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Abstract

Tissue inhibitors of matrix metalloproteinase (TIMPs) are multifunctional proteins with both matrix metalloproteinases (MMPs) inhibitory effects and growth regulatory activity. TIMP-4, a novel human tissue inhibitor of metalloproteinase, was identified and characterized. Enzymatic kinetic studies revealed IC_{50} values of 19, 3, 45, 8, and 83 nM for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, respectively. TIMP-4 has a strong inhibitory effect on the invasion of human breast cancer cells across reconstituted basement membranes. To determine if TIMP-4 can modulate the *in vivo* growth of human breast cancers, we administrated TIMP-4 both locally by transfection of the gene into breast cancer cells and systemically by a gene therapy approach with intramuscular injection of TIMP-4 expression plasmid. Overexpression of TIMP-4 locally in breast cancer cells inhibited the invasion of the cells in the *in vitro* and tumor growth in nude mice. However, here we report that systemic administration of human TIMP-4 by electroporation-mediated intramuscular injection of naked TIMP-4 DNA stimulates tumorigenesis of human breast cancer cells in nude mice. In consistence with tumor stimulatory effect, TIMP-4 upregulates Bcl-2 and Bcl-X_L protein. TIMP-4 also protects apoptosis in human breast cancer cells *in vitro* and mammary tumors *in vivo*. A synthetic MMP inhibitor BB-94 did not have such anti-apoptotic effect. Analyses of TIMP-4 expression in human mammary specimens indicate that TIMP-4 protein is increased in mammary carcinoma cells compared with normal mammary epithelial cells. These data indicate an anti-apoptotic activity in breast cancer cells and a tumor-stimulating effect of TIMP-4 when administrated systemically.

TABLE OF CONTENTS

I. BACKGROUND AND SIGNIFICANCE

The 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 and 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-regulations 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 inhibit primary tumor growth (8-13).

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

Although the inhibitory effects of TIMP on tumor growth and metastasis was achieved by local expression of the TIMP genes into tumor cells, most MMPs and TIMPs are not expressed in genetically altered cancer cells but synthesized and secreted by adjacent stromal fibroblasts (26-28). Potential therapeutic application of TIMPs for cancer treatment is limited (a) by the lack of a method for systemic administration of TIMPs which can reach distant tumor locations and (b) the lack of systemic assessment of the balanced net effects 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, and therefore lead one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. So far, the effect of systemic administration of TIMP on tumorigenesis has not been determined. 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 effects of systemic TIMP-4 gene delivery on mammary tumorigenesis. Unexpectedly, we demonstrated for the first time that systemic delivery of TIMP-4 by intramuscular administration of naked TIMP-4 DNA significantly stimulated mammary tumorigenesis *in vivo*.

II. WORK ACCOMPLISHED

Specific Aim 1: Screening of TIMP-4 expression in a variety of human breast tissues to more fully evaluate the biological relevance of the TIMP-4 on breast cancer progression. Finished (see second year report)

Specific Aim 2: To study the relevance of the TIMP-4 transfection to the invasion and metastasis of breast cancer cells in nude mice. Finished (see second report).

Specific Aim 3: To prepare the active recombinant TIMP-4 proteins. Finished (see second year report).

NEW STUDIES

1. 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 on human mammary specimens including both normal glands and malignant carcinomas. A total of 40 human breast specimens were analyzed. A strong TIMP-4 staining (Fig. 1A) was observed in 9 out of 10 breast carcinomas. We also selected 15 human breast sections that contain both infiltrated breast cancer cells and residual normal breast epithelial cells in order to get an assessment of TIMP-4 protein expression in normal vs. cancer cells in the same tissue section. While 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. 1C & D).

In order to localize the cellular source of the TIMP-4 expression in mammary gland, we performed *in situ* hybridization analysis. Fourteen out of fifteen *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 TIMP4 mRNA in most breast cancer cell lines by Northern blot (30).

2. Systemic tumor stimulating effect of intramuscularly administered TIMP-4 gene. Since 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 intramuscular administration of TIMP-4 expression plasmid. Based on published data on intramuscular delivery of DNA plasmid (31), we selected two doses (50 and 150 μg) of TIMP-4 expression plasmid for intramuscular injection followed by electroporation. Sera were collected prior to the injection, and at 4, 8, 13, and 21 days following the injection. TIMP-4 protein levels were determined by Western blot analysis. For the dose of 50 μg plasmid, serum levels of TIMP-4 were slightly increased over control at day 4 (data not shown). However, at the dose of 150 μg plasmid, serum levels of TIMP-4 were significantly increased. As seen in Fig. 2A, while there was a minimal TIMP-4 protein in the plasma prior to the injection, when 150 μg plasmid was administrated, the amount of TIMP-4 was increased 3 fold at

4 days following 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 intramuscular sites of nude mice five 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 6 x 10^5 of cells 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 sizes of tumors in TIMP-4 injected mice were 283%, 327%, 253%, and 171% of that in control mice at days 7, 11, 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), while very weak TIMP-4 signal was detectable in xenografts from control mice (Fig. 2C).

In a separate experiment, we tested if systemic administration of TIMP-4 gene could increase the tumor incidence when the number of injected tumor cells was reduced. As shown in Fig. 2E, when the number of injected MDA-MB-435 cells was reduced from 6 x 10^5 to 2 x 10^5, the tumor incidences was greatly reduced from 80% (13 out of 16) to 5% (1 out of 18) at week 1 and from 90% (15 out of 16) to 40% (7 out of 18) at week 2, respectively. However, when TIMP-4 was pre-administrated to the mice inoculated with 2 x 10^5 tumor cells, a significant increase in tumor incidence and a decrease in tumor latency were observed. The tumor incidences were increased from 5% in control mice to 44% (7 out of 16) in TIMP-4 treated mice at week 1 and from 40% in control mice to 88% (14 out of 16) in treated mice at week 2, respectively.

3. TIMP-4 enhanced survival of breast cancer cells *in vitro* and breast tumor xenograft *in vivo*. To study if TIMP-4 mediated stimulation of tumorigenesis is mediated by its antiapoptotic effect or growth stimulation, we selected MDA-MB-435 and MDA-MB-453 cells for testing direct effect of exogenously added recombinant human TIMP-4 on cell survival in response to adriamycin treatment. Dose dependence experiments (0.1-1 μM) showed that adriamycin induced 8% of cell death at the dose of 0.1 μM and over 95% of cell death at the dose of 1 μM (data not shown). As shown in Fig. 3, in the absence of TIMP-4, 19% and 25% of MDA-MB-435 and MDA-MB-453 cells remained viable after 48 hrs treatment with 0.5 μM of adriamycin, respectively. However, in the presence of TIMP-4 (80 nM), cell survival increased to 61% for MDA-MB-435 cells and 65% for MDA-MB-453 cells, respectively. Synthetic MMP inhibitor BB-94 (5 μM) had no significant effect on adriamycin-induced apoptosis (data not shown). To determine whether cell proliferation may be affected by TIMP-4, both MDA-MB- 435 and MDA-MB-453 cells were treated with 80 nM of TIMP-4 for two days followed by [^{3}H]thymidine labeling for 6 hrs. The incorporation of [^{3}H]thymidine was measured by standard scintillation counting. TIMP-4 had no significant effect on the proliferation of MDA-MB-435 and MDA-MB-453 cells.

The effect of systemic administration of TIMP-4 gene on apoptosis was investigated in MDA-MB-435 human breast carcinoma xenograft model. Since the most possible anti-apoptotic effects of TIMP-4 *in vivo* is present during the early phase of tumor onset and growth, mice bearing established tumors from both control and TIMP-4 injected mice were sacrificed at day 7 after tumor cell injection. PCI-neo-TIMP4 plasmid or empty vector as control were injected 5 days before tumor cells inoculation and 2 days after tumor cells injection. As shown in Fig. 4A, the tumor sizes increased significantly in TIMP-4 treated mice vs. control mice. Immunohistochemical analyses of

tumors showed that there was no significant difference in proliferative index between control and TIMP-4 treated mice (Fig. 4B). TUNNEL assay revealed that apoptotic cells in tumor decreased 3-fold in TIMP-4 treated mice(Fig. 4C). Thus, TIMP-4 protects breast cancer cells from apoptosis both *in vitro* and *in vivo*.

4. Effect of TIMP-4 on apoptotic proteins. To further investigate the role of TIMP-4 in regulation of apoptosis in breast cancer cells, we transfected MDA-MB-435 human breast cancer cells with full-length TIMP-4 cDNA and named these transient transfected cells as TIMP4-435 cells. We investigated Bcl-2, Bcl-X_L, Bax, Fas, and FasL protein expression in TIMP4-435 vs. control cells by Western blot. While Bax, Fas, and FasL proteins were not detectable in the cells, both Bcl-2 and Bcl-X_L protein expression were significantly increased in TIMP4-435 cells (Fig. 5). In the absence of adriamycin, TIMP-4 stimulated Bcl-X_L expression 20-fold and Bcl-2 expression 4-fold. In the presence of adriamycin, more than 12-fold stimulation in TIMP4-435 cells vs. control cells were observed for Bcl-X_L; while very weak Bcl-2 expression was observed in control cells treated with adriamycin for 6 hrs, a significant amount of Bcl-2 protein was present in TIMP4-435 cells treated with adriamycin at the same time point.

5. Effect of systemic administration of TIMP-4 on serum endostatin levels. MMP may negatively regulate angiogenesis by converting matrix proteins to angiogenic inhibitors. Systemic inhibition of tumor growth by administration of endostatin gene has been previously demonstrated (31). Since endostatin is converted from type 18 collagen, which may be mediated by MMPs (32), we wondered if 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 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 (Table 2). The increased endostatin expression in TIMP-4 injected mice may be due to the larger tumor size of the TIMP-4 injected mice compared with that of control mice.

III. SUMMARY OF KEY DATA:

1. Systemic administration of TIMP-4 by electroporation-mediated intramuscular injection of naked TIMP-4 DNA resulted in a sustained plasma TIMP-4 level and a significant **stimulation** of breast cancer xenografts in nude mice.
2. In consistence with tumor-stimulating effect, TIMP-4 stimulates Bcl-2 and Bcl-X_L protein expression and protects breast tumor cells *in vitro* and mammary tumor xenografts *in vivo* from apoptosis.
3. In consistence with tumor-stimulating effect, analyses of clinical mammary specimens demonstrated a significant increased TIMP-4 protein in malignant mammary epithelial cells vs. normal cells.

IV. SIGNIFICANCES:

1. Extracellular matrix (ECM)-degrading matrix metalloproteinases (MMPs) lead to ECM turnover, a key event in cancer growth and progression. The tissue inhibitors of matrix metalloproteinase

(TIMPs) limit the activity of MMPs, which suggests their use for **cancer gene therapy**. It is intriguing that our systemic evaluation of biological effect of TIMP-4 on stimulation of mammary tumorigenesis is in contrast with the previously demonstrated anti-tumor and anti-metastatic effect of TIMP.

2. It should be noted that the previously established dominant anti-tumor effect of TIMP is primarily based on local expression of TIMP gene in cancer cells but not by systemic evaluation of TIMP function. Since TIMPs are primarily expressed in stromal cells but not in malignant epithelial cells, local expression of TIMP in cancer cells may not mimic stromal-epithelial interaction *in vivo*.
3. TIMP has both MMP inhibitory effect and the growth promotion and anti-apoptotic activity. A systemic assessment of the balanced net effects between their tumor suppressing MMP inhibitory effect and the cell survival pro-tumor activity is required to address the potential TIMP mediated tumor gene therapy. Our data indicated that the anti-apoptotic effect of TIMP-4 plays a key role in TIMP-4 mediated tumor stimulation. Therefore, systemic administration of TIMP clinically to block MMP may not exert tumor-suppressing effect as people expected but may promote disseminated cells to establish overt metastases or to promote residual minimal disease recurrence. In this regard, the potential cancer treatment by targeting TIMP warrants further investigation.
4. The increased TIMP expression was demonstrated in a variety of different tumors. Although TIMP-1 and TIMP-2 were shown to reduce tumor cell invasion and through MMP inhibition. However, immunohistochemical studies showed that increased TIMP-1 expression is often associated with negative prognosis in many human solid tumors, including metastatic breast cancer, colorectal cancer, gastric carcinoma, lymphoma, and non-small cell lung carcinoma. It was demonstrated that the clinical outcome of breast cancer is more closely related to the presence of TIMP2 in the peri-tumoral stroma than to the corresponding MMPs. Our systemic evaluation of biological effect of TIMP-4 on stimulation of mammary tumorigenesis provides the basses for unexpected results of these clinical studies as well as a new conceptual basis for design of strategies that use MMP inhibitors as a probe for proteinase function in cancer gene therapy. Such an approach will be useful for evaluating the functions of other members of TIMP and MMP families.

V. PUBLICATIONS RELATED TO DAMD17-96-1-6261

1. Mingsheng Wang, Yiliang E. Liu, John Greene, Shijie Sheng, Alexander Fuchs, Eliot M. Rosen, Y. Eric Shi. Inhibition of Tumor Growth and Metastasis of Human Breast Cancer Cells Transfected with Tissue Inhibitor of Metalloproteinase 4. ***Oncogene***, 14 (23): 2767-2774, 1997.
2. Yiliang E. Liu, Mingsheng Wang, John Greene, Jeffery Su, Stephen Ullrich, Hui, Li, Shijie Sheng, Preston Alexander, Qingxiang Amy Sang, and Y. Eric Shi. Preparation and characterization of recombinant TIMP-4 protein. ***J. Biol. Chem.***, 272 (30): 20479-20483, 1997.

VI. REFERENCES:

1. Heppner, K. J., Matrisian, L. M., Jensen, R. A., and Rodgers, W. H. Expression of matrix metalloproteinase family members in breast cancer represents a tumor induced host response. Am. J. Pathol., *14:* 273-282, 1996.
2. Himelstein, B. P., Canete-Soler, R., Bernhard, E. J., Dilks, D. W., and Muschel, R. J. Metalloproteinases in tumor progression: the contribution of MMP-9. Invasion. Metastasis, *14:*

246-258, 1994.
3. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. & Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature, *348:* 699-704, 1990.
4. Basset, P., Wolf, C., Rouyer, N., Bellocq, J. P., Rio, M. C., and Chambon, P. Stromelysin-3 in stromal tissue as a control factor in breast cancer behavior. Cancer, *7:* 1045-1049, 1994.
5. Poulsom, R., Hanby, A. M., Pignatelli, M., Jeffery, R. E., Longcroft, J. M., Rogers, L., and Stamp, G. W. Expression of gelatinase A and TIMP-2 mRNAs in desmoplastic fibroblasts in both mammary carcinomas and basal cell carcinomas of the skin. J. Clin. Pathol., *46:* 429-436 ,1993.
6. Naylor, M. S., Stamp, G. W., Davies, B. D., and Balkwill, F. R. Expression and activity of MMPS and their regulators in ovarian cancer. Int. J. Cancer, *58:* 50-56, 1994.
7. Tsuchiya, Y., Sato, H., Endo, Y., Okada, Y., Mai, M., Sasaki, T., and Seiki, M. Tissue inhibitor of metalloproteinase 1 is a negative regulator of the metastatic ability of a human gastric cancer cell line, KKLS, in the chick embryo. Cancer. Res., *53:* 1397-1402, 1993.
8. Imren, S., Kohn, D. B., Shimada, H., Blavier, L., and DeClerck, Y. A. Overexpression of tissue inhibitor of metalloproteinases-2 retroviral-mediated gene transfer in vivo inhibits tumor growth and invasion. Cancer Res., *56:* 2891-2895, 1996.
9. Watanabe, M., Takahashi, Y., Ohta, T., Mai, M., Sasaki, T., and Seiki, M. Inhibition of metastasis in human gastric cancer cells transfected with tissue inhibitor of metalloproteinase 1 gene in nude mice. Cancer, *77:* 1676-1680, 1996.
10. Khokha, R. et al. Suppression of the tumorigenic and metastatic abilities of murine B16-F10 melanoma cells in vivo by the overexpression of the tissue inhibitor of the metalloproteinases-1. J. Natl. Cancer. Inst., *86:* 299-304, 1994.
11. Khokha, R., Zimmer, M. J., Wilson, S. M., and Chambers, A. F. Up-regulation of TIMP-1 expression in B16-F10 melanoma cells suppresses their metastatic ability in chick embryo. Clin.Exp. Metastasis, *10:* 365-370, 1992.
12. DeClerck, Y. A., Perez, N., Shimada, H., Boone, T. C., Langley, K. E., and Taylor, S. M. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res., *52,* 701-708, 1992.
13. Wang M., Liu Y.E., Greene J., Sheng S., Fuchs A., Rosen E.M., Shi Y.E. Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. Oncogene, *14*: 2767-2774, 1997.
14. Khokha, R., Waterhouse, P., Yagel, S., Lala, P.K., Overall, C.M., Norton, G. & Denhardt, D.T. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science, *243*: 947-950, 1989.
15. Denhardt, D. T., Khokha, R., Yagel, S., Overall, C. M., and Parhar, R. S. Oncogenic consequences of down-modulating TIMP expression in 3T3 cells with antisense RNA. Matrix Suppl., *1:* 281-285,1992.
16. Mohanam, S., Wang, S.W., Rayford, A., Yamamoto, M., Sawaya, R., Nakajima, M., Liotta, L.A., Nicolson, G.L, Stetler-Stevenson, W.G. & Rao, J.S. Expression of tissue inhibitors of metalloproteinases: negative regulators of human glioblastoma invasion in vivo. Clin. Exp. Metastasis, *13:* 57-62, 1995.
17. Chesler, L., Golde, D. W., Bersch, N., and Johnson, M. D. Metalloproteinase inhibition and erythroid potentiation are independent activities of tissue inhibitor of metalloproteinases-1. Blood, *68*: 4506-4515, 1995.

18. Stetler-Stevenson, W. G., Bersch, N., and Golde, D. W. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. FEBS. Lett., *296,* 231-234, 1992.
19. Bertaux, B., Hornebeck, W., Eisen, A. Z., Dubertret, L. Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. J Invest Dermatol., *97*: 679-685, 1991.
20. Corcoran, M. L., and Stetler-Stevenson, W. G. Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism. J. Biol. Chem., *270*: 13453-13459, 1995.
21. Hayakawa, T., Yamashita, K., Ohuchi, E., and Shinagawa, A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). J. Cell Sci., *107*: 2373-2379, 1994.
22. Guedez L., Stetler-Stevenson W.G., Wolff L., Wang J., Fukushima P., Mansoor A., and Stetler-Stevenson M. In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. J. Clin. Invest., *102*: 2002-2010, 1998.
23. Li G., Fridman R., and Kim H.C. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. Cancer Res., *59*: 6267-6275, 1999.
24. Alexander C.M., Howard E.W., Bissell M.J., and Werb Z. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. J. Cell Biol., *135*, 1669-1677, 1996.
25. Ahonen M., Baker A.H., Kahari. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res., *58*: 2310-2315, 1998.
26. Coussens L.M., and Web Z. Matrix metalloproteinases and the development of cancer. Chem. Biol., *3*, 895-904, 1996.
27. Shi YE. and Liu YE. In: *Epithelial Mesenchymal Interactions in Cancer,* Goldberg, I.D. and Rosen, E.M. (Eds) (1995) p215-234.
28. Sternlicht M.D., Lochter A., Sympson C.J., Huey B., Rougier J.P., Gray J.W., Pinkel D., Bissell M.J., Werb Z. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell, *98*: 137-146, 1999.
29. Liu Y.E., Wang M., Greene J., Su J., Ullrich S., Li H., Sheng S., Alexander P., Sang Q.A., and Shi Y.E. Preparation and characterization of recombinant TIMP-4 protein. J. Biol. Chem., *272*: 20479-20483, 1997.
30. Greene J., Wang M., Liu Y.E., Raymond L.A., Rosen E.M., and Shi Y.E. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase-4 (TIMP4). J. Biol. Chem., *271*: 30375-30380, 1996.
31. Blezinger P., Wang J., Gondo M., Quezada A., Mehrens D., French M., Singhal A., Sullivan S., Rolland A., Ralston R., and Wang M. Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene. Nature Biotech., *17*: 343, 1999.
32. Wen W, Moses M.A., Wiederschain D., Arbiser J.L., and Folkman J. The generation of endostatin is mediated by elastase. Cancer Res., *59*: 6052, 1999.

Figure Legends

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**) A strong TIMP-4 protein staining in a highly infiltrating breast carcinoma. (**B**) As a negative control, no signal of TIMP-4 protein was detected in the same sample when the section was stained with non-immunized control IgG. (**C**) A strong positive TIMP-4 staining in infiltrated malignant breast cancer cells (arrows) in comparison with a much weak TIMP-4 staining in surrounding residual normal mammary lobule epithelial cells. (**D**) Relatively high levels of TIMP-4 staining in malignant breast cancer cells *(arrow- head)* compared with surrounding residual normal mammary ductal epithelial cells (arrow). (**E**) A strong positive staining of TIMP-4 protein in malignant epithelial cells; in contrast, non-detectable or much less TIMP-4 staining was observed in stromal cells surrounding ductal carcinoma in situ (DCIS). (**F**) In the same slide as E, TIMP-4 mRNA was detected in stromal cells surrounding the DCIS 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 anti-sense probe.

Fig. 2. Effects of systemic administration of TIMP-4 gene on mammary tumor growth and incidence. (**A**). Serum derived TIMP-4 protein following intramuscular injection of the plasmid. Ten μl aliquots of sera collected before and at 4, 8, 13, and 21 days following a single intramuscular injection of 150 μg TIMP-4 expression plasmid were subjected to western blot analysis with anti-TIMP-4 antibody. Figure represents a time course of serum TIMP-4 levels in one mouse. Similar TIMP-4 levels were observed in all 5 mice tested for each time point. (**B**) Time course of TIMP-4 stimulated mammary tumorigenesis. The data were plotted based on experiment 1 in Table 1. The number of cells injected was 6×10^5. (**C-D**) Accumulation of TIMP-4 on tumor xenograft sections. Mice were sacrificed at day 10 after tumor cell injection (15 days after administration of TIMP-4 gene). Tumor sections collected from both control plasmid and TIMP-4 expression plasmid injected mice were subjected to immunohistochemical analysis with TIMP-4 antibody. While there was a minimal TIMP-4 protein on tumor xenograft from control mice (**C**), tumor xenograft from TIMP-4 plasmid injected mice showed strong TIMP-4 immunoreactivity (**D**). (**E**). Tumor incidences. MDA-MB-435 cells were injected either at 6×10^5 or at 2×10^5 cells per injection. TIMP-4 administration was described in Table 1. Tumor incidences were analyzed in three groups. Control 1, 8 control mice were injected with 6×10^5 cells/injection. Control 2, 9 control mice were injected with 2×10^5 cells/injection. TIMP-4, 8 TIMP-4 treated mice were injected with 2×10^5 cells/injection. For all three groups, each mouse received 2 injections.

Fig. 3. Effects of rTIMP-4 protein on Adriamycin-induced apoptosis. MDA-MB-453 (A) and MDA-MB-435 (B) cells were grown in 96 well plates. Each treatment group consisted of ten wells. Cells were pretreated with or without 80 nM recombinant human TIMP-4 for 24 hours and then treated with 0.5 μM of Adriamycin for 12 hours. Cells were harvested 2 days after adriamycin treatment. Survivals of untreated cells (control) were taken as 100 percent and all other survival ratios were normalized to the control. Data represent the mean ± SD of ten cultures.

Fig. 4. Effects of systemic administration of TIMP-4 on apoptosis and proliferation of breast cancer xenograft. (A). Tumor size from control and TIMP-4 treated mice at 7 days. Intramuscular injection

of control and TIMP-4 plasmids and tumor cells injection were described in Table 1. The average tumor size was increased 5-fold ($p<0.001$) in the TIMP-4 treated mice vs. control mice. (B). Histological analysis sections of tumors from TIMP-4-treated and control human breast carcinomas were analyzed for proliferation (PCNA). There was no significant difference in the proliferative index of tumor cells in treated versus untreated tumors. (C). Histological sections of tumors were also analyzed for apoptosis (TUNEL assay). The apoptotic index of the tumor cells decreased 3-fold ($p<0.01$) in the TIMP-4 treated mice.

Fig. 5. Analyses of the bcl-2 and bcl-X_L protein expression. Both control MDA-MB-435 cells and TIMP-4 transfected cells were treated with 1 μM adriamycin for 6 hours. Total cellular proteins were isolated before and after adriamycin treatment, normalized, and 40 μg of total protein were subjected to Western blots. Bcl-2 protein was detected as 28KD band by mouse anti-bcl-2 monoclonal antibody. Bcl- X_L protein was detected as 30KD band by rabbit polyclonal antibody. Expression of β-actin was used as a control for protein loading.

Table 1. Effects of systemic administration of TIMP-4 plasmid on mammary tumorigenesis

		Tumor Vol (^{3}mm)			
		Day 7	*Day 10*	*Day 15*	*Day 21*
Experiment	*Treatment group*				
1	Control	17 ± 5	26 ± 9	80 ± 29	314 ± 68
	TIMP-4	51 ± 9	85 ± 9	202 ± 34	537 ± 86
	P value	.006	.007	.013	.04
2	Control	21 ± 6		85 ± 19	293 ± 46
	TIMP-4	45 ± 8		164 ± 34	538 ± 70
	P value	.04		.02	.01
3	Control	14 ± 6		91 ± 32	337 ± 99
	TIMP-4	41 ± 10		182 ± 63	521 ± 102
	P value	.02		.024	.04

TIMP-4 or control plasmid (150 μg) were injected intramuscularly with electroporation five days before injection of cells and every 10 days thereafter. Six hundred thousand of 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 micro-metastasis were determined as described in Materials and Methods. Volumes are expressed as means ± SEs (number of tumors assayed). For each experiment, there were total 10 injections for 5 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 21 day and no lung metastases were observed.

Table 2. Effect of systemic administration of TIMP-4 gene on serum endostatin level

Treatment	Tumor volume (mm^3)	Endostatin level (ng/ml)
Control mice	855.4 ± 161.5	67.5 ± 7.3
TIMP4 injected mice	1040.8 ± 101.2	95.8 ± 10.9

From experiment 1 in Table 1, sera were isolated from control and TIMP-4 mice at 28 days following injection of breast cancer cells. There were five mice in each group. Twenty μl of serum from each mouse was subjected to endostatin ELISA analysis (Chemikine™ Mouse Endostatin™ EIA Kit, Chemicon International, Inc. CA) according to the manufacture's guideline. The data represent the mean of five mice in each group.

FIGURE 1

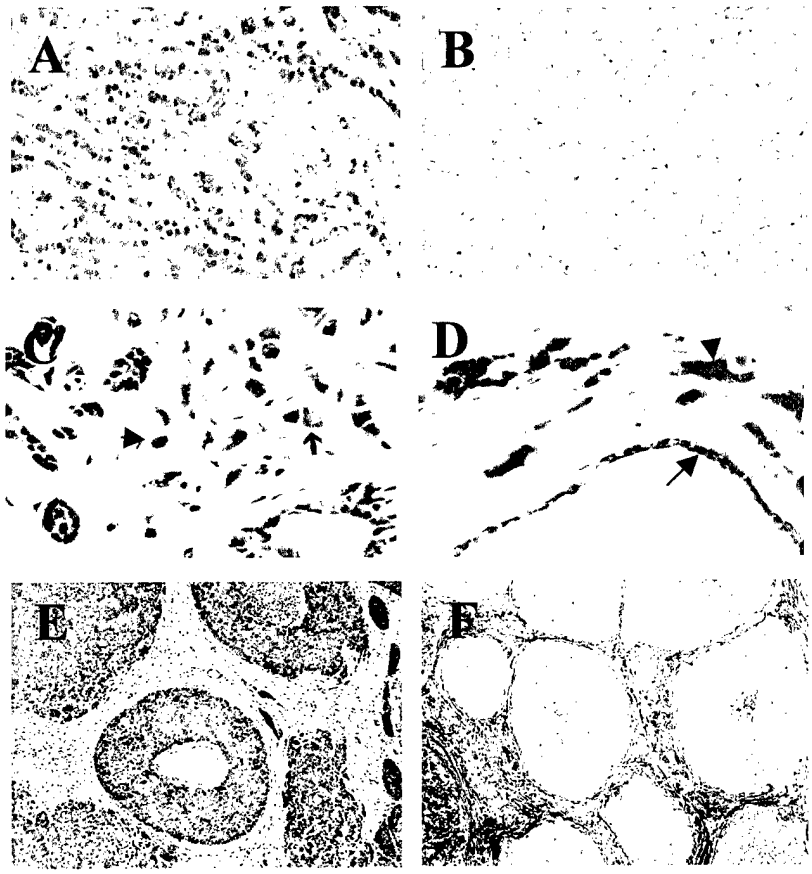

FIGURE 2

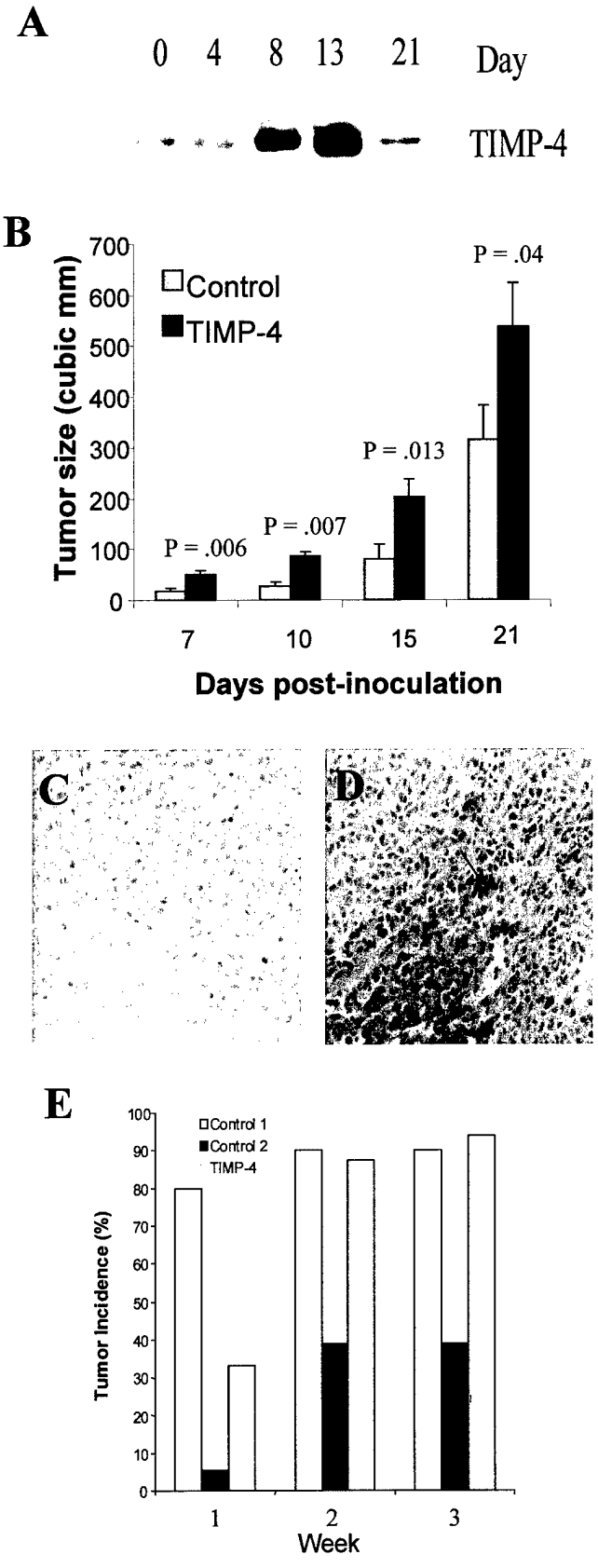
A
0 4 8 13 21 Day
TIMP-4
B
Tumor size (cubic mm)
700
600
500
400
300
200
100
0
Control
TIMP-4
P = .006
P = .007
P = .013
P = .04
7 10 15 21
Days post-inoculation
C
D
E
Tumor Incidence (%)
100
90
80
70
60
50
40
30
20
10
0
Control 1
Control 2
TIMP-4
1 2 3
Week

FIGURE 3

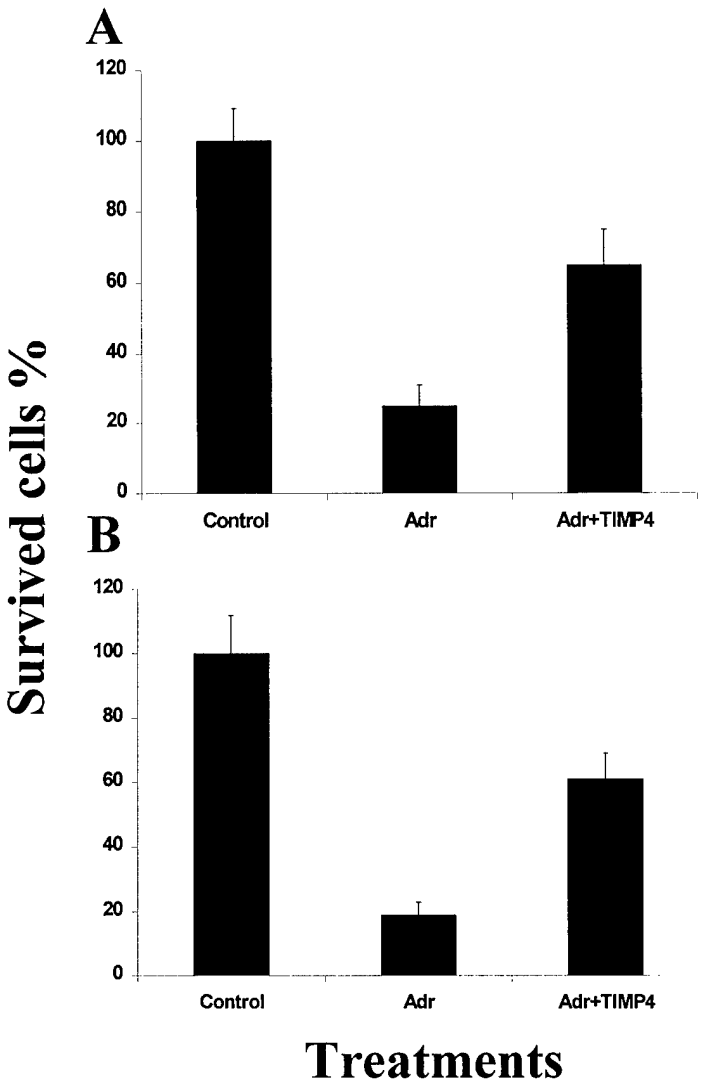
A
120
100
80
60
40
20
0
Control
Adr
Adr+TIMP4
B
120
100
80
60
40
20
0
Control
Adr
Adr+TIMP4
Survived cells %
Treatments

FIGURE 4

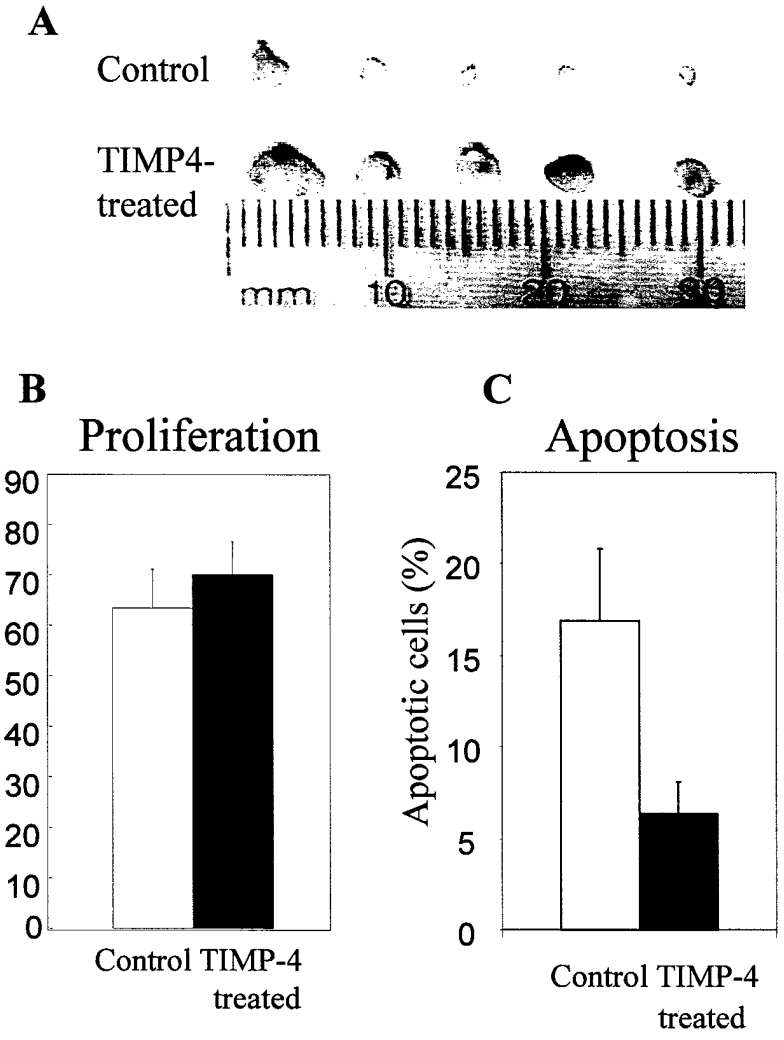
A
Control
TIMP4-treated
mm
10
20
30
B
Proliferation
PCNA (%)
90
80
70
60
50
40
30
20
10
0
Control
TIMP-4 treated
C
Apoptosis
Apoptotic cells (%)
25
20
15
10
5
0
Control
TIMP-4 treated

FIGURE 5