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Abstract

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 inhibited the invasion potential of the cells in the *in vitro* invasion assay. When injected orthotopically into nude mice, TIMP-4 transfectants were significantly inhibited in tumor growth by 4-10-fold in primary tumor volumes; and in an axillary lymph node and lung metastasis as compared with controls. However, administration of TIMP-4 by electroporation-mediated intramuscular injection of TIMP-4 expression plasmid DNA resulted in a sustained plasma TIMP-4 level and a significant stimulation of mammary xenografts in nude mice. Our data demonstrate differential effects of TIMP-4 on mammary tumorigenesis, which may have significant impact on potential therapeutic application of MMP inhibitors in cancer therapy.

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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). Therefore, 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. In fact, tumor invasion and metastasis can be inhibited by up-regulation of TIMP expression in tumor cells (7-12). Alternatively, down-regulation of TIMP-1 and TIMP-2 have been reported to contribute significantly to the tumorigenic and invasive potentials (13-15). In addition to inhibiting tumor cell invasion and metastasis, overexpression of TIMPs also inhibit primary tumor growth including c-Ha-ras transfected rat embryo fibroblasts (8), melanoma cells (9), and colon carcinoma cells (10).

TIMPs have been shown to be multifunctional factors. Contrasting with their anti-MMP activity, TIMPs also promote cell growth. The stimulating effect on cell growth was initially recognized when TIMP-1 and TIMP-2 were identified having erythroid-potentiating activities (16-17). It is now clear that TIMP-1 and TIMP-2 are mitogenic for non-erythroid cells, including normal keratinocytes (18), fibroblasts (19), lung adenocarcinoma cells (20), and melanoma cells (20). In addition, the recent evidence indicates that TIMP family is involved in apoptosis. While TIMP-1 and TIMP-2 have antiapoptotic effect and enhance tumor cell survival (21-24), TIMP-3 induces apoptosis (25-26).

Although the inhibitory effects of TIMP on tumor growth and metastasis was achieved by local expression of the TIMP gene into tumor cells, most MMPs and TIMPs are not expressed in genetically altered cancer cells but synthesized and secreted by adjacent stromal fibroblasts (27-29). 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. So far, the effect of systemic administration of TIMP on tumorigenesis has not been determined. 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 (see last 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 (see last year report).

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

NEW STUDIES

TIMP-4 enhances survival of breast cancer cells. Because previous studies suggested an antiapoptotic role for TIMP-1 and TIMP-2 ( ), we wished to examine whether the increased TIMP-4

protein accumulation on malignant mammary epithelial cells plays a role in protection of the cells. In this regard, we selected MDA-MB-435 and MDA-MB-453 cells because of lack of endogenous TIMP-4 expression ( ) for testing direct effect of exogenously added TIMP-4 on cell survival in response to adriamycin treatment. Dose dependence experiments (0.1-1 μM) showed adrimycin 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. 1, in the absence of TIMP-4, 18% of MDA-MB-435 cells remained viable after 48 hrs treatment with 0.5 μM of adrimycin. However, in the presence of TIMP-4 (80 nM), cell survival increased to 58% for MDA-MB-435 cells. Synthetic MMP inhibitor BB-94 (5 μM) had no significant effect on adriamycin-induced apoptosis. To eliminate the possibility that TIMP-4-induced cell survival was due to its mitogenic activity, MDA-MB- 435 cells were treated with 80 nM of TIMP-4 for two days followed by ^{3}H-thymidine incorporation for 6 hrs. No significant growth alternation was observed (data not shown).

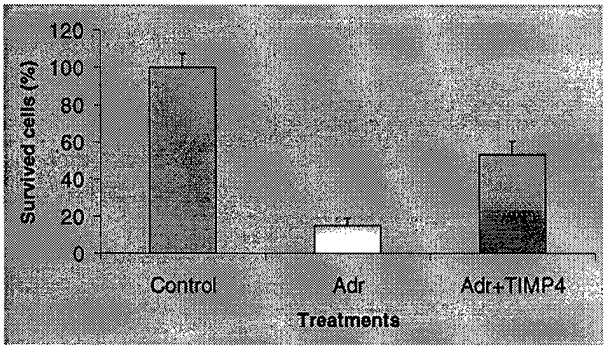

FIGURE 1 Effects of rTIMP-4 protein and Adriamycin on MDA-MB-435 cells. MDA-MB-435 cells were grown in 96 well plates. Each treatment group consisted of ten wells. Survival of untreated cells (control) were taken as 100 percent and all other survival ratios were normalized to this group. Cells were treated with 0.5 uM of Adriamycin with or without of 80 nM rTIMP-4.

Intramuscular delivery of TIMP-4 plasmid. Since TIMP-4 protected breast cancer cells from apoptosis *in vitro*, we were interested in whether the similar pro-tumor effect could be achieved *in vivo* by gene therapy approach through intramuscular administration of TIMP-4 expression plasmid. One hundred and fifty micrograms of TIMP-4 expression plasmid were administrated by intramuscular injection and followed by electroporation. Plasma was collected prior to the injection, and at 7 and 14 days following the injection. TIMP-4 protein levels determined by Western blot analysis. As seen in Figure 2, while there was no detectable TIMP-4 protein in the plasma prior to the injections, significant amount of TIMP-4 was detected in the plasma at 7 days following the injection and continued to be present at 14 days post injection.

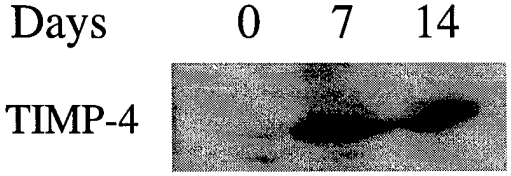

FIGURE 2 Western analysis of TIMP-4 in plasma. Intramuscular injection of plasmid. Fifty microliters (150 μg) of TIMP-4 plasmid DNA (TIMP-4 cDNA in pCI-neo mammalian expression vector, Promega Corporation, Madison, WI) or plasmid DNA alone were injected into the bilateral tibialis anterior muscles of 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 pulse of 200 V each were delivered to the injection site at a rate of one pulse per second, each pulse lasting for 50 ms. Then, three pulses of the opposite polarity were applied. Five μl aliquots of plasma collected before and 7 and 14 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 time course of plasma TIMP-4 levels. Similar TIMP-4 levels were observed in all 5 mice tested for each time point.

Stimulation of mammary tumorigenesis by intramuscularly administered of naked TIMP-4 DNA. We injected nude mice with either TIMP-4 plasmid or control plasmid three days before inoculation of MDA-MB-435 cells and every 10 days thereafter. After a lag phase of 6-9 days, tumors in TIMP-4 injected mice increased in volume at an exponential rate. In contrast, the tumors in control mice had a significantly lower growth rate compared with that of TIMP-4 injected mice (table 1). At 7 weeks following tumor cell injection, the size of tumors in TIMP-4 injected mice were increased 87.5% and 71.2% of that in control mice in tow separate experiments.

Experiment	Group	Tumor incidence	Tumor vol (mm3)	Stimulation rate (%)	p value
1	Control	9/10	578.9±74.9		
	TIMP4	10/10	1229.4±194.4	87.5	<0.05
2	Control	5/6	313.8±67.8		
	TIMP4	6/6	537.3±86.4	71.2	<0.05

Table 1. Effect of intramuscular injection of TIMP-4 plasmid on mammary tumorigenesis. MDA-MB-435 cells were injected at day one into the mammary fat pads, and tumor volume was determined. Volumes are expressed as means±s.e.s (number of tumors assayed). Experiment 1, 600,000 cells were injected for each injection and total 10 injections for five mouse in each group, Experiment 2, 150,000 cells were injected and total 6 injections for 3 mouse in each group. Mice were sacrificed 35 days after injection. Statistical comparison for primary tumors was analysed by Students t test.

III. SUMMARY OF KEY DATA:

1. TIMP-4 has antiapoptotic effect on breast cancer cells.

2. Intramuscular gene thearpy with TIMP-4 plasmid. Administration of TIMP-4 by electroporation-mediated intramuscular injection of TIMP-4 expression plasmid DNA resulted in a sustained plasma TIMP-4 level and a significant growth stimulation of MDA-MB-435 xenografts in nude mice.

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