, MD). Sprouting and complexity of network formation were evaluated by counting the number of polygonal chambers.
TIMP-1 Overexpression Results in Increased Image and Statistical Analysis

Each membrane were counted (under a light microscope) in at least 5 fields. Mean colony size was determined by measuring the diameter of 10 colonies per image obtained at 4× magnification in each well. Experiments were performed in triplicate.

Matrigel Invasion Assay

Invasion assay was conducted using Biocoat Matrigel Invasion Chambers with 8-μm pores (Becton Dickinson) according to the manufacturer’s instructions. Briefly, 2.5 × 10^4 cells were resuspended in fresh serum-free medium and seeded into the upper chamber, whereas the lower chamber contained medium with 10% fetal bovine serum. After 48 hours, uninvaded cells were scraped off, and membranes were fixed in 4% paraformaldehyde and stained with crystal violet. The cells on each membrane were counted (under a light microscope) in at least 5 fields.

Image and Statistical Analysis

Image Pro image analysis system was used to make all quantitative assessments on tissue sections and in vitro assays. Any image manipulation to facilitate the image analysis process was uniform across all experiments in that in vitro assay. All studies were carried out in blinded fashion. Statistical analysis was undertaken using IBM SPSS version 22. Significance was assessed using an α level of 0.05, unless otherwise noted.

RESULTS

Characterization of TIMP-1–Overexpressing Clones

H2009 cells were transfected with the vector pBK-CMV-hTIMP-1. Several clones that expressed high levels of TIMP-1 RNA and protein were isolated. HB-6 and HB-1 clones were selected for further investigation based on their significant overexpression of TIMP-1 message, as confirmed by reverse transcription–polymerase chain reaction (Fig. 1A) and Western blot analysis (Fig. 1B). The in vitro growth rate of these clones was similar to those of parental cell lines and was assessed using an MTT (3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide) solution (Sigma, St Louis, MO). Mean colony count was based on the numbers from the 3 wells. Mean colony size was uniform across all experiments in that in vitro assay.

TIMP-1 Overexpression Induces Angiogenesis In Vitro

Serum-free conditioned medium from H2009 cells, EV-3, and HB-6 or HB-1 clones was added at equal protein concentrations to HUVECs in medium, overlaid on Matrigel-coated wells, and monitored for a period of 3 days. The number of junctional points and the extent of chamber formation were measured by image analysis in multiple wells in triplicate. To examine differences between groups, we used 1-way analysis of variance. A Tukey-Kramer multiple comparison procedure was used to control the overall α level to examine post hoc pairwise differences between groups. Wells with SFCM from HB-6 and HB-1 clones, compared with wells with SFCM from H2009 cells and empty vectors, showed a marked increase in angiogenic activity both in the number of endothelial chambers and in the number of junctional points formed in the endothelial network (Fig. 5A). The numbers of junctional points within the network formed by endothelial cell processes connecting with each other showed a greater than 1.5-fold increase in HB-6 and HB-1 compared with controls, H2009, and EV-3, with no significant difference between the parental cell line H2009 and the empty vector clone EV-3: HB-6 (mean, 62.8) versus control (mean, 50.2; p < 0.009); HB-1 (mean, 65.5) versus tumors was greater in the HB-1 clone; 84% (n = 11 of 13) had histologically verifiable neoplasm compared with only 50% (n = 6 of 12) in H2009-injected animals (Fig. 2A). The percentage of animals developing large tumors (i.e. tumors that occupied between 25% and 50% of the hemisphere) within the area encompassed by the external capsule was higher in HB-1–injected mice. Almost 62% of HB-1 mice had large tumors, as defined above, versus 27% of H2009 mice (p < 0.05) (Fig. 2B).

Morphologic Features of Implanted Tumors

Tumors resulting from stereotactic implantation of HB-1 cells were frequently larger than those from H2009 cells. Overall histologic appearance (i.e. of moderately differentiated epithelial neoplasms) was similar. Tumors were well demarcated from adjacent brain tissue, which showed reactive changes (Figs. 3A, B). In comparison with H2009 tumors, HB-1 neoplasms were more infiltrative, often growing with finger-like extensions rather than as entirely discrete masses (Fig. 3C). In some animals, HB-1 tumors grew at multiple sites; occasional HB-1 tumors (n = 3) developed as multifocal neoplasms (distinctly separate from each other) and even involved the noninjected hemisphere (Fig. 3D). Some HB-1 tumors (n = 3) extended downward to involve the skull base and sinuses or extended upward to infiltrate the skull and scalp, with invasion of bone and muscle (Figs. 3E, F).

Tumor Vasculature

Immunohistochemistry using the vessel marker CD31 confirmed the increased angiogenic profiles initially observed on routine hematoxylin and eosin–stained sections in H2009 versus HB-1 tumors (Fig. 4A). There was a greater than 2-fold increase in the number of vessels in HB-1 tumors (mean, 22.1) versus H2009 tumors (mean, 9.2; p < 0.0001) (Fig. 4B).

TIMP-1 Overexpression Induces Angiogenesis In Vivo

HB-6 and HB-1 clones were added at equal protein concentrations to HUVECs in medium, overlaid on Matrigel-coated wells, and monitored for a period of 3 days. The number of junctional points and the extent of chamber formation were measured by image analysis in multiple wells in triplicate. To examine differences between groups, we used 1-way analysis of variance. A Tukey-Kramer multiple comparison procedure was used to control the overall α level to examine post hoc pairwise differences between groups. Wells with SFCM from HB-6 and HB-1 clones, compared with wells with SFCM from H2009 cells and empty vectors, showed a marked increase in angiogenic activity both in the number of endothelial chambers and in the number of junctional points formed in the endothelial network (Fig. 5A). The numbers of junctional points within the network formed by endothelial cell processes connecting with each other showed a greater than 1.5-fold increase in HB-6 and HB-1 compared with controls, H2009, and EV-3, with no significant difference between the parental cell line H2009 and the empty vector clone EV-3: HB-6 (mean, 62.8) versus control (mean, 50.2; p < 0.009); HB-1 (mean, 65.5) versus tumors was greater in the HB-1 clone; 84% (n = 11 of 13) had histologically verifiable neoplasm compared with only 50% (n = 6 of 12) in H2009-injected animals (Fig. 2A). The percentage of animals developing large tumors (i.e. tumors that occupied between 25% and 50% of the hemisphere) within the area encompassed by the external capsule was higher in HB-1–injected mice. Almost 62% of HB-1 mice had large tumors, as defined above, versus 27% of H2009 mice (p < 0.05) (Fig. 2B).
control (mean, 50.2; p < 0.001); HB-6 (mean, 62.8) versus H2009 (mean, 43.9; p < 0.0001); HB-1 (mean, 65.5) versus HB-6 (mean, 62.8; p < 0.0001); HB-1 (mean, 65.5) versus EV-3 (mean, 46.1; p = 0.0438). Values are presented as the means of at least 3 independent experiments (Fig. 5B).

The numbers of polygonal chambers formed by seeded endothelial cells supplemented with SFCM from experimental groups were increased by almost 2-fold in the HB-6 and HB-1 groups compared with controls, H2009, and EV-3: HB-6 (mean, 40.9) versus control (mean, 33.9; p < 0.013); HB-1 (mean, 41.4) versus control (mean, 33.9; p < 0.007); HB-6 (mean, 40.9) versus H2009 (mean, 26.7; p < 0.0001); HB-1 (mean, 41.4) versus H2009 (mean, 26.7; p < 0.0001); HB-6 (mean, 40.9) versus EV-3 (mean, 28.7; p < 0.0001); HB-1 (mean, 41.4) versus EV-3 (mean, 28.7; p < 0.001). Endothelial cells cultured with SFCM from HB-6 and EV-3 did not have significantly different mean numbers: EV-3 (mean, 28.7) and H2009 (mean, 26.7; p = 0.270) (Fig. 5C).

**FIGURE 1.** (A) HB-1 and HB-6 clones demonstrate higher message levels of TIMP-1 on reverse transcription–polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used for internal normalization. H2009, lung adenocarcinoma; HEV3, empty vector; HB-1 and HB-6, transfected H2009 cell lines. (B) Protein expression of TIMP-1 in transfected clones is verified by Western blot analysis. Protein samples from SFCM were subjected to Western blot analysis with anti–TIMP-1 antibodies. HB-1, HB-2, HB-4, and HB-6 are transfected H2009 cell lines. (C) The transfection process did not alter the proliferation of HB-1 and empty vectors (EV-1 and EV-3) at 48 hours. Baseline value is arbitrarily taken as 100 from H2009 data and compared with determined relative proliferation.

**TIMP-1–Overexpressing Clones Exhibit Increased Anchorage-Independent Growth**

Normal cells, compared with tumorigenic cells, require anchorage dependence for growth and undergo apoptosis shortly after loss of adhesion. Cell anchorage–independent growth (soft agar colony formation) assays were carried out to examine whether HB-6 and HB-1 clones exhibited increased tumorigenesis relative to H2009 and EV-3 cells. The TIMP-1–overexpressing clones HB-6 and HB-1 affect anchorage-independent growth. Both the number of colonies formed and the mean size of colonies were increased in the experimental groups. Representative images showing greater numbers and sizes of cell colonies in soft agar in HB-6 and HB-1 versus H2009 and EV-3 are shown in Figure 6A. The numbers of colonies increased considerably in wells with HB-6 and HB-1 cells versus wells with H2009 and EV-3 cells: HB-6 (mean, 25.8) versus H2009 (mean, 15.8; p < 0.0001); HB-1 (mean, 27.2) versus H2009 (mean, 15.8; p < 0.0001); HB-6 (mean, 25.8) versus EV-3 (mean, 16.3; p < 0.0001); HB-1 (mean, 27.2) versus EV-3 (mean, 16.3; p < 0.0001). There was no significant difference in the mean numbers of colonies between EV-3 and parental H2009 cells (p = 0.759) (Fig. 6B).

In addition, the mean colony size (determined by measuring the mean diameter in digital units) of 10 colonies per 4× image in each well was also significantly increased in the same groups: HB-6 (mean, 175.2) versus H2009 (mean, 137.2; p < 0.0001); HB-1 (mean, 185.5) versus H2009 (mean, 137.2; p < 0.0001); HB-6 (mean, 175.2) versus EV-3 (mean, 135.9; p < 0.0001); HB-1 (mean, 185.5) versus EV-3 (mean, 135.9; p < 0.0001) (Fig. 6C). The increased numbers and larger colonies seen in this anchorage-independent growth in soft agar are...
interpreted as defining enhanced tumorigenesis and onco-
genic transformation, consistent with aggressive growth patterns identified in vivo.

TIMP-1–Overexpressing Cells Exhibit an Increase in Invasion Profile

In the invasion assay, the number of cells increased almost 3-fold in wells with HB-6 and HB-1 cells versus wells with H2009 and EV-3 cells: HB-6 (mean, 98.2) versus H2009 (mean, 39.3; \( p < 0.0001 \)); HB-1 (mean, 137.6) versus H2009 (mean, 39.33; \( p < 0.0001 \)); HB-6 (mean, 98.2) versus EV-3 (mean, 37.7; \( p < 0.0001 \)); HB-1 (mean, 137.6) versus EV-3 (mean, 37.7; \( p < 0.0001 \)). H2009 and EV-3 cells did not have significantly different numbers of invading cells (\( p = 0.840 \)) (Figs. 7A, B). This assay lends further support to our in vivo observation that TIMP-1 makes cells more invasive.

DISCUSSION

The processes involved in the establishment of CNS metastases include a series of sequential steps, each one playing a critical role in its contribution to the final result (20). The implantation model used in these studies is an example of orthotopic brain metastasis and is a local growth model (21) that focuses primarily on tumor interactions with the host environment as the tumor seeks to proliferate and establish itself while concurrently developing its independent vascularity.

Tissue inhibitor of matrix metalloproteinase-1 is a well-documented mitogen. It was first identified as an erythrocyte-potentiating protein that could stimulate the growth and differentiation of erythroid precursors (22–27). Tissue inhibitor of matrix metalloproteinase-1 has since been shown to exhibit growth-promoting activity in a wide range of cell types, including normal keratinocytes, fibroblasts, breast cancer cells, and osteosarcoma cells (28–34), and tumor-promoting activity (22, 23). Equally important has been its role in inhibiting cell growth (35). For many years, the traditional notion in tumor biology has been that TIMP serves as inhibitors of the invasive process. These classic views were supported by several reports on experimental models depicting TIMP-mediated inhibition of tumor growth (36–39). However, in recent years, this notion has become moot because many studies have documented the role of TIMP in tumor invasion, apoptosis, and angiogenesis (40, 41).

The present study was undertaken to define the role of TIMP-1 overexpression in the CNS microenvironment with its unique ECM composition. Our studies show increased tumor growth in the brain after stereotactic implantation of lung carcinoma cells that overexpress TIMP-1. Our results clearly demonstrate increased size and number of HB-1 tumors in mice, exhibiting an aggressive growth pattern with finger-like projections rather than the discrete mass usually formed by H2009 cells. In colony formation assays, we identified an increase in the number of colonies formed by HB-6 and HB-1 cells and an increase in invasion through Matrigel in a Boyden chamber assay, which is again attributed to TIMP-1 overexpression in these clones. Our study is in contrast to that of Kruger et al (42), who showed that overexpressing TIMP-1 conferred resistance to brain metastasis. It should be pointed out that they had used a mesenchymal tumor cell line in contrast to our epithelial cell line. In addition, the TIMP-1 used in that study was not tumor-derived; it has been shown that the TIMP-1 genotype of the tumor—not that of the host—influences tumor invasion (43). In addition, this group has also shown that TIMP-1 promotes liver metastasis (44). These studies further contribute to our understanding of the more complex roles that TIMP, in this case TIMP-1, play in the context of interactions within the host environment of the CNS.

The second significant observation in this study was the impact of TIMP-1 overexpression on angiogenesis both in
vivo and in vitro. The development and establishment of vascular supply are long-established key elements of the growth, maturation, and maintenance of tissue under physiologic conditions (45). Angiogenesis is required in various pathologic processes such as wound healing (46), rapid growth of solid tumors (47, 48), and a variety of other clinical conditions (45, 49–51). Similarly, vascular proliferation also forms a key component of increased malignancy in gliomas (52–54). In the framework of neoplasia, blood vessels are required within primary and metastatic tumors for the delivery of oxygen and nutrients needed to promote tumor growth. The establishment of metastasis only seems to become biologically significant

FIGURE 3. (A) Tumors resulting from stereotactic implantation of HB-1 cells were frequently larger than those resulting from H2009 cells. Overall histologic appearance was similar, with characteristic moderately differentiated epithelial neoplasm. (B) Tumors were well demarcated from adjacent brain tissue, which showed reactive changes. (C) HB-1 tumors were more infiltrative than H2009 cell tumors and often grew with finger-like extensions, rather than as entirely discrete masses. (D) Occasional HB-1 tumors (n = 3) developed as multifocal neoplasms distinctly separate from each other (arrows). (E, F) Some HB-1 tumors (n = 3) often overstepped cranial boundaries, extending downward to involve the skull base and sinuses, with invasion of bone and muscle. (A–F) Hematoxylin and eosin–stained sections (original magnification: 12.5×, 200×, 200×, 12.5×, 100×, and 200×, respectively).
when the implanted cell colony establishes its own blood supply derived from the host site (55). Angiogenesis thus plays an important role in promoting the aggressive and metastatic behavior of tumors. In this context, the role of TIMP-1 has been ambiguous. Some studies have documented the role of TIMP-1 as proangiogenic (e.g. enhanced vascular endothelial growth factor expression has been demonstrated with increased vascularization of tumors after TIMP-1 overexpression in mammary carcinoma cells) (56). Tissue inhibitor of matrix metalloproteinase-1 has also been reported to promote vascular endothelial growth factor-induced neovascularization in the retina (57). Contrary viewpoints have been expressed in several studies that documented TIMP-1 as playing an antiangiogenic role (58, 59). Tissue inhibitors of matrix metalloproteinase may be proangiogenic simply by negating the effects of matrix metalloproteinases, which break down the ECM and release antiangiogenic factors such as angiostatin and tumstatin (60). The present study adds to this view with our observations documenting a proangiogenic role for TIMP-1. This was evidenced by highly vascularized tumors in animals after stereotactic implantation of TIMP-1-overexpressing cells and by increased formation of more complex endothelial networks in vitro after addition of SFCM from the same HB-1 cells and SFCM from another TIMP-1-overexpressing clone, HB-6.

Evidence defining the functions of TIMP beyond their classic role of inhibiting matrix metalloproteinases (e.g. in cell proliferation, tumor growth and metastasis, apoptosis and

FIGURE 4. (A) Increased angiogenic profiles in HB-1 versus H2009 tumor implants, as observed by immunohistochemistry using the vessel marker CD31. Original magnification: 20×. (B) Mean vessel densities determined by counts of discrete CD31-immunoreactive vessels within the tumors. There was a greater than 2-fold increase in the mean number of vessels in HB-1 tumors versus H2009 tumors (p < 0.0001). Box plot defining range and median (bar), with mean values for each group, are shown. Asterisk represents points that are designated statistical outliers.
FIGURE 5. Wells plated with Matrigel and seeded with endothelial cells (HUVECs) were incubated with SFCM from HB-6 and HB-1 cells and compared with those receiving SFCM from empty vector cells (EV-3) and H2009 cells. (A) Similarly plated HUVECs grown in EGM-2 without addition of SFCM from any cell group also served as controls. There was a marked increase in angiogenic activity with SFCM from HB-6 and HB-1 cells versus H2009, EV-3, or control, as evidenced by increased junctional points and chambers. (B) Box plots defining range and median (bar), with mean values listed for each group, are shown. Junctional points were increased in the HB-6 and HB-1 groups by almost 1.5-fold compared with H2009, EV-3, and control. Endothelial cells cultured in SFCM from EV-3 versus H2009 did not have a significantly different mean number of junctional points. (C) Similarly, the number of polygonal chambers formed within the endothelial network showed a marked (almost 2-fold) increase in the experimental group versus control H2009 and EV-3. Endothelial cells cultured with SFCM from EV-3 and H2009 did not have significantly different values. Box plots defining range and median (bar), with mean values shown for each group. Values are the means of at least 3 independent experiments.
angiogenesis) has continued to accumulate. Several investigators have attributed these diverse functions of TIMP to matrix metalloproteinase-independent processes, including those that may be attributed to the presence of putative TIMP receptors on the cell surface. These functions vary positively or negatively, affecting different cell types, and the presence of cell surface receptors could explain why TIMP expression causes inhibition of growth in some tumors but results in growth and metastasis in other tumors. Similarly, angiogenesis and apoptosis are positively or negatively affected, as reviewed by Jiang et al (61). In this context, α3β1 integrin and vascular endothelial growth factor receptor-2 have been identified as TIMP-2 and TIMP-3–interacting proteins on cell surfaces, respectively (62, 63). Recent studies have reported signaling functions of TIMP-1 through receptors such as CD63/integrin β1, integrin αvβ3, and pro–matrix metalloproteinase-9/CD44. These receptors have been shown to be involved in the antiapoptotic role of TIMP-1 via upregulation of focal adhesion kinase, Akt, and extracellular signal–regulated kinase signaling (64–67). The promotion of liver metastasis by TIMP-1 has been shown to occur through the induction of the hepatocyte growth factor signaling pathway (44). Tissue inhibitor of matrix metalloproteinase-1 has also been shown to translocate to the nucleus (68, 69) and may play a role in signaling in the nucleus. Thus, a number of mechanisms beyond simply inhibition of matrix metalloproteinases have been reported and may be at play in this study.

Tumor cell interactions within the host microenvironment, modulated by the ECM, matrix metalloproteinases and TIMP, and the cytokine and angiogenesis pathways, are key considerations in metastasis. Tissue inhibitors of matrix metalloproteinase have frequently been considered to regulate the proteolytic activity of matrix metalloproteinases relative to the ECM (their primary function). However, it is now evident that these molecules may have far-reaching effects and may truly be multifunctional proteins with an extensive repertoire of functional interactions that affect tumor infiltration and growth, apoptosis, and angiogenesis. Deciphering the complex interactions of TIMP, particularly those that may occur by matrix metalloproteinase–independent pathways, raises new

FIGURE 6. Overexpression of TIMP-1 affects cell anchorage–independent growth (soft agar colony formation assay). The number of colonies formed and the mean size of colonies were increased. (A) Images of representative cell colonies in soft agar, visually depicting the increased number and size of colonies in TIMP-1–overexpressing clones HB-6 and HB-1 versus H2009 and empty vector cells (EV), are shown at low magnification. (B) The number of colonies was significantly increased in wells with HB-6 and HB-1 cells versus H2009 and EV-3 cells; EV-3 and H2009 cells did not have significantly different numbers of colonies. (C) The mean colony size (determined by measuring the mean diameter in digital units) of 10 colonies per 4 × image in each well increased almost 1.5-fold in wells with HB-6 and HB-1 clones versus wells with H2009 and EV-3 cells. There were no significant differences between parental H2009 and EV-3 cells.
considerations for understanding the biology of tumor microenvironment interactions and, consequently, for identifying new therapeutic targets.

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FIGURE 7. The TIMP-1-overexpressing clones HB-6 and HB-1 display increased invasion through Matrigel. (A) Visual representation of increased numbers of HB-6 and HB-1 cells invading into the central portion of the chambers in comparison with H2009 and empty vector cells (EV-3). (B) Quantitative analysis confirming that the number of invading cells increased almost 3-fold in wells with HB-6 and HB-1 versus wells with H2009 and EV-3 cells. EV-3 and H2009 cells did not have significantly different invasion characteristics.


