Stimulation of Mammary Tumorigenesis by Systemic Tissue Inhibitor of Matrix Metalloproteinase 4 Gene Delivery

Yangfu Jiang, Mingsheng Wang, Mahmut Y. Çeliker, Yiliang E. Liu, Qing Xiang Amy Sang, Itzhak D. Goldberg, and Y. Eric Shi

Abstract

Tissue inhibitors of matrix metalloproteinase (TIMPs) are multifunctional proteins with both matrix metalloproteinase (MMP) inhibitory effects and growth-regulatory activity. TIMPs inhibit MMP activity, suggesting a use for cancer gene therapy. However, here we report that systemic administration of human TIMP-4 by electroporation-mediated i.m. injection of naked TIMP-4 DNA stimulates tumorigenesis of human breast cancer cells in nude mice. Consistent with tumor stimulation, TIMP-4 up-regulates Bcl-2 and Bcl-XL protein. TIMP-4 also inhibits apoptosis in human breast cancer cells in vitro and mammary tumors in vivo. A synthetic MMP inhibitor BB-94 did not have such antiapoptotic effect. Analysis of TIMP-4 expression in human mammary specimens indicates that TIMP-4 protein is increased in mammary carcinoma cells compared with normal mammary epithelial cells. These data indicate an antiapoptotic activity in breast cancer cells and a tumor-stimulating effect of TIMP-4 when administered systemically.

Introduction

Overproduction and unrestrained activity of MMPs have been linked to malignant conversion of tumor cells. Augmented MMP activity is associated with the metastatic phenotype of carcinomas (1–4). Decreased production of TIMP could also result in greater MMP activity, leading to increased invasive potential of cancer cells (5, 6). In fact, tumor invasion and metastasis can be inhibited by up-regulation of TIMP expression in tumor cells (7–13). Alternatively, down-regulation of TIMP-1 and TIMP-2 have been reported to contribute significantly to the tumorigenic and invasive potentials (14–16). In addition to inhibiting tumor cell invasion and metastasis, overexpression of TIMPs in tumor cells also inhibits primary tumor growth (8–13).

TIMPs have been shown to be multifunctional factors. In addition to their anti-MMP activity, TIMPs also regulate cell growth. The stimulatory effect on cell growth was initially recognized when TIMP-1 and TIMP-2 were identified having erythroid-potentiating activities (17, 18). It is now clear that TIMP-1 and TIMP-2 are also mitogenic for nonerythroid cells, including normal keratinocytes, fibroblasts, lung adenocarcinoma cells, and melanoma cells (19–21). In addition, recent evidence indicates that the TIMP family is involved in apoptosis. Whereas it has been demonstrated that TIMP-1 and TIMP-2 have antiapoptotic effects in mammary epithelial cells and lymphocytes (22–24), TIMP-3 induces apoptosis (25). The role of TIMP-4 on apoptosis has not been identified.

Although the inhibitory effect of TIMP on tumor growth and metastasis was achieved by local expression of the TIMP genes in tumor cells, most MMPs and TIMPs are not expressed in genetically altered cancer cells but rather synthesized and secreted by adjacent stromal fibroblasts (26–28). Potential therapeutic applications of TIMPs for cancer treatment is limited by the lack of a method for systemic administration of TIMPs, which can reach distant tumor locations and by the lack of systemic assessment of the net effect between their tumor-suppressing MMP inhibitory effect and the cell survival pro-tumor activity. An imbalance between MMPs and TIMPs in favor of enzymatic inhibition might be important in inhibiting tumor angiogenesis and malignant progression. It was reported that i.p. injection of recombinant TIMP-1 inhibited the invasion of murine melanoma cells and reduced lung colonization, whereas recombinant TIMP-1 had no significant effect on the tumor growth (29, 30). Our previous study indicated that TIMP-4 inhibited tumor growth and metastasis when it was transfected into human breast cancer cells (13). In the current study, we investigated the effect of systemic TIMP-4 gene delivery on mammary tumorigenesis. Unexpectedly, we demonstrated for the first time that systemic delivery of TIMP-4 by i.m. administration of naked TIMP-4 DNA significantly stimulated mammary tumorigenesis in vivo.

Materials and Methods

Preparation of Recombinant Human TIMP-4 Protein and Anti-TIMP-4 Antibody. rhTIMP-4 protein and anti-TIMP-4 antibody were prepared as described previously (31). Briefly, rhTIMP-4 was expressed in baculovirus-infected insect cells and purified to homogeneity by a combination of cation exchange, hydrophobic, and size-exclusion chromatography. The purified protein migrated as a single 

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3 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitors of MMP; rhTIMP, recombinant human; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; PCNA, proliferating cell nuclear antigen.
nistics, Germany) assay were performed according to the manufacturers’ instructions. The proliferative index (PCNA) and the apoptotic index (TUNEL) were evaluated by the percentage of cells scored under a light microscope at 200-fold magnification.

In Situ Hybridization. Deparaffinized and acid-treated sections (5-μm thick) were treated with proteinase K, prehybridized, and hybridized overnight with digoxigenin-labeled antisense transcripts from a TIMP-4 cDNA insert. The TIMP-4 antisense probe is a 550-bp fragment from nucleic acids 130 to 683. The full-length TIMP-4 cDNA was cut by KpnI and SmaI; the 550-bp insert was subcloned into Bluescript II plasmid, and the resulting plasmid was named as Bluescript TIMP4-B. The 550-bp antisense probe was generated by SmaI digestion of Bluescript TIMP4-B plasmid, followed by T7 polymerase. After hybridization, RNase treatment and three stringent washes were carried out, sections were incubated with mouse antidigoxigenin antibodies (Boehringer), followed by incubation with biotin-conjugated secondary rabbit anti-mouse antibodies (Dako). The colorimetric detection was performed by a standard indirect streptavidin-biotin immunoreaction method using Universal LSAB kit (Dako) according to the manufacturer’s instructions.

i.m. Injection of Plasmid and Electroporation. Fifty μl (150 μg) of TIMP-4 plasmid DNA (TIMP-4 cDNA in pCI-neo mammalian expression vector; Promega Corp., Madison, WI) or empty vector (the parental plasmid) as a control was injected into the bilateral tibialis anterior muscles of 6-week-old of female nude mice using a disposable insulin syringe with a 25-gauge needle. For electroporation, a pair of electrode needles was inserted into the muscle with a 5-mm gap within the DNA injection sites, and electric pulses were delivered using an electric pulse generator Electro Square Porator ECM 830 (Genetronics, Inc., San Diego, CA). Three pulses of 150 V each were delivered to the injection site at a rate of one pulse/second, each pulse lasting for 50 ms. Then, three pulses of the opposite polarity were applied.

Tumor Growth in Athymic Nude Mice. Nude mouse tumorigenic assay was performed as we described previously (13). Briefly, parental MDA-MB-435 cells were grown to 80–90% of confluence in 150-cm² dishes and were harvested by incubation with 5 mM EDTA in PBS. EDTA was neutralized with medium containing serum. The cells were washed twice with serum-free medium, counted, and resuspended in serum-free Iscove’s modified medium at a concentration of 5 × 10⁶ cells/ml. Approximately 0.5 × 10⁶ cells (0.1 ml) were injected into 5–6-week-old female athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. The animals were ear tagged. Primary tumor growth was assessed by measuring the volume of each tumor at weekly intervals. Tumor size was determined at intervals by three-dimensional measurements (mm) using a caliper.

Cell Death Assay. Human breast cancer MD-MBA-435 cells were maintained in monolayer culture in Iscove’s modified medium containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The effect of recombinant human TIMP-4 protein on cell death in response to Adriamycin was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulphonyl]1H-tetrazo-

ium-5-carboxanilide inner salt assay (Roche Molecular Biochemicals). Briefly, cells were cultured in 96-well plates at 3000 cells/well. Cells were incubated with or without 80 nM recombinant human TIMP-4 protein (29). After 24 h, cells were treated with 0.5 μM of Adriamycin. Forty-eight h later, cells were incubated at 37°C with 100 μl of 2,3-bis[2-methoxy-4-nitro-5-

sulphonyl]1H-tetrazolium-5-carboxanilide inner salt labeling mixture for 4 h; absorbance was measured with ELISA plate reader at 490 nm.

Immunoblot Analysis. Equal amounts of protein were subjected to 12% SDS-PAGE and transferred to Hybond-C membrane (Amersham, Arlington Heights, IL). Antibodies against bcl-2, bcl-XL, bax, Fas, and Fas ligand were purchased from Oncogene Research Products. Western blot analyses were carried out using the appropriate antibody as noted in the figure legends, and protein bands were visualized with horseradish peroxidase-conjugated anti-

IgG antibody and enhanced chemiluminescence according to the manufacturer’s recommendations (Amersham).

Results

Expression of TIMP-4 in Human Breast Samples. To assess the biological relevance of TIMP-4 on mammary tumors, we first analyzed TIMP-4 protein expression in human mammary specimens including both normal glands and malignant carcinomas. A total of 40

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**Fig. 1. Expression of TIMP-4 protein and mRNA in human mammary gland.** Cells stained brown indicate TIMP-4 expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained epithelial and stromal cells. Sections in A–E are immunohistochemical staining, and section F is in situ hybridization. A, strong TIMP-4 protein staining in a mammary ductal carcinoma in situ. B, as a negative control, no signal of TIMP-4 protein was detected in the same sample when the antibody was preincubated with TIMP-4 protein. C, strong positive TIMP-4 staining in infiltrated malignant breast cancer cells (arrows) in comparison with much weaker TIMP-4 staining in surrounding residual normal mammary lobule epithelial cells. D, relatively high levels of TIMP-4 staining in malignant breast cancer cells (arrowhead) compared with surrounding residual normal mammary ductal epithelial cells (arrow). E, strong positive staining of TIMP-4 protein in malignant epithelial cells; in contrast, nondetectable or much less TIMP-4 staining was observed in stromal cells surrounding ductal carcinoma in situ. F, in the same slide as E, TIMP-4 mRNA was detected in stromal cells surrounding the ductal carcinoma in situ but not in malignant epithelial cells. The section was also hybridized with the sense probe, and no detectable background staining was observed at the same conditions for the antisense probe.
human breast cancer specimens were analyzed. A strong TIMP-4 staining (Fig. 1A) was observed in 9 of 10 breast carcinomas. We also selected 15 human breast sections that contain both infiltrated breast cancer cells and residual normal breast epithelial cells to assess TIMP-4 protein expression in normal versus cancer cells in the same tissue section. Whereas minimal TIMP-4 staining was observed in residual normal lobular and ductal breast epithelial cells, a significant TIMP-4 staining was observed in the infiltrating malignant breast cancer cells (Fig. 1, C and D).

To localize the cellular source of the TIMP-4 expression in mammary gland, we performed in situ hybridization analysis. Fourteen of 15 in situ hybridization analyses showed that TIMP-4 mRNA was expressed in stromal cells but not in epithelial cells. Although TIMP-4 mRNA was detected in the stromal fibroblasts surrounding the carcinoma (Fig. 1F), in the same sample, the TIMP-4 protein was primarily localized on the malignant breast epithelial cells (Fig. 1E). The expression of TIMP-4 mRNA in stroma is consistent with the inability to detect the TIMP-4 mRNA in most breast cancer cell lines by Northern blot (32).

**Systemic Tumor Stimulating Effect of i.m. Administered TIMP-4 Gene.** Because TIMP-4 is synthesized in mammary stromal cells, secreted, and accumulated on cancer cells, local expression of TIMP-4 in cancer cells by gene transfection may not mimic the in vivo stromal-epithelial interactions. To examine the functions of increased TIMP-4 protein expression on mammary tumorigenesis and to study the effect of systemic administration of TIMP-4 on tumor growth, we undertook a gene therapy approach through i.m. administration of TIMP-4 expression plasmid. On the basis of published data on i.m. delivery of DNA plasmid (33), we selected two doses (50 and 150 μg) of TIMP-4 expression plasmid for i.m. injection, followed by electroporation. Sera were collected prior to the injection and at 4, 8, 13, and 21 days after the injection. TIMP-4 protein levels were determined by Western blot analysis. For the dose of 50 μg of plasmid, serum levels of TIMP-4 were slightly increased over control at day 4 (data not shown). However, at the dose of 150 μg of plasmid, serum levels of TIMP-4 were significantly increased. As seen in Fig. 2A, although there was a minimal TIMP-4 protein in the plasma prior to the injection, when 150 μg of plasmid was administrated, the amount of TIMP-4 was increased 3-fold at 4 days after the injection. A significant amount TIMP-4 protein was observed at 8 and 13 days with a 19- and 29-fold increase over control, respectively.

We injected either TIMP-4 plasmid or control plasmid into i.m. sites of nude mice 5 days before inoculation of MDA-MB-435 breast cancer cells and every 7 days thereafter. Three independent experiments were done to confirm reproducibility, and the data from these experiments are summarized in Table 1. When 5 × 10⁵ of cells were injected, tumors developed after a lag phase of 5–7 days. There was no significant difference in tumor incidence among control and TIMP-4 injected mice. However, the tumor volume of TIMP-4-injected mice was significantly larger than that of empty vector injected mice. Fig. 2B shows a representative of experiment 1. The size of tumors in TIMP-4-injected mice were 283, 327, 253, and 171% of that in control mice at days 7, 10, 15, and 21, respectively. Consistent with this data, a strong immunohistochemical staining of TIMP-4 was observed in breast cancer xenografts from TIMP-4-injected mice (Fig. 2D), whereas very weak TIMP-4 signal was detectable in xenografts from control mice (Fig. 2C).

In a separate experiment, we tested whether systemic administration of the TIMP-4 gene could increase the tumor incidence when the number of injected tumor cells was reduced. When the number of injected MDA-MB-435 cells was reduced from 5 × 10⁵ to 2 × 10⁵, the tumor incidences were greatly reduced from 80% (13 of 16) to 5% (1 of 18) at week 1 and from 90% (15 of 16) to 40% (7 of 18) at week 2, respectively. However, when TIMP-4 was preadministered to the mice inoculated with 2 × 10⁵ tumor cells, a significant increase in
Table 1 Effects of systemic administration of TIMP-4 plasmid on mammary tumorigenesis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment group</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>17 ± 5</td>
<td>26 ± 9</td>
<td>80 ± 29</td>
<td>314 ± 68</td>
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<tr>
<td></td>
<td>TIMP-4</td>
<td>51 ± 2</td>
<td>85 ± 9</td>
<td>202 ± 34</td>
<td>537 ± 86</td>
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<td></td>
<td></td>
<td>0.006</td>
<td>0.007</td>
<td>0.013</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>21 ± 6</td>
<td>85 ± 19</td>
<td>293 ± 46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIMP-4</td>
<td>45 ± 8</td>
<td>164 ± 34</td>
<td>538 ± 70</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0.04</td>
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<td>0.01</td>
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<tr>
<td>3</td>
<td>Control</td>
<td>14 ± 6</td>
<td>91 ± 32</td>
<td>337 ± 99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIMP-4</td>
<td>41 ± 10</td>
<td>182 ± 63</td>
<td>521 ± 102</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.02</td>
<td>0.024</td>
<td>0.04</td>
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</tbody>
</table>

TIMP-4 or control plasmid (150 µg) was injected intramuscularly with electroporation 5 days before injection of cells and every 7 days thereafter. Six hundred thousand MDA-MB-435 cells (150 µl) were injected at day one into the mammary fat pads of 6-week-old female nude mice, and tumor volumes and lung micrometastasis were determined as described in “Materials and Methods.” Volumes are expressed as means ± SE (number of tumors assayed). For each experiment, there were a total 10 injections for five mice in each group, and each mouse received two injections. Statistical comparisons were made using the two-tailed Student’s t-test. Mice were sacrificed at day 21, and no lung metastases were observed.

Discussion

The blocking of MMP activity by TIMPs might be important in inhibiting tumorigenesis and subsequent malignant progression. However, some TIMP members also have a growth-stimulating and anti-apoptotic effect, which may promote tumorigenesis. These conflicting facts prompted us to investigate the role of systemic expression of TIMP-4 on mammary tumorigenesis. The significance of the current study is 4-fold: (a) more TIMP-4 protein accumulated in mammary cancer cells versus normal cells in clinical specimens; (b) TIMP-4 stimulates Bcl-2 and Bcl-XL protein expression and protects breast tumor cells in vitro and mammary tumor xenografts in vivo from apoptosis; (c) electroporation-mediated i.m. delivery of naked TIMP DNA can provide a sustained high level of TIMP-4 protein in serum; and (d) results in a significant stimulation of the growth of breast cancer cells.

The observation of increased TIMP-4 protein in malignant cancer...
cells versus normal cells in human mammary specimens prompted us to investigate the role of TIMP-4 on mammary tumorigenesis. In contrast to the previous approach of local expression of TIMP-4 by transfection of cancer cells with the gene (13), we undertook a systemic gene therapy approach of i.m. injection of naked TIMP-4 DNA. This approach is based on two rationales: (a) local expression of TIMP in cancer cells may not mimic stromal-epithelial interaction in vivo, because TIMP-4 mRNA is expressed in stromal cells but not in malignant epithelial cells; and (b) delivery of heterologous genes into skeletal muscles has been shown to be able to provide sustained production of proteins (33). Our data show that TIMP-4 protein can be systemically produced in muscle cells by a single injection of naked DNA, followed by electroporation. The production of TIMP-4 and its release into the circulation were sustained for at least 2 weeks. Furthermore, a significant amount of TIMP-4 protein was accumulated on tumor xenografts. Unexpectedly, systemic administration of TIMP-4 resulted in a significant stimulation of primary mammary tumorigenesis.

It is intriguing that systemic delivery of TIMP-4 via i.m. injection of DNA results in a stimulatory effect on mammary tumorigenesis, which is in contrast to the previously demonstrated antitumor and antitumor effect of TIMPs (7–13). The TIMP-4-mediated, tumor-stimulating effect can be viewed from two perspectives; (a) systemic overexpression of TIMP-4 locally in every breast cancer cell would generate abundant inhibitory proteins and create a microenvironment in the tumor-stromal interface where the pro-tumor MMP activity is blocked. In contrast, in the i.m. gene therapy approach, TIMP-4 protein has to cross a vast amount of extracellular matrix proteins and circulation before reaching the target tumor cells. Therefore, the amount of TIMP-4 bioavailable to the tumor cells may be much lower than that from locally expressed TIMP-4 in transfected cells. In addition, when the TIMP-4 gene was administrated i.m., the anti-MMP function of circulating TIMP-4 may be neutralized in part by circulating MMPs, and therefore, the balance was shifted in favor of its antia apoptotic activity when it reached to the tumor site.

Regulation of apoptosis by TIMPs has been reported. Whereas TIMP-3 induces apoptosis, TIMP-1 and TIMP-2 have an antia apoptotic effect. As for the mammary gland, TIMP-1 inhibits apoptosis of human breast epithelial cells in vitro (23) and rescues mammary epithelial cell from apoptosis in transgenic mice (24). Consistent with this antia apoptotic effect of TIMP-1 on the mammary gland, we reported here a similar apoptosis-inhibiting effect of TIMP-4 on human breast cancer cells as well as on mammary tumor xenografts. On the apoptotic pathways, TIMP-1, which is up-regulated by Bcl-2, seems to function downstream of Bcl-2 (23). TIMP-4 stimulates Bcl-2 and Bcl-XL expression, which may contribute to the inhibition of apoptosis. By using a TIMP-4 affinity column, we have identified a putative TIMP-4-binding protein on the cell surface (data not shown). We are currently in the process of cloning this putative TIMP-4 binding protein and to study the mechanism of TIMP-4-mediated regulation of apoptosis.

MMPs and TIMPs also play a complex role in regulating angiogenesis. On one hand, MMP may trigger an angiogenic switch. On the other hand, it can convert matrix proteins into angiogenic inhibitors. Systemic inhibition of tumor growth by administration of the endostatin gene has been demonstrated previously (33). Because endostatin is converted from type 18 collagen, which may be mediated by MMPs (34), we wondered whether TIMP-4-induced tumor stimulation is mediated by reduction of endostatin levels as result of inhibition of MMPs. Levels of mouse endostatin in sera isolated from tumor-bearing control mice and TIMP-4-injected mice were determined.

- **Adriamycin**
  - 0 h
  - 6 h
  - rTIMP-4 (nM)
  - Bcl-2
  - Bcl-XL
  - β-Actin

![Fig. 5. Analyses of the bcl-2 and bcl-XL protein expression. Both control MDA-MB-435 cells and rhTIMP-4-treated cells were treated with 1 μM Adriamycin for 6 h. Total cellular proteins were isolated before and after Adriamycin treatment and normalized, and 40 μg of total protein were subjected to Western blots. Bcl-2 protein was detected as a Mr 28,000 band by mouse anti-bcl-2 monoclonal antibody. Bcl-XL protein was detected as a Mr 30,000 band by rabbit polyclonal antibody. Expression of β-actin was used as a control for protein loading.](image-url)
using a commercially available ELISA kit. This assay showed that TIMP-4-induced tumor stimulation was not associated with a decrease in endostatin levels but rather a slight increase in endostatin level (data not shown). The increased endostatin expression in TIMP-4-injected mice may be attributable to the larger tumor volume of the TIMP-4-injected mice compared with that of control mice.

Although TIMPs have been shown to inhibit tumor invasion and metastasis, the data presented here demonstrated a higher level of TIMP-4 expression in breast carcinoma cells than in normal breast epithelial cells. The increased TIMP-4 expression was demonstrated in a variety of different tumors. TIMP-1 expression is often associated with poor prognosis in many human solid tumors, including metastatic breast cancer (35–38), colorectal cancer (39), gastric carcinoma (40), lymphoma (41), and non-small cell lung carcinoma (42). It has been demonstrated that the outcome of patients with breast cancer is more closely related to the presence of TIMP-2 in the peritumoral stroma than to the corresponding MMPs (35–37). It is not easy to understand why the elevated content of TIMP expression is associated with malignant cancer cells. One explanation is that the increased expression of TIMPs may be reciprocally related to the increased expression of MMPs during the tumor-mediated degradation of extracellular matrix. Therefore, elevated level of TIMP in the invasive breast carcinomas may represent one of the subsequent acute host responses to the remodeling stimuli in an attempt to regulate the local tissue degradation. Alternatively, the high level of TIMP expression in breast cancer may favor the proposed MMP-independent growth regulatory and apoptotic regulatory functions. Our evaluation of biological effect of TIMP-4 on stimulation of mammary tumorigenesis provides the rationale for unexpected results of these clinical studies. Our data indicated that the antiapoptotic effect of TIMP-4 plays a key role in TIMP-4 mediated tumor stimulation. Therefore, systemic administration of full-length TIMP protein or DNA clinically to block the biological effect of TIMP-4 on stimulation of mammary tumorigenesis ...
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