Elevated Tissue Inhibitor of Metalloproteinase 1 RNA in Colorectal Cancer Stroma Correlates with Lymph Node and Distant Metastases

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ABSTRACT

Tissue inhibitor of metalloproteinase (TIMP) inhibits the proteolytic activity of several metalloproteinase centrally involved in tumor invasion and metastases. The purpose of this study was to determine the origin of TIMP-1 mRNA production in both human colorectal cancer (CRC) and metastatic liver lesions as well as define the relationships between TIMP-1 RNA expression and standard clinicopathological variables of CRC. Total cellular RNA, extracted from 56 CRC and 10 liver metastases, were examined by Northern blot hybridization. The mean-normal mucosa fold increase of TIMP-1 RNA was significantly elevated in both CRC (12.1 ± 1.7) and liver metastases (10.0 ± 3.6). No relationship was noted between TIMP-1 expression and tumor size, location or differentiation. Based on lymph node metastases status, significantly higher TIMP-1 RNA levels were found in CRC with metastases than in those without metastases (15.6 ± 3.3 versus 7.9 ± 1.3) (P = 0.04). Similarly, TIMP-1 RNA levels were higher in primary CRC with distant metastases than those without distant metastases (17.6 ± 4.1 versus 9.3 ± 1.9) (P = 0.04). In situ hybridization localized TIMP-1 mRNA predominantly in tumor stroma within spindle fibroblast-like cells rather than in cancer cells themselves. The correlation between the increased TIMP-1 mRNA level and advanced CRC stage noted in this study reflects a possible growth-promoting function for TIMP-1 in human CRC.

INTRODUCTION

Tumor metastases consists of a series of complex events including degradation and turnover of basement membrane and extracellular matrix (1, 2). This process requires numerous degradative enzymes including members of the MMP family. MMPs are zinc-dependent enzymes, secreted in latent proenzyme forms (1), whose activities have been shown to correlate with the metastatic phenotype (3). MMPs are regulated at numerous levels including via natural tissue inhibitor proteins such as TIMP (1, 2). Thus far, three TIMP genes (TIMP-1, TIMP-2, and TIMP-3) have been identified (4–6).

TIMP-1, a glycoprotein with an apparent molecular size of 28.5 kDa, first isolated from rabbit bone (7, 8), is produced by variety of human tissues and human tumor cell lines (5, 9–12). TIMP-1 inhibits the proteolytic activity of several MMPs including collagenase, gelatinase, and stromelysin and forms a 1:1 complex with activated interstitial collagenase. An inverse correlation between TIMP-1 levels and the metastatic potential of murine and human tumor cells (13) as well as recombinant TIMP-1 inhibition of metastatic lung colonization from i.v. injected B16-F10 tumor cells (14) suggest a possible therapeutic role for TIMP-1. However, since TIMP-1 is highly homologous with EPA, an autocrine growth factor for the erythroid leukemia cell line K562 (15), it has been suggested that TIMP-1 may also function as a growth factor. This notion is supported by our previously noted increase of TIMP-1 expression in a limited series of CRC specimens (16, 17).

Because of TIMP’s intriguing bifunctional properties, this study was undertaken to (a) document the origin of TIMP-1 mRNA production in both human CRC and metastatic liver lesions and (b) to correlate relative TIMP-1 RNA expression with standard clinicopathological variables.

MATERIALS AND METHODS

Tissue Processing

Surgical samples were obtained from the operating room immediately after resection in accordance with the guidelines of the human tissue review committee of Memorial Sloan-Kettering Cancer Center. They were quick frozen in liquid nitrogen and stored at -80°C until processed. Samples were handled and stored under strict RNase-free conditions. Specimens were from the tumor edge, avoiding its necrotic center. Normal mucosa specimens were obtained from the surgical resection margin by sharp dissection between the mucosa and the muscularis mucosa (18).

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3 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CRC, colorectal cancer; EPA, erythroid-potentiating activity; β2-M, β2-microglobulin.
Case Material
Specimens consisted of (a) primary CRC and paired adjacent mucosa and (b) liver metastases from CRC and paired normal liver. None of the patients had received prior radiation or chemotherapy.

The Surgical Pathological Laboratory of Memorial Sloan-Kettering Cancer Center performed routine histopathological examination on the resected specimens using hematoxylin and eosin staining. All patients were staged according to the TNM classification system (19).

DNA and RNA Probes
A probe of the human TIMP-1 gene, a 900-bp EcoRI/KpnI restriction endonuclease DNA fragment (20), and a cDNA probe of β2-M (21), which is equally expressed in both proliferating and nonproliferating cells, were performed. DNA fragments were purified by low melting agarose gels and recovered using GeneClean (BIO 101 Inc., La Jolla, CA). Probes were radioactively labeled with [35S]UTP using a random, primed DNA labeling kit (Ref. 22; Boehringer Mannheim Biochemical).

For in situ hybridization studies, sense and antisense 35S-labeled RNA probes were prepared from human TIMP-1 cDNAs cloned in Bluescript KS+ (Stratagene). TIMP-1 sense probes were made by T3 polymerase following digestion with KpnI, and antisense probes were generated by T7 polymerase after EcoRI template digestion. Transcribed RNA was labeled with [α-35S]UTP (1200/Ci/mmol, Dupont NEN). DNA template was removed by incubating with 1 p4sacp RNase-free DNase. Ten μg tRNA were added and samples were extracted with phenol-chloroform. RNA probes were hydrolyzed with sodium carbonate buffer (pH 10.2) for 60 mm at 60°C, neutralized, and ethanol precipitated. Probes were redissolved in 10 mM DTT at a final concentration of 1.5 ng/μl and stored at −20°C.

Northern Blot Hybridization
Total RNA Isolation. Frozen tissue specimens were homogenized in 4 μl guanidinium thiocyanate, and total RNA and DNA were separated by ultracentrifugation through a cesium chloride cushion, as previously demonstrated (16, 18).

RNA Analyses. Ten μg of total cellular RNA/lane were denatured in 50% formamide and 6% formaldehyde for 15 min at 65°C, chilled on ice, and separated on a 1.0% agarose/6.8% DNAse. Fragments were transferred to a Duralon-UV membrane (Stratagene) by capillary blotting in 10X SSC for 16 h. Membranes were UV cross-linked with 120,000 μJ/cm2 using a UV Stratallinker 1800 (Stratagene, La Jolla, CA). Blots were prehybridized (42°C, 50% formamide, 10% dextran sulfate, 1% SDS, 1 μM NaCl, and 100 μg/ml denatured salmon sperm DNA) for 5 h and then hybridized overnight in the same solution with a 32P-labeled probe. The blots were washed to a final stringency of 65°C in 0.1X SSC and 0.1% SDS. Autoradiography was performed at −80°C with an intensifying screen. Ethidium bromide staining of gels revealed equal RNA loading. To further ensure that comparable amounts of RNA from both tumor and adjacent normal tissue had been transferred, blots were rehybridized with a β2-M probe (21).

Densitometric Quantitation. The extent of hybridization was quantitated with an LKB XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden; Ref. 18). β2-M mRNA transcripts were used as internal controls. The fold increase of the 0.9-kb TIMP-1 RNA in each tumor relative to its corresponding adjacent normal tissue was calculated after normalizing for β2-M RNA expression:

\[
T/N=(T_{TIMP-1};T_{β2-M})/(N_{TIMP-1};N_{β2-M})
\]

where T is tumor and N is normal tissue.

In Situ Hybridization
Tissue Fixation and Embedding. Specimens were obtained immediately after resection and fixed in RNase-free 4% paraformaldehyde overnight at 4°C, sequentially dehydrated with 50, 70, 85, 95, and 100% ethanol, and embedded in paraffin as described previously (23).

Hybridization. Paraffin sections (5–10-μm-thick) were dried overnight at 42°C and deparaffinized in xylene. The tissue was rehydrated by passing it through graded ethanol. Slides were incubated with proteinase K, and then washed in freshly prepared triethanolamine buffer containing 0.25% acetic anhydride. The labeled probes were diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.1 μM DTT, and Denhardt’s solution. The slides were covered and incubated at 57°C overnight in a 50% formamide, 2X SSC humidified chamber.

Washes. After hybridization, the coverslip was gently removed in 4X SSC and 10 mM DTT. Slides were washed at room temperature in 4X SSC for 1 h, then in 2X SSC, 50% formamide, and 10 mM DTT solution at 68°C for 40 min. Slides were treated with 20 μg/ml RNase-A in 20 mM Tris, pH 7.5, 0.5 M NaCl, and 1 mM EDTA at 37°C for 30 min, and then washed in the same buffer without RNase-A for 30 min. The final wash was in 2X SSC, 50% formamide, and 1 mM DTT at 68°C for 40 minutes. 2X SSC for 5 min at room temperature, and 0.1X SSC for 15 min at 50°C. Slides were dehydrated through graded ethanol and air dried.

Autoradiography. Autoradiographic detection of the hybrids was carried out by dipping in NTB-2 emulsion (Eastman Kodak, Rochester, NY) at 42°C under soft light and dried at room temperature for at least 2 h. The slides were placed in a light-tight box at 4°C for 1 to 2 weeks. After exposure, slides were subsequently developed in Kodak D-19 developer for 4 min at 15°C, washed in water, and fixed in Kodak fixer. Slides were counterstained with hematoxylin and eosin and then mounted for light microscopy. Silver grains were visualized by dark-field microscopy.

Statistical Analyses
The difference of standardized TIMP-1 between tumor and paired normal tissue was assessed by the paired t test. The relationship between TIMP-1 and clinical variables among two groups was analyzed using the Student t test. The difference in TIMP-1 among multigroups was studied using the method for ANOVA.

RESULTS
Characteristics of Patients. This study includes 56 primary CRC patients (2 cases with synchronous liver metastases).
TIMP-1 RNA overexpression was lower in rectal cancers than colon cancers. The relationship between TIMP-1 RNA overexpression and sex, age, tumor size, and location is shown in Table 1. There was no significant association between TIMP-1 RNA level and sex, age, tumor size, and location. Although the TIMP-1 RNA level progressively increased in going from the primary CRC group with no metastases to the group with lymph node metastases and distant metastases, a significant correlation was noted only between the Dukes’ A-B and Dukes’ C stages, respectively. Although the TIMP-1 RNA level progressively increased in going from the primary CRC group with no metastases to the group with lymph node metastases and distant metastases, a significant correlation was noted only between the Dukes’ A-B and Dukes’ D stages (P < 0.05).

TIMP-1 RNA overexpression in primary CRCs was greater in CRCs with mesenteric lymph node involvement (N_1-3) (15.6 ± 3.3) than those without lymph node metastases (N_0) (7.9 ± 1.3; P = 0.04). Furthermore, patients with distant metastases (M_1) (liver, lung) also demonstrated a statistically significant elevation of TIMP-1 RNA levels in the primary CRC when compared to patients without distant metastases (M_0) (17.6 ± 4.1 versus 9.3 ± 1.9; P = 0.04).

Localization of TIMP-1 in CRCs and Liver Metastases. In situ hybridization analysis was performed using paraffin-embedded tumor sections from 26 CRCs, 3 adenomas, and 10 liver metastases from CRC. Sections from tumor and corresponding normal tissue were hybridized with 35S-labeled TIMP-1. The presence of silver grains in dark-field micrography were representative of positive in situ hybridization. All 26 CRCs and 10 liver metastases were positive for TIMP-1 expression. Intensity of staining varied between different cases and in different areas of the same tumor. However, relative to adenomas and corresponding normal mucosa from the resection margin, TIMP-1 RNA expression was limited to stroma cells immediately surrounding cancer cells. The control sense probes for TIMP-1 showed only background autographic signals (data not shown). Since silver grain signals occurred only with the antisense probe and none were detected with the sense probe, the hybridization was felt to be specific. Fig. 3 exhibits TIMP-1
**Table 1** Correlation between TIMP-1 overexpression in primary colorectal cancers and clinicopathological parameters

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<th>Parameter</th>
<th>No. of cases</th>
<th>Sex</th>
<th>%</th>
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<sup>a</sup> T test for comparison of two groups, F test for comparison of more than two groups.

<sup>b</sup> P values is significantly different.

mRNA expression in cancer stroma and not in surrounding normal mesenchymal cells and mucosa.

In primary CRC, as shown in Fig. 4, TIMP-1 signals were strongest within the stroma cells of tumors, while the cancer epithelial cell signals were very weak or absent. In liver metastases, shown in Fig. 5, TIMP-1 signals were similarly seen predominantly in the peritumor stroma cells. Furthermore, Figs. 4 and 5 demonstrate that TIMP-1-labeled cells are clearly spindle fibroblast-like stroma cells.

**DISCUSSION**

Our results clearly demonstrate that TIMP-1 RNA is significantly overexpressed in all stages of primary CRC when compared to corresponding adjacent normal tissue. The striking correlation between TIMP-1 RNA overexpression in primary CRC with mesenteric lymph node and distant organ metastases is unique and suggests a possible central role for TIMP-1 in CRC invasion and metastases.

Previous studies (16, 17) have demonstrated, in a small series of specimens, elevated TIMP-1 RNA (16) and protein (17) levels in human CRC. In addition, we demonstrated that the extent of TIMP-1 mRNA overexpression was greatest in the more advanced CRCs, based on Dukes' staging system. In the present study, we demonstrate that TIMP-1 RNA is predominantly produced by the CRC stromal cells, rather than CRC themselves, and that the extent of this overexpression correlates very closely with the presence of mesenteric lymph node involvement and distant metastases, but not with the extent of bowel wall invasion (T level). The clinical significance of this observation lies on the potential utility of TIMP-1 measurements in primary CRC in order to differentiate, among similar T level CRCs, those patients that may or may not have synchronous lymph node involvement and distant metastases. This form of a priori knowledge could have a profound impact on how we manage CRC patients, in particular rectal cancers where sphincter-saving surgery could be selectively performed on patients.
**Fig. 3** In situ hybridization using a $^{35}$S UTP-labeled TIMP-1 antisense RNA probe on colon cancer. A and B, dark-field and corresponding bright-field micrographs, respectively. × 250. In dark-field micrographs, TIMP-1 RNA expression corresponds to silver grains. As noted, the intensity of TIMP-1 RNA expression is greatest in the pericancer stroma. Higher magnification of area indicated by arrows in B is shown in C. × 750. Ca, cancer; Mc, mesenchymal cells; Mu, mucosa.
TIMP-1 RNA Expression in CRC and Liver Metastases

with rectal cancers known to be free of regional and distant metastases.

Biologically, however, these findings are inconsistent with the notion that TIMP-1 acts as a functional inhibitor of tumor cell invasion. Investigators have previously demonstrated an inverse correlation between the TIMP-1 level and metastatic potential in vitro and in several human and mouse tumor models (13, 14, 24–28). Reduction of TIMP-1 activity via transfection of antisense TIMP-1 into noninvasive Swiss 3T3 cell lines increased in vitro amnion invasion and exhibited greater metastatic ability in vivo (13). Conversely, i.p. injection of recombinant human TIMP-1 (rTIMP-1) to mice inhibited lung colonization of iv delivered B16-F10 melanoma cells (14). Administration of recombinant TIMP-1 has also been shown to reduce the lung colonization of highly invasive ras-transformed rat embryo fibroblasts (28). More recently, transfection of B16-F10 melanoma cells resulting in overproduction of TIMP-1 results in a significant reduction in their in vitro invasive ability. This reduction correlated with the level of TIMP-1 overexpression (25). Furthermore, a TIMP-1 up-regulated cell line grew more slowly in culture when compared to the parental B16-F10 cell (24).

Overexpression of TIMP-1 mRNA has also been reported in non-Hodgkin’s lymphoma (29). A subgroup of high-grade immunoblastic lymphomas expressed high levels of TIMP-1 mRNA, whereas lower grade follicular lymphomas expressed low levels of TIMP-1 transcripts. In addition, long-term survival past 1 year was limited to those individuals whose tumors contained lower levels of TIMP-1 mRNA (29).

A possible explanation for elevated TIMP-1 RNA expression in CRC with regional and distant metastases is that metastatic CRC cells induce surrounding stroma cells such as macrophages to produce MMPs. Elevated local MMP concentration would then lead to increased TIMP-1 production. As a result, high levels of TIMP-1 may reflect high levels of metalloproteinase in tumor tissue. This is supported by the previously noted increased level of MMPs in advanced CRC (30–32). Another possibility is that TIMP-1 may possess growth-promoting function in addition to proteinase inhibitory activity in CRC.

TIMP-1 shares sequence identity with the EPA gene (20). EPA is a 28-kDa glycoprotein with in vitro erythroid burst-promoting activity and growth factor properties (15, 33). TIMP-1 is also an effective in vivo stimulator of erythropoiesis (34, 35). Several studies have shown that TIMP-1 has growth-promoting properties (15, 36, 37). TIMP-1 stimulates the proliferation of human skin keratinocytes and gingival fibroblasts (37). Hayakawa et al. (38) have found that TIMP-1 accounts for a significant portion of the growth factor activity of serum: it has the capability of stimulating a wide range of human and bovine cell lines, including those derived from tumors (human breast:

Fig. 4 Detection of TIMP-1 mRNA by in situ hybridization in primary colon cancer. Sections were hybridized with a 35S-labeled antisense RNA probe specific for TIMP-1 mRNA. In multiple sections (A-D), positive TIMP-1 mRNA expression is principally in fibroblast-like stroma cells. Bars, 25 μm.
Fig. 5 Detection of TIMP-1 mRNA by in situ hybridization in liver metastases from CRC. Sections were hybridized with a 35S-labeled antisense RNA probe specific for TIMP-1 mRNA. In multiple sections (A-D), positive TIMP-1 RNA expression is principally in fibroblast-like stroma cells. Bars, 25 μm.

We have demonstrated that TIMP-1 RNA is overexpressed in the peritumor stroma of CRC and liver metastases when compared to corresponding adjacent normal tissue. To our knowledge, this is the first report of quantitative analysis of TIMP-1 RNA levels in liver metastases from human CRC. In addition, we note a striking correlation between peritumor stroma TIMP-1 RNA overexpression and synchronous lymph node and distant metastases. These data suggest an important role for stromal production of TIMP-1 in CRC progression and metastasis. Our results demonstrate the potential identification of patients with synchronous CRC metastases via measurements of TIMP-1 RNA levels in primary CRC. Prospective studies are in progress evaluating the sensitivity and specificity of such measurements.

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906 TIMP-1 RNA Expression in CRC and Liver Metastases


Elevated tissue inhibitor of metalloproteinase 1 RNA in colorectal cancer stroma correlates with lymph node and distant metastases.


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