Tissue Inhibitor of Metalloproteinases-1 Promotes Liver Metastasis by Induction of Hepatocyte Growth Factor Signaling


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Abstract

Balanced expression of proteases and their inhibitors is one prerequisite of tissue homeostasis. Metastatic spread of tumor cells through the organism depends on proteolytic activity and is the death determinant for cancer patients. Paradoxically, increased expression of tissue inhibitor of metalloproteinases-1 (TIMP-1), a natural inhibitor of several endometalloproteinases, including matrix metalloproteinases and a disintegrin and metalloproteinase-10 (ADAM-10), in cancer patients is negatively correlated with their survival, although TIMP-1 itself inhibits invasion of some tumor cells. Here, we show that elevated stromal expression of TIMP-1 promotes liver metastasis in two independent tumor models by inducing the hepatocyte growth factor (HGF) signaling pathway and expression of several metastasis-associated genes, including HGF and HGF-activating proteases, in the liver. We also found in an in vitro assay that suppression of ADAM-10 is in principle able to prevent shedding of cMet, which may be one explanation for the increase of cell-associated HGF receptor cMet in livers with elevated TIMP-1. Similar TIMP-1-associated changes in gene expression were detected in livers of patients with metastatic colorectal cancer. The newly identified role of TIMP-1 to create a prometastatic niche may also explain the TIMP-1 paradoxon. [Cancer Res 2007;67(18):8615–23]

Introduction

Metastasis and progressive scattering of tumor cells throughout tissues is the main cause of organ failure and subsequent death of cancer patients. Proteolytic remodeling of components of the extracellular matrix (ECM) in tissue interstitium is a prerequisite of the metastatic process (1). Matrix metalloproteinases (MMP) have long been thought to be major regulators of the pericellular protease-dependent steps of tumor progression (2). Altered expression profiles of some MMPs have been correlated with poor clinical prognosis for several human tumor types (3). Based on these facts, the historical view of MMPs and cancer was that they exerted proinvasive and prometastatic activity by mere ECM remodeling (1). Thus, it was hypothesized that therapeutic broad-spectrum inhibition of MMPs would result in antimetastatic activity (1). Indeed, under certain experimental circumstances, overexpression of the endogenous broad-spectrum metalloproteinase [including a disintegrin and metalloproteinase-10 (ADAM-10); ref. 4] inhibitor tissue inhibitor of metalloproteinases-1 (TIMP-1; ref. 5) can reduce tumor cell invasion (6, 7).

In contrast to these observations are other results, which are actually in agreement with clinical studies showing a correlation between elevated TIMP-1 expression and poor prognosis in many human cancer types (5, 8), where elevated levels of TIMP-1 either had no effect on metastasis (9) or led to increased incidence (10, 11) or growth (12) of primary tumors. These effects of TIMP-1 on cancer development have been attributed to its known proproliferative, antiapoptotic (5), or proangiogenic (13) activities, which are necessary but not sufficient for metastasis.

Tumor cell invasion and metastasis are the primary determinants of the survival of cancer patients (13). Signaling pathways leading to the expression of proteolytic enzymes have been suggested to be key mediators of invasive growth (14). Hepatocyte growth factor (HGF/scatter factor) signaling regulates a multitude of downstream prometastatic effector molecules, such as urokinasetype plasminogen activator (uPA), uPA receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), and MMPs (15–17). HGF stimulates metastasis of diverse tumor cells (15) and elevated levels of circulating HGF (18) or aberrant activity of cMet (14) has been detected in patients with many cancer types, including non-Hodgkin’s lymphoma (19) and colorectal cancer (14). Although elevated TIMP-1 expression in these patients correlates with decreased survival, a link between TIMP-1 and metastasis-promoting pathways has previously not been described. Here, we address this issue and report that elevated stromal levels of TIMP-1 induce HGF signaling, leading to enhanced expression of other metastasis-promoting genes. Consequently, susceptibility of the liver for metastasis of tumor cell lines of different origin was shown to be increased, assigning a new role to TIMP-1 as a factor creating a prometastatic niche.

Materials and Methods

Cell culture and adenoviruses. HEK 293 (20), HT1080lacZ-K15 (21), I-CL5s (22), and 293T cells (23) were cultured as described previously.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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NIH3T3 cells were cultured similarly to HT1080lacZ-K15 (21). Amino acids 1 to 126 of the human TIMP-1 cDNA (N-TIMP-1; ref. 24) were cloned into pSecTag2/Hygro A (Invitrogen). A DNA fragment consisting of Ig leader sequence and N-TIMP-1 cDNA or full-length human TIMP-1 cDNA (pGEMbTIMP-1; ref. 25), respectively, was obtained and cloned into the adenoviral shuttle plasmid pKAl (26). Recombinant adenoviruses were generated, purified, and plaque titrated as described previously (26). As control, Ad70-3, an adenovirus without transgene, was used (27). Functionality of adenoviral constructs was tested in vitro (data not shown). Adenoviral infection in vitro was done as described previously (26).

**Experimental metastasis assays.** Pathogen-free, athymic CD1<sup>nu/nu</sup> mice (Charles River) were injected i.v. with 2 x 10<sup>7</sup> plaque-forming units of Ad70-3, AdTIMP-1, and AdN-TIMP-1, respectively, or vehicle (1% glycerin in PBS) alone. Three days later, 5,000 HT1080lacZ-K15 cells were injected i.v. Alternatively, inoculation of tumor cells preceded the adenoviral transfer by 2 days (Fig. 1C). Mice were sacrificed and their livers and lungs were removed at 6 days (L-C1.5s) or 21 days (HT1080lacZ-K15) after inoculation. The right lobe of each lung and three samples of each liver, with a volume of $\sim$ 0.5 cm$^3$, were snap frozen in liquid nitrogen for biochemical analysis, and left lung lobes and remaining liver were stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal; Roche Diagnostics) as described (22). Blue multicellular foci on the surface of the livers (L-C1.5s) or lungs (HT1080lacZ-K15) were counted. Total metastasis burden was assessed by quantitative real-time PCR (RT-PCR) for lacZ. All animal experiments were done in compliance with the guidelines of the Tierschutzgesetz des Landes Oberbayern.

**Immunostaining.** Fixed sections of livers of the different groups were paraffin embedded for immunostaining. Dewaxing, rehydration, blocking, and antigen retrieval of liver sections (4 μm) were done as described (28). Sections were incubated with primary antibodies (proliferating cell nuclear antigen [PCNA] antibody, 1:2,000, Novocastra Laboratories Ltd.; cMet antigen [PCNA] antibody, 1:2,000, Santa Cruz Biotechnology) for 20 h at 4°C in PBS, 0.1% (v/v) Tween 20. StreptAB Complex/HRPDuett Mouse/Rabbit kit (Dako Cytomation) and 3,3′-diaminobenzidine (DAB) substrate (DAKO Liquid DAB+ Substrate-Chromogen Solution, DAKO Corp.) were used for detection. Sections were counterstained with Mayer’s hemalaun (Merck).

For detection of cMet activation, unfixed fresh liver samples were

**Figure 1.** TIMP-1–mediated reduction of formation of macrometastases but induction of liver-specific secondary spread of tumor cells. A and B, CD1<sup>nu/nu</sup> mice received the respective adenovirus 3 d before inoculation of L-C1.5s (A) or HT1080lacZ-K15 cells (B), respectively. Six or 21 d, respectively, after tumor cell inoculation, mice were sacrificed and livers and lungs were removed. A, top left, number of X-Gal–stained liver metastases overexpressing human TIMP-1 or N-TIMP-1 compared with the virus control group. Columns, mean; bars, SE. Add70-3: 67.6 ± 7.73, n = 5; AdTIMP-1: 38.50 ± 8.14, n = 6, P = 0.03; AdN-TIMP-1: 32.83 ± 8.32, n = 6, P = 0.009. Close-up pictures of the surfaces of representative X-Gal–stained L-C1.5s metastasis-bearing livers (bottom) show increased micrometastasis in livers with elevated levels of TIMP-1 or N-TIMP-1. Top right, the total L-C1.5s tumor burden in livers overexpressing the TIMP-1 variants was significantly increased compared with the virus control as detected by quantitative RT-PCR of lacZ in metastasis-bearing livers. Columns, mean of relative lacZ mRNA amount versus 18S RNA; bars, SE. Add70-3: 0.22 ± 0.04, n = 3; AdTIMP-1: 1.99 ± 0.80, n = 4, P = 0.044; AdN-TIMP-1: 1.96 ± 0.76, n = 4, P = 0.041. B, significant reduction of HT1080 lung metastasis by overexpression of human TIMP-1. Top left, columns, mean of the number of X-Gal–stained metastases over the whole surface of the left lung lobe; bars, SE. Add70-3: 38.1 ± 16.1, n = 16; AdTIMP-1: 2.6 ± 1.4, n = 10; P = 0.018. Significant increase of HT1080 metastasis in livers with elevated levels of TIMP-1. Top right, columns, mean of relative lacZ mRNA amount versus 18S RNA; bars, SE. Add70-3: 0.018 ± 0.002; AdTIMP-1: 0.040 ± 0.007, n = 3, P = 0.04. Bottom, close-up pictures of the surfaces of representative X-Gal–stained HT1080 metastasis-bearing liver. C, effects of TIMP-1 elevation after tumor cell inoculation on liver metastasis. CD1<sup>nu/nu</sup> mice received the respective adenovirus 2 d after inoculation of L-C1.5s. Six days after tumor cell inoculation, mice were sacrificed and livers were removed and X-Gal stained. The number of macrometastases (>0.2 mm) was counted on the whole surface of the liver. Top left, columns, mean; bars, SE. Add70-3: 45 ± 4, n = 4; AdTIMP-1: 44 ± 3, n = 4, P = 0.044. Top right, columns, mean of relative lacZ mRNA amount versus 18S RNA; bars, SE. Add70-3: 0.29 ± 0.16, n = 4; AdTIMP-1: 2.25 ± 0.62, n = 4. Bottom, X-Gal–stained livers. Representative pictures of each group. Bar, 200 μm. Asterisks, macrometastases; arrows, micrometastases.
TIMP-1 Promotes Liver Metastasis via HGF Signaling

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In inhibition studies, the signal induced by a specific kinase inhibitor.

Results

Reduction of experimental macrometastases but induction of scattered micrometastases in the liver by TIMP-1. To achieve elevated TIMP-1 expression in the host, we transduced full-length cDNAs of TIMP-1 by i.v. inoculation of adenoviral vectors into mice. Efficient adenoviral transduction of liver cells was determined by quantitative RT-PCR (Supplementary Fig. S1). Maintenance of transgene overexpression achieved by the adenoviral vectors until the end of all following metastasis assays was verified (Supplementary Fig. S1). To test the effects of elevated stromal TIMP-1 on tumor cell invasion, we used the well-established syngeneic in vivo metastasis assay with lacZ-tagged lymphoma cells (L-CL5s), which form hepatic metastatic foci following i.v. injection. Colonies that we defined as macrometastases in livers reached the cutoff size of 0.2 mm in this assay at day 6 after tumor cell inoculation. At this time point, the combination of successful extravasation right after tumor cell inoculation and proliferation of the extravasated tumor cells for at least six days resulted in multicellular foci of this size. Detected micrometastases (<0.2 mm) resulted from additionally invaded tumor cells if their number exceeds the 5,000 inoculated tumor cells.

Three days after adenoviral gene transfer, TIMP-1–transduced and control mice were challenged with L-CL5s tumor cells. The pattern of metastasis in TIMP-1–transduced mice was significantly altered compared with the control virus–transduced mice: Elevated levels of TIMP-1 correlated with significant reduction of the number of macrometastases (by 43%; \( P = 0.003 \); Fig. 1A, top left). However, on the level of micrometastases, no decrease but a drastic increase of scattered infiltrating tumor cells was detected in livers with elevated TIMP-1 (Fig. 1A, bottom). In one photographic frame (1.3 mm × 1.5 mm), already 623 micrometastatic foci at the liver surface were visible (Fig. 1A, bottom), showing that the number of scattered micrometastases in the entire liver by far exceeded the number of inoculated cells (5,000). Quantification of the tumor cell...
tag lacZ revealed an overall increase of total metastasis burden in livers transduced with TIMP-1 cDNA compared with the control ($P = 0.044$; Fig. 1A, top right), reflecting induction of micrometastasis.

To determine whether inhibition of macrometastasis but promotion of micrometastasis was a function of the metalloproteinase-inhibitory activity of TIMP-1, we investigated the effect of the N-terminal domain of human TIMP-1 (N-TIMP-1) harboring the metalloproteinase-inhibitory bioactivity on liver metastasis. Indeed, elevated levels of N-TIMP-1 significantly suppressed macrometastasis by 51% ($P = 0.009$; Fig. 1A) and promoted micrometastasis ($P = 0.041$; Fig. 1A), indicating that N-TIMP-1 is sufficient to evoke the described effects.

We used lacZ-tagged human fibrosarcoma cells (HT1080lacZ-K15) that typically form large pulmonary metastatic foci and only few single tumor cells as micrometastases, but no macrometastases, in livers (26) to exclude that the TIMP-1 effect was cell type dependent. After challenge of CD1<sup>nu/nu</sup> mice with HT1080lacZ-K15, significant reduction of lung metastasis by 93% was observed in TIMP-1–transduced animals compared with mice with virus control ($P = 0.018$; Fig. 1B, top left). Again, in livers with elevated TIMP-1 levels, a significant increase of the metastatic burden was found ($P = 0.04$; Fig. 1B, top right). No induction of HT1080lacZ-K15 micrometastasis was detected in lungs (data not shown). In the above experiments, no differences in metastasis between control virus–transduced mice and mice treated with vehicle alone were found, excluding effects of viral transduction itself (data not shown).

**Effects of TIMP-1 elevation on liver metastasis after tumor cell inoculation.** To determine whether TIMP-1 affects liver metastasis on two different levels, [i.e., on the level of initial manifestation and subsequent growth (leading to formation of macrometastases) or subsequent infiltration (micrometastases)], we inoculated L-CL.5s cells into CD1<sup>nu/nu</sup> 2 days before adenoviral transfer of TIMP-1 cDNA. The number of macrometastatic colonies was not affected compared with the control (Fig. 1C, top left). However, we still observed induction of micrometastasis compared with the control (Fig. 1C, bottom), resulting in a significantly increased total metastasis burden in livers with elevated TIMP-1 (Fig. 1C, top right). This indicates that micrometastases take advantage of the TIMP-1–modulated host microenvironment. In contrast, macrometastasis formation was only reduced when TIMP-1 was already elevated in the host at the time point of extravasation right after tumor cell inoculation (Fig. 1A).

**Proliferation activity of TIMP-1–induced scattered liver micrometastases.** To assess if the increased presence of invaded L-CL.5s cells in the liver was a function of TIMP-1–induced proliferation, we determined their proliferative status by examining PCNA expression. Indeed, infiltrating T-cell lymphoma cells in livers with elevated TIMP-1 or N-TIMP-1 were proliferatively active (Fig. 2A), indicating a proproliferative effect of the TIMP-1–modulated environment on these cells, accounting for the increase of total metastasis burden in the liver. A proproliferative environment was further indicated by the presence of proliferating hepatocytes in these groups (Fig. 2A, arrows). In the control livers, proliferation was restricted to the micrometastases (Fig. 2A, left).

**TIMP-1 induces HGF signaling in the liver.** To elucidate a molecular mechanism responsible for the TIMP-1–induced proproliferative and proinvasive environment in the liver, we tested for expression of HGF, also known as scatter factor. Elevated levels of HGF correlated with the TIMP-1–induced scattered metastatic metastasis-free ($P = 0.041$; Fig. 1C, bottom inset, controls without primary antibody for each slide. Black arrows, additionally stained micrometastases. D, TIMP-1–induced activation of cMet. Cryosections of metastasis-bearing (top) and metastasis-free (bottom) livers, respectively, were immunohistochemically analyzed for phosphorylated cMet (phospho cMet). Counterstaining was done with DAPI. Asterisks, lumen of blood vessels; arrows, staining of phosphorylated cMet. Bar, 100 μm.

**Figure 2.** Induction of hepatocyte proliferation and HGF signaling by elevated levels of TIMP-1. A, PCNA staining of L-CL.5s metastasis-bearing liver sections (4 μm) 9 d after adenoviral gene transfer and 6 d after L-CL.5s inoculation. Representative sections. Asterisks, PCNA-positive hepatocytes; arrows, PCNA-positive micrometastases. B, left, HGF protein level 9 d after gene transfer and 6 d after L-CL.5s inoculation. HGF protein in pools of three livers per group was quantified by ELISA (Addl70-3: 30.3 pg/mg; AdTIMP-1: 75.8 pg/mg; AdN-TIMP-1: 80.6 pg/mg). Right, HGF protein in metastasis-free livers 3 d after gene transfer. HGF protein in pools of three livers per group was quantified by ELISA (Addl70-3: 25.2 pg/mg; AdTIMP-1: 41.1 pg/mg; AdN-TIMP-1: 45.7 pg/mg). C, increased cMet expression in AdTIMP-1–transduced livers. Paraffin-embedded sections of L-CL.5s metastasis-bearing livers 9 d after adenoviral gene transfer and 6 d after tumor cell inoculation were immunohistochemically analyzed for cMet localization. Bottom inset, stronger staining for cMet was found within metastases compared with liver parenchyma; top inset, controls without primary antibody for each slide.

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phenotype (Fig. 2B, left). In fact, increase of HGF expression was already a component of the TIMP-1–altered liver environment as it could already be detected 3 days after adenoviral gene transfer (i.e., at the time point of tumor cell challenge; Fig. 2B, right). In addition, cMet, the tyrosine kinase receptor of HGF, was elevated throughout the tissue of metastasis-bearing livers transduced by either TIMP-1 species (Fig. 2C) and in macrometastases (Fig. 2C, insets).

Elevated expression of either TIMP-1 variant led to markedly increased HGF signaling, as detected by immunohistochemical staining of phosphorylated cMet throughout the liver parenchyma (Fig. 2D). In control animals, activated cMet was very low even in macrometastases (Fig. 2D, top). Importantly, this TIMP-1–mediated induction of HGF signaling was already present 3 days after TIMP-1 gene transfer (Fig. 2D, bottom), at the time point of tumor cell challenge, suggesting that a HGF pathway–connected metastatic niche has been formed.

Causal relationship between HGF signaling and TIMP-1–induced scattering of experimental liver metastasis. In TIMP-1–transduced animals, the scattered metastasis phenotype was already apparent 3 days after inoculation of L-CL5s cells (Fig. 3A, top). To investigate whether HGF signaling was essential for formation of scattered metastasis, we first transduced mice with AdTIMP-1 or Addl70-3 virus, treated the mice with a cMet inhibitor, and challenged with L-CL5s cells. Kinase inhibition prevented formation of micrometastasis (Fig. 3A, bottom), indicating that HGF signaling was essential for TIMP-1–induced micrometastatic spread. The TIMP-1–induced significant increase of total metastasis burden compared with the control (P < 0.001; Fig. 3B) was significantly reduced by cMet inhibition (P < 0.001; Fig. 3B). The number of multicellular metastatic foci was significantly reduced by elevated levels of TIMP-1 compared with the control (P = 0.048; Fig. 3C), indicating that formation of macrometastasis is not affected by HGF signaling.

Overexpression of TIMP-1 reduces metalloproteinase activity and increases activity of other proteases in the liver. As proteolytic activity is one prerequisite of metastasis, we next assessed whether TIMP-1–related changes in metastasis rely on modulated proteolytic activity. In situ zymography revealed gelatinolytic activity in macrometastatic foci in Addl70-3–transduced livers but not in foci of AdTIMP-1–transduced animals (Fig. 4A, left). As this was inhibitable by the canonical metalloproteinase inhibitor 1,10-phenanthroline (Fig. 4B, left), we showed that the achieved TIMP-1 levels indeed inhibited MMP activity in vivo. No MMP-9–derived proteolytic activity was detected in livers with elevated TIMP-1 (Fig. 4C), indicating the expected functional activity of TIMP-1 to inhibit proMMP-9 activation.

The gelatinolytic activity detectable in TIMP-1–elevated liver parenchyma distants from metastases (Fig. 4A, right) was by and large metalloproteinase independent, as it was only slightly inhibitable by 1,10-phenanthroline (Fig. 4B). Addition of broad-spectrum cysteine protease inhibitor E64 (34) or broad-spectrum serine protease inhibitor aprotinin (35) to the in situ zymography DQ-overlay revealed the remaining gelatinolytic activity as partly derived from serine and cysteine proteases (Fig. 4D). Elevated levels of N-TIMP-1 had the same effects (data not shown). These data indicate that increased expression of TIMP-1 in the liver led to an increase of metalloproteinase-independent proteolytic activity.

**Figure 3.** Reduction of TIMP-1–induced micrometastatic spread by cMet inhibition. A to C, C57BL/6nu/nu mice were transduced with AdTIMP-1 or Addl70-3. Three days later, mice were treated with a cMet-specific kinase inhibitor [2-(2,6-dichloro-4-[2-hydroxyethoxy]phenyl)-4-[(3-benzoxylphenyl)-5-[(3-hydroxypropyl-amino)- pyrimidin-4-yl]-N-H-imidazole] or vehicle alone and challenged by inoculation of L-CL5s cells. Three days after tumor cell inoculation, mice were sacrificed and livers were removed and X-Gal stained. A, pictures of the surface of representative livers of each treatment group. Bar, 0.1 mm. B, total metastasis burden in the liver as detected by quantitative RT-PCR of lacZ. Columns, mean; bars, SE. Addl70-3/vehicle: 0.059 ± 0.021; AdTIMP-1/vehicle: 0.728 ± 0.233; Addl70-3/cMet inhibitor: 0.040 ± 0.016; AdTIMP-1/cMet inhibitor: 0.051 ± 0.030; all n = 3. C, number of multicellular metastatic foci was counted on the whole surface of X-Gal–stained livers. Columns, mean; bars, SE. Addl70-3/vehicle: 55 ± 10; AdTIMP-1/vehicle: 36 ± 2; Addl70-3/cMet inhibitor: 42 ± 9; AdTIMP-1/cMet inhibitor: 25 ± 6; all n = 5.
expression (relative mRNA expression: 21.22 ± 1.89, n = 6, P < 0.001; Fig. 5). This difference also correlated with shorter time interval between detection of primary tumor and liver metastases [26.83 ± 5.81 months (n = 6) versus 13.75 ± 2.62 months (n = 8); P = 0.049].

Inhibition of cMet shedding by TIMP-1. Interestingly, although cMet protein was increased in livers with elevated TIMP-1 (Fig. 2C), mRNA expression of cMet was not up-regulated in these livers, neither at the time point of tumor cell inoculation (Fig. 5) nor in metastasis-bearing livers (Supplementary Fig. S2). Reduced shedding of cMet by TIMP-1–induced inhibition of metalloproteinase activity may account for the accumulation of cMet protein, but it was thus far unknown that TIMP-1 can inhibit its shedding. As this hypothesis cannot be addressed in vivo, we chose a murine nontumorigenic cell line expressing inhibitable metalloproteinases as well as cMet, thus providing all prerequisites for a proof-of-concept study. Overexpression of either TIMP-1 or N-TIMP-1 in NIH3T3 cells (Fig. 6A) resulted in significantly augmented membrane-bound cMet protein in these cells compared with the virus control (both P < 0.026; Fig. 6B), although RNA expression of cMet was not increased (both P > 0.33; Fig. 6C).

TIMP-1 indeed inhibited cMet shedding, as reduced levels of soluble cMet were detected in supernatant of cells overexpressing TIMP-1/N-TIMP-1 compared with the control (both P < 0.033; Fig. 6D). The elevated level of cell-associated cMet in cells overexpressing TIMP-1/N-TIMP-1 correlated with increased phosphorylation of cMet (Supplementary Fig. S3).

Activity of ADAM-10 regulates cMet shedding. Knockdown of ADAM-10 mRNA expression in NIH3T3 cells by 88% (Fig. 6A) led to a significant increase of membrane-bound cMet protein compared with the control (P = 0.018; Fig. 6B), whereas cMet mRNA expression was not enhanced (P = 0.2; Fig. 6C). ADAM-10 knockdown was verified on protein level (Supplementary Fig. S4). A significant reduction of shed cMet in the supernatant of cells with reduced ADAM-10 expression was detected (P = 0.021; Fig. 6D), indicating that depletion of ADAM-10 was sufficient to accumulate cMet on the cell surface. In addition, suppression of ADAM-10 mRNA expression significantly increased cMet signaling (Supplementary Fig. S3).

Together, these data suggest a molecular pathway regulated by TIMP-1 that could lead to a microenvironment with higher susceptibility to metastases.
Discussion

This study shows that host-derived TIMP-1 can promote liver metastasis by induction of HGF signaling. Until recently, the dominant concept of natural inhibitors of tumor-associated proteases focused on their capacity to repress activity of ECM remodeling enzymes, such as metalloproteinases. This view has undergone revision based on increased awareness of “degradome” functions of metalloproteinases and the tumor microenvironment and recognition of their participation during many stages of tumor progression (3).

The original hypothesis that TIMP-1 overexpression would yield a reduction in the number of successfully extravasated tumor cells into a target organ was based on numerous reports on the importance of MMPs for metastasis in many tumor models (36). In agreement with these and our previous studies indicating the importance of gelatinase inhibition for formation of macrometastases of the lymphoma model (28, 29), elevated host levels of TIMP-1 prevented macrometastases only when it was already increased at the time point of tumor cell inoculation. This confirms the already known effect of metalloproteinase inhibition by TIMP-1 on the level of extravasation.

In contrast to this inhibitory action on initial invasion, we reveal here for the first time an unexpected metastasis-promoting feature of TIMP-1 (i.e., induction of scattered invasion of tumor cells in liver parenchyma). The bulk of these micrometastases cannot derive from augmented extravasation of the L-CL5s cells because the number of the proliferative active and scattered micrometastases by far exceeded the number of inoculated tumor cells. Previous studies have reported that, even when metalloproteinases are required for metastasis in a particular tumor model, TIMP-1 does not always suppress metastasis (9, 37), supporting the idea that TIMP-1 possesses a broad range of biological activities, some of which may be independent of its metalloproteinase-inhibitory function (11, 38–40). Those variations of antitumorigenic efficacy of TIMP-1 have been suggested to depend on the concentrations of TIMP-1 (10, 41) or related to the proproliferative and antiapoptotic features of TIMP-1 (11, 12, 38).

The new aspect is that we found a proinvasive feature of TIMP-1 when elevated in the liver based on TIMP-1–induced stimulation of HGF signaling and downstream expression of metastasis-promoting genes. The N-terminal domain of TIMP-1, which includes the metalloproteinase-inhibitory domain, was sufficient to exhibit this effect, indicating that broad-spectrum inhibition of metalloproteinases is responsible for this scattering. In agreement with this notion, treatment with a synthetic broad-spectrum metalloproteinase inhibitor also leads to liver-specific promotion of metastases.

Figure 5. Common TIMP-1–associated alterations in gene expression in mouse and human. Mice were transduced with either control virus (Addl70-3) or AdTIMP-1. Three days later, mice were sacrificed and livers were removed. Normal liver samples of patients with metastatic colorectal cancer were obtained at least 2 cm distant from colorectal liver metastases during curative surgical interventions. Total RNA was isolated from murine and human liver samples and gene expression was assessed by quantitative RT-PCR. Human samples were initially tested for TIMP-1 expression and then divided into two subgroups according to their TIMP-1 expression levels (low TIMP-1: 98.9 ± 19.8, n = 6; high TIMP-1: 98.9 ± 19.8, n = 6; P < 0.001). All samples were tested for uPA, uPAR, PAI-1, tPA, matriptase, PCNA, MMP-2, MMP-9, ADAM-10, cathepsin G (Cath G), neutrophil elastase (NE), and Met mRNA expression. Columns, mean of gene expression in murine (Addl70-3 (n = 4) versus AdTIMP-1 (n = 3); uPA: 100 ± 16.3% versus 193.3 ± 20.3%; uPAR: 100 ± 15.3% versus 557.9 ± 63.2%; PAI-1: 100 ± 21.2% versus 5,083.0 ± 3,052.9%; tPA: 100 ± 18.4% versus 1,673.8 ± 465.2%; matriptase: 100 ± 4.4% versus 451.5 ± 33.8%; PCNA: 100 ± 82.7% versus 554.6 ± 80.3%; MMP-9: 100 ± 47.2% versus 494.1 ± 34.6%; MMP-2: 100 ± 45.9% versus 289.4 ± 63.2%; ADAM-10: 100 ± 35.7% versus 542.8 ± 397.4%; cathepsin G: 100 ± 52.6% versus 2,040.0 ± 721.1%; neutrophil elastase: 100 ± 66.0% versus 1,024.3 ± 541.1%; Met: 100 ± 9.3% versus 109.6 ± 8.9%) and human (low TIMP-1: uPA: 100 ± 16.6% versus 615.5 ± 61.2%; uPAR: 100 ± 20.7% versus 691.6 ± 131.1%; PAI-1: 100 ± 15.7% versus 107.1 ± 182%; tPA: 100 ± 10.8% versus 116.9 ± 18.6%; matriptase: 100 ± 18.1% versus 213.0 ± 24.4%; PCNA: 100 ± 24.3% versus 231.7 ± 50.7%; MMP-9: 100 ± 29.5% versus 2,146.8 ± 396.4%; MMP-2: 100 ± 30.6% versus 695.3 ± 590.9%; ADAM-10: 100 ± 11% versus 150.6 ± 33.9%; cathepsin G: 100 ± 31.9% versus 379.5 ± 107.8%; neutrophil elastase: 100 ± 75.5% versus 1,621.6 ± 339.2%; Met: 100 ± 17.0% versus 111.5 ± 27.6%) livers; bars, SE. *, P < 0.05.
metastasis in several xenograft models as well as in the syngeneic T-cell lymphoma model (42, 43). In addition, in the present study, promotion of liver metastasis was found, whereas lung metastasis was significantly reduced. Either TIMP-1 was not able to induce HGF signaling in the lung or induction of HGF signaling in the lung tissue has different effects on the formation of a premetastatic niche than in the liver parenchyma. However, several studies revealed that inhibition or overexpression of proteases or protease families evolves different effects on metastasis to liver versus lung (26, 42–44). This leads to the hypothesis that metastasis to the liver requires a different repertoire of proteases than metastasis to the lung and that the different microenvironments present in these organs react differentially on changed proteolysis. The underlying mechanism of the organ specificity of the premetastatic effect of TIMP-1 will need to be determined in future studies.

Upon elevation of TIMP-1, we observed augmented HGF as well as cMet protein in livers. Simultaneous increase of HGF and functional cell-associated cMet can explain the overall increase of HGF/cMet signaling. Elevated presence of the active form of HGF, which is required for signaling (15), can derive from the observed up-regulation of the HGF-activating proteases uPA and matriptase (45, 46) in the liver. Active matriptase and uPA (also activated by matriptase; ref. 46) can lead to an additional increase of proteolytic activity by initiating a protease activation cascade, including the activation of plasminogen by uPA and of some pro-MMPs by active plasmin (47). Elevated levels of TIMP-1 in the liver also increased expression of cathepsin G, also a gelatinolytic enzyme (48), as well as expression of neutrophil elastase, which also enhances plasminogen activation (49). Such proteases are likely the source of the increased nonmetalloproteinase-derived proteolytic activity observed in metastasis-bearing liver sections with augmented TIMP-1 levels in situ. Interestingly, the TIMP-1–provoked gene expression signature of the experimental model resembled in some instances (matriptase, uPA, uPAR, PCNA, MMP-9, and cathepsin G) the signature observed in liver samples of colorectal cancers in patients with elevated levels of TIMP-1, which is a hint on general molecular mechanisms provoked by elevated stromal TIMP-1. In the future, stable knockdown of cMet in host and/or tumor cells will further characterize the role of TIMP-1–induced cMet signaling in liver metastasis.

A link between metalloproteinase inhibition and enhancement of HGF/cMet signaling was revealed by an in vitro biochemical assay: Elevated levels of TIMP-1/N-TIMP-1 preserved activatable cMet on cell surfaces by reduced cMet shedding. Interestingly, suppression of ADAM-10 also prevented shedding of cMet, indicating that ADAM-10 is involved in the regulation of cMet shedding. Sheddases are already known for their capacity to regulate cell surface–associated tyrosine kinase receptors (50). The data indicate that preservation of cMet is induced by TIMP-1.
or N-TIMP-1 and may be a consequence of direct inhibition of ADAM-10 and thereby loss of its sheddase activity (4) or indirectly by inhibition of ADAM-10 activation.

Many tumor cell lines respond to elevated levels of HGF (scatter factor) with increased invasive behavior (15, 16). Indeed, the altered host environment fed back to the inoculated tumor cell lines, which expressed functional cMet (Supplementary Fig. S5). Thus, increased scattering of tumor cells in the liver parenchyma seems to be based on a reaction of the tumor cells to the TIMP-1-provoked change of homeostasis in the host. Identification of this TIMP-1–provoked prometastatic niche reveals a novel role for TIMP-1 during cancer progression and illuminates the intricacy of protease and protease inhibitor networks that regulate the penultimate step of cancer evolution and are one explanation for the association between tumor aggressiveness and increased levels of TIMP-1 in cancer patients.

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