

Tissue Inhibitor of Matrix Metalloproteinase-1 Suppresses Apoptosis of Mouse Bone Marrow Stromal Cell Line MBA-1

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Received: 18 April 2005 / Accepted: 16 February 2006 / Online publication: 10 May 2006

Abstract. We investigated the action of tissue inhibitor of metalloproteinase-1 (TIMP-1) on apoptosis and differentiation of mouse bone marrow stromal cell line MBA-1. TIMP-1 did not affect alkaline phosphatase (ALP) activity, suggesting that it is not involved in osteoblastic differentiation in MBA-1 cells. However, TIMP-1 inhibited MBA-1 apoptosis induced by serum deprivation in a dose-dependent manner. Our study also showed increased Bcl-2 protein expression and decreased Bax protein expression with TIMP-1 treatment. TIMP-1 decreased cytochrome *c* release and caspase-3 activation in MBA-1 cells. TIMP-1 activated phosphatidylinositol 3-kinase (PI3-kinase) and c-Jun N-terminal kinase (JNK), and the PI3-kinase inhibitor LY294002 or the JNK inhibitor SP600125 abolished its antiapoptotic activity. To investigate whether antiapoptotic action of TIMP-1 was mediated through its inhibition on MMP activities, we constructed mutant TIMP-1 by site-directed mutagenesis, which abolished the inhibitory activity of MMPs by deletion of Cys1 to Ala4. Wild-type TIMP-1 and mutant TIMP-1 expression plasmids were transfected in MBA-1 cells, and results showed that mutant TIMP-1 still protected the induced MBA-1 cell against apoptosis. These data suggest that TIMP-1 antiapoptotic actions are mediated via the PI3-kinase and JNK signaling pathways and independent of TIMP-1 inhibition of MMP activities.

Key words: Tissue inhibitor of metalloproteinase-1 — Bone marrow stromal cell — Apoptosis

Tissue inhibitors of metalloproteinases (TIMPs) are the natural protease inhibitors of matrix metalloproteinases (MMPs), which belong to a family of endopeptidases marked by their ability to degrade extracellular matrix [1–4]. In the TIMP family, TIMP-1 is the predominant form expressed by bone cells [2]. Recently, it was shown that TIMP-1 overexpression increased bone density in the mouse [5]. Although the ability of TIMP-1 to inhibit MMP activation could be partially accounted for by

increased bone density, the mechanism remains unknown.

Besides the ability to inhibit the activity of MMPs, TIMP-1 has additional physiological functions. Recently, TIMP-1 has been shown to display antiapoptotic activity on B cells, Burkitt's lymphoma cell lines, a breast cell line, and erythroid cells [6–9]. Studies have shown that some biological activity of TIMP-1 appeared to be a direct cellular effect mediated by cell-surface "receptors" or "binding sites" and was independent of their functions as MMP inhibitors [10, 11]. Liu et al. [12] revealed that TIMP-1 inhibition of apoptosis in human breast epithelial cells was involved in signaling related to focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK), rather than in regulation of cell-extracellular matrix interactions via MMP activity. In a study on bone metabolism, Sobue et al. [3] demonstrated that TIMP-1 directly stimulates the bone-resorption activity of isolated mature osteoclasts at physiological concentrations; this stimulatory action is likely to be independent of TIMP-1's ability to inhibit MMPs.

Here, we investigated the action of TIMP-1 on apoptosis and differentiation of the mouse bone marrow stromal cell line MBA-1.

Materials and Methods

Antibodies and Reagents

We used the following antibodies and reagents: recombinant human TIMP-1 (Calbiochem, San Diego, CA, USA); recombinant human bone morphogenetic protein-2 (BMP-2; Peprotech Asia); DMEM (Sigma, St. Louis, MO, USA); fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY); anti-mouse β -actin polyclonal antibody (Sigma); anti-Bcl-2, Bax, caspase-3, cytochrome *c*, phosphorylated phosphatidylinositol 3-kinase (p-PI3-kinase) p85 α , PI3-kinase p85 α , phospho-c-Jun N-terminal kinase (p-JNK), JNK, phospho-p38, p38, phosphorylated extracellular signal-regulated kinase (p-ERK)1/2, ERK1/2 antibody, antimouse immunoglobulin G (IgG) peroxidase conjugate, and antirabbit IgG peroxidase conjugate antibodies (Santa Cruz Biotechnology, Waltham,

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MA); antiphosphotyrosine monoclonal antibody (mAb) clone 4G10 (Upstate Biotechnology, Lake Placid, NY); immobilized protein G-agarose beads (Pierce, Rockford, IL); alkaline phosphate (ALP, Sigma); osteocalcin (OC), radioimmunoassay kit (DiaSorin, Stillwater, MN); and PD098059, SB203580, LY294002, and SP600125 (Calbiochem).

Mouse Marrow Stromal Cell Line MBA-1 Cell Culture

The mouse bone marrow cell line MBA-1 was kindly provided by Professor Shamay (Department of Histology and Cell Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel). Benayahu et al. [13] showed that MBA-1 cells constitutively express collagen I, noncollagenous proteins, and ALP mRNA and can form primary mineralized bone. Maturation of MBA-1 cells *in vitro* was accompanied by low expression of mRNA for procollagen I or by a marked increase in osteonectin and osteopontin mRNA levels [13]. Thus, the ability to follow the expression of these genes through bone formation *in vitro* has been demonstrated. Our study showed the ability of mineralized matrix formation. Treatment with mineralization medium [Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 50 µg/mL ascorbic acid + 10 mM β-glycerophosphate] began on culture day 4, and cells were recultured for 21 days. Mineralization of cells was evaluated with 1% alizarin red stain after fixation in 95% ethanol.

MBA-1 cells were plated in a 25 cm² flask in phenol red-free DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL ascorbic acid. The cells were plated in culture plates for 1 day and then treated with vehicle, dimethylsulfoxide (DMSO) or 200–1,000 ng/ml TIMP-1 for 48 hours in serum-free medium. The cells were harvested for apoptotic analysis and Western blotting.

ALP Activity Assay

Cells were distributed in six-well plates at a density of 1×10^5 /well for 1 day and then treated in the absence or presence of 200–1,000 ng/mL TIMP-1 for 48 hours in serum-free medium. Cell layers were scraped into solution containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% NaN₃, and 1 µg/mL aprotinin. After the lysates were homogenized by sonication for 20 seconds, ALP activity was measured using an ALP kit. To normalize the protein expression to total cellular proteins, a fraction of lysate solution was used in a Bradford protein assay.

Cell Apoptosis Measurement

Apoptosis was assessed directly by measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histone in the cytoplasm) using a Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche, Mannheim, Germany), according to the kit protocol. Briefly, cells were plated at a density of 1×10^4 /well in 24-well plates for 1 day, followed by culture in serum-free medium for 48 hours in the absence or presence of 200–1,000 ng/mL TIMP-1 and 800 ng/mL TIMP-1 for 4–48 hours. The cell layers were rinsed with phosphate-buffered saline (PBS) and extracted with 0.5 mL of lysis buffer after a 30-minute incubation at 4°C. The cell lysates were then centrifuged for 10 minutes at 15,000 rpm, and the aliquots of aqueous supernatant were tested for apoptosis using the Cell Death Detection kit.

We tested the effects of these signal inhibitors on apoptosis. The cells were serum-deprived in the absence or presence of 800 ng/mL TIMP-1 for 48 hours and treated with various inhibitors of signal transduction, 10 µM PD098059 (specific inhibitor of ERK/MAPK), 10 µM SB203580 (specific inhibitor of p38 kinase), 50 nM LY294002 (inhibitor of PI3-kinase), 20 µM SP600125 (JNK inhibitor II), or vehicle (DMSO), then analyzed by Cell Death Detection ELISA.

Western Blot Analysis

MBA-1 cells were plated in six-well plates for 1 day, followed by culture in serum-free medium for 48 hours in the absence or presence of 200–800 ng/mL TIMP-1. Cell layers were homogenized with Triton lysis buffer [50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% Triton X-100, 0.02% NaN₃, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 µg/mL aprotinin, and 1 µg/mL aminoethylbenzenesulfonyl fluoride (ABSF)]. Lysates were centrifuged for 15 minutes at 12,000g to remove debris. Protein concentrations were determined using the Bradford protein assay. Protein from each cell layer homogenate (40 µg) was loaded onto a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk, membranes were incubated with antimouse Bcl-2 mAb, antimouse Bax mAb, or antirabbit caspase-3 antibody. The membrane was reprobed with peroxidase-conjugated secondary antibodies. Blots were processed using an enhanced chemiluminescence kit and exposed to film, then analyzed by densitometry.

Phosphorylation levels of p38, ERK, and JNK were examined by Western blot to evaluate the role of the MAPK viability signal pathway. First, MBA-1 cells were treated with 800 ng/mL TIMP-1 for the desired times. Then, cells were washed quickly with cold PBS containing 5 mM EDTA and 0.1 mM Na₃VO₄ and lysed with a lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM NaH₂PO₄, 10% glycerol, 2 mM Na₃VO₄, 10 mM NaF, 1 mM ABSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin. Protein concentrations were determined using the Bradford protein assay. Western blots were performed as above with anti-p-JNK, JNK, p-p38, p38, p-ERK, and ERK antibodies.

Analysis of Cytochrome *c* Release

Release of cytochrome *c* from mitochondria into cytosol was measured by Western blot. Briefly, cells were treated with or without 200–800 ng/mL TIMP-1 and homogenized with Triton lysis buffer as described above. Cell lysates were centrifuged at 100,000g for 30 minutes to yield soluble cytosolic fraction (supernatant). Supernatants were then subjected to Western blot analysis as described above with antirabbit cytochrome *c* antibody.

Detection of PI3-Kinase Phosphorylation

Cells were lysed in a radioimmunoprecipitation assay buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), 100 mM NaCl, 0.1% deoxycholic acid, 10% Nonidet P-40, 1 mM EDTA, 1 mM ethyleneglycoltetraacetic acid (EGTA), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM sodium vanadate, and 50 mM sodium fluoride] at 4°C for 30 minutes. Lysates were centrifuged for 15 minutes at 12,000g to remove debris and immunoprecipitated using antiphosphotyrosine mAb clone 4G10 and immobilized protein G-agarose beads. Immunoprecipitates were washed three times with radioimmunoprecipitation assay buffer and resolved by 8% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Tyrosine-phosphorylated PI3-kinase proteins were detected by Western blot using p-PI3-kinase p85α antibody. The amount of immunoprecipitated protein was determined by Western blot analysis using a primary antibody that recognizes PI3-kinase p85α.

Plasmid Constructs and Transfection

Mouse wild-type full-length TIMP-1 cDNA (WT TIMP-1) was constructed by polymerase chain reaction (PCR) using primers 5'-GAAGCTTGCCACCATGATGGCCCCCTTT-3' and 5'-GGGATCCTCATCGGGCCCCAAGGGAT-3'. HindIII sites

were introduced to 5' ends and *Bam*HI sites were introduced to 3' ends of the mouse full-length TIMP-1 cDNA. WT TIMP-1 was incubated with Taq polymerase and adenosine triphosphate for 30 minutes to add the A's at the tail; the cDNA was then cloned using pGEM-T vector systems (Promega, Madison, WI). Mutant TIMP-1 cDNA (Mut TIMP-1) [14, 15], in which Cys1-Sre2-Cys3-Ala4 was deleted to abolish the inhibiting activity of MMPs, was constructed by PCR using primers 5'-CCACCCACCCACAGACAGCCTTC-3' and 5'-GGCC TTACTGGAAGCTATCAG-3'. The reaction was performed using the pGEM-T vector as the template. DNA products were phosphorylated by T4 polynucleotide kinase (Takara, Otsu Shiga, Japan), and self-ligation of the linear DNA product was then transformed in JM109. The WT and Mut TIMP-1 fragments were excised from the pGEM-T vector with restriction enzymes *Bam*HI and *Hind*III. These fragments were isolated by agarose gel electrophoresis, and the purified fragments were ligated to pCDNA3.1. DNA sequencing analysis confirmed the fidelity of these constructs.

WT TIMP-1 and Mut TIMP-1 overexpressing clones were produced through stable transfection. Briefly, the plasmids of WT TIMP-1, Mut TIMP-1, and pCDNA3.1 control were respectively transfected into MBA-1 cells using lipofectamine 2000 (Invitrogen, La Jolla, CA) according to the manufacturer's protocol. Stably transfected cells were subjected to 400 µg/mL G418 antibiotic selection for 14 days, and at least six colonies from each transfection were isolated for further analysis. The overexpression of WT TIMP-1 and Mut TIMP-1 was confirmed by Western blot with anti-TIMP-1 antibody, and levels of TIMP-1 in medium were measured using an ELISA kit (Amersham, Piscataway, NJ). Apoptosis was assayed by ELISA as above.

MMP Inhibition Study: WT TIMP-1 and Mut TIMP-1 Transfection

The inhibitory action of WT TIMP-1 and Mut TIMP-1 on MMP activity was evaluated using an MMP-2 and MMP-9 activity assay system (Amersham). Briefly, medium of cultured WT TIMP-1 and Mut TIMP-1 overexpressing clones was collected and subjected to MMP-2 and MMP-9 activity analysis.

Statistical Analyses

Data are presented as means ± standard deviation (SD). Comparisons were made with one-way analysis of variance. All experiments were repeated at least twice, and representative experiments are shown.

Results

Effect of TIMP-1 on ALP Activity in MBA-1 Cells

MBA-1 cells were cultured in medium containing 10% FBS, 50 µg/mL ascorbic acid, and 10 mM β-glycerol-phosphate and formed the mineralization nodules on culture day 21 (data not shown), showing that the cells can differentiate into osteoblast-like cells. MBA-1 cells were treated in the absence or presence of 200–1,000 ng/mL TIMP-1 for 48 hours in serum-free medium. However, TIMP-1 showed no effects on ALP activity (Table 1).

TIMP-1 Protected MBA-1 Cells against Serum Deprivation-Induced Apoptosis

The results demonstrated that TIMP-1 inhibited MBA-1 apoptosis induced by serum deprivation in a time-

Table 1. Effects of TIMP-1 on ALP activity in cultured MBA-1 cells

	ALP activity [nmol/(min·mg protein)]
Control	12.5 ± 3.1
TIMP-1	
400 ng/mL	14.1 ± 3.9
600 ng/mL	13.1 ± 3.1
800 ng/mL	12.3 ± 4.3
1,000 ng/mL	10.4 ± 3.8

MBA-1 cells were treated with vehicle or TIMP-1 at 400–1,000 ng/mL for 48 hours. Cell lysates were homogenized, and ALP activity was measured using an ALP kit. Results are expressed as means ± SD ($n = 5$)

dependent manner (Fig. 1A). At 48 hours of culture, apoptotic cells at 200 ng/mL TIMP-1 (1.36 ± 0.22 ELISA absorbance units) were fewer than in controls (2.43 ± 0.25 , $P < 0.05$) in a dose-dependent manner, showing a maximal apoptotic effect at 800 ng/mL (0.30 ± 0.16 , $P < 0.001$) after 48 hours of incubation (Fig. 1B). There were also statistically significant difference between the 200, 600, 800, and 1,000 ng/mL TIMP-1 treatment groups.

Effect of TIMP-1 on Bcl-2 and Bax Protein Expression, Cytochrome *c* Release, and Caspase-3 Activity in MBA-1 Cells

Western blot was performed to examine the role of TIMP-1 on Bcl-2 and Bax protein expression in MBA-1 cultures. TIMP-1 dose-dependently induced Bcl-2 protein expression but downregulated Bax protein expression (Fig. 2).

Cytochrome *c* was released into cytoplasm in the serum-free culture; however, release was inhibited after addition of TIMP-1 (Fig. 2). Activated caspase-3 was also markedly decreased in TIMP-1-treated cells (Fig. 2).

TIMP-1 Activated JNK and PI3-Kinase Signaling Pathways in MBA-1 Cells

TIMP-1 had no effect on p38 and ERK phosphorylation, whereas it enhanced the levels of phosphorylated JNK1/2. This effect was observed at 5 minutes and reached the maximal level of p-JNK1/2 at 30 minutes after TIMP-1 treatment (Fig. 3A). The levels of p-PI3-kinase in MBA-1 cells rapidly increased after 10 minutes of TIMP-1 incubation (Fig. 3A). These data demonstrated that TIMP-1 activated JNK and PI3-kinase signaling pathways in MBA-1 cells.

TIMP-1-mediated cell apoptosis was reduced by inhibitor of PI3-kinase LY294002 in MBA-1 cells (Fig. 3B). Similarly, inhibition of the MAPK pathway reduced MBA-1 cell apoptosis in the presence of SP600125 but not in the presence of PD98059 and SB203580.

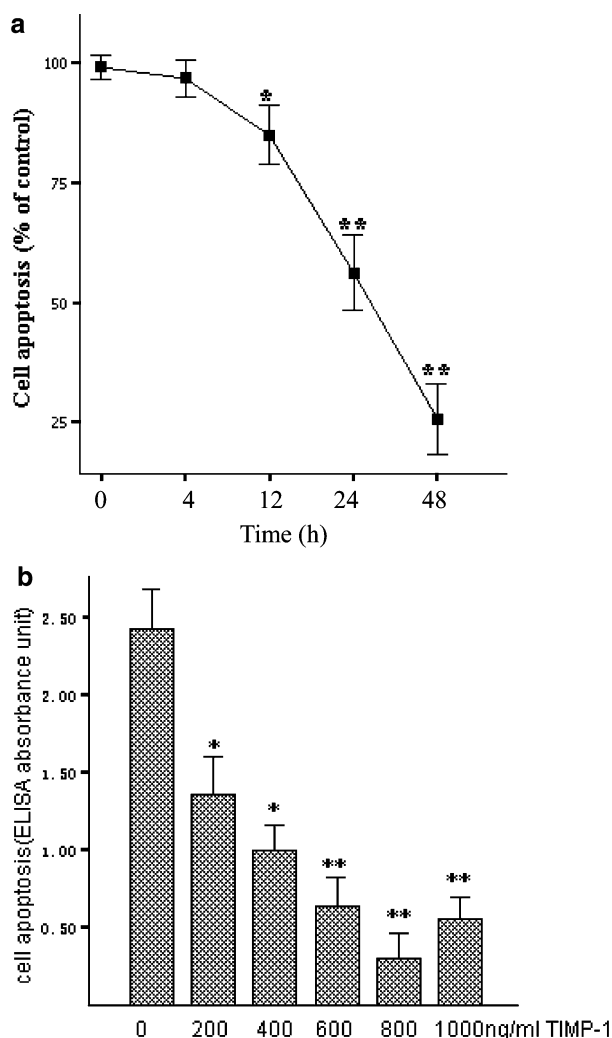


Fig. 1. Effect of TIMP-1 on MBA-1 cell apoptosis by Cell Death ELISA Detection. Cells were exposed to 800 ng/mL TIMP-1 for 4–48 hours and to 200–1,000 ng/mL TIMP-1 in serum-free medium for 48 hours. Apoptosis was assessed using a Cell Death Detection kit and expressed as ELISA absorbance units. (A) Time-course effects of TIMP-1 on cell apoptosis. Dots represent the percentage viability at various time points. * $P < 0.05$ and ** $P < 0.001$ compared with control. (B) Dose-response effects of TIMP-1 on cell apoptosis. Bars represent means \pm SD ($n = 6$). * $P < 0.05$ and ** $P < 0.001$ compared with control.

Effects of various inhibitors of signal transduction on Bcl-2 and Bax expression by TIMP-1 were also evaluated (Fig. 4). TIMP-1-induced Bcl-2 expression was reduced by LY294002 and SP600125 but not PD98059 and SB203580. The decreased Bax expression by TIMP-1 was weakened in the presence of LY294002 and SP600125 but not PD98059 and SB203580 (Fig. 4).

Effects of WT TIMP-1 and Mut TIMP-1 Transfection on MMP Inhibitory Activity and Cell Apoptosis

Western immunoblot was performed to examine the expression of TIMP-1 in the medium of transfection

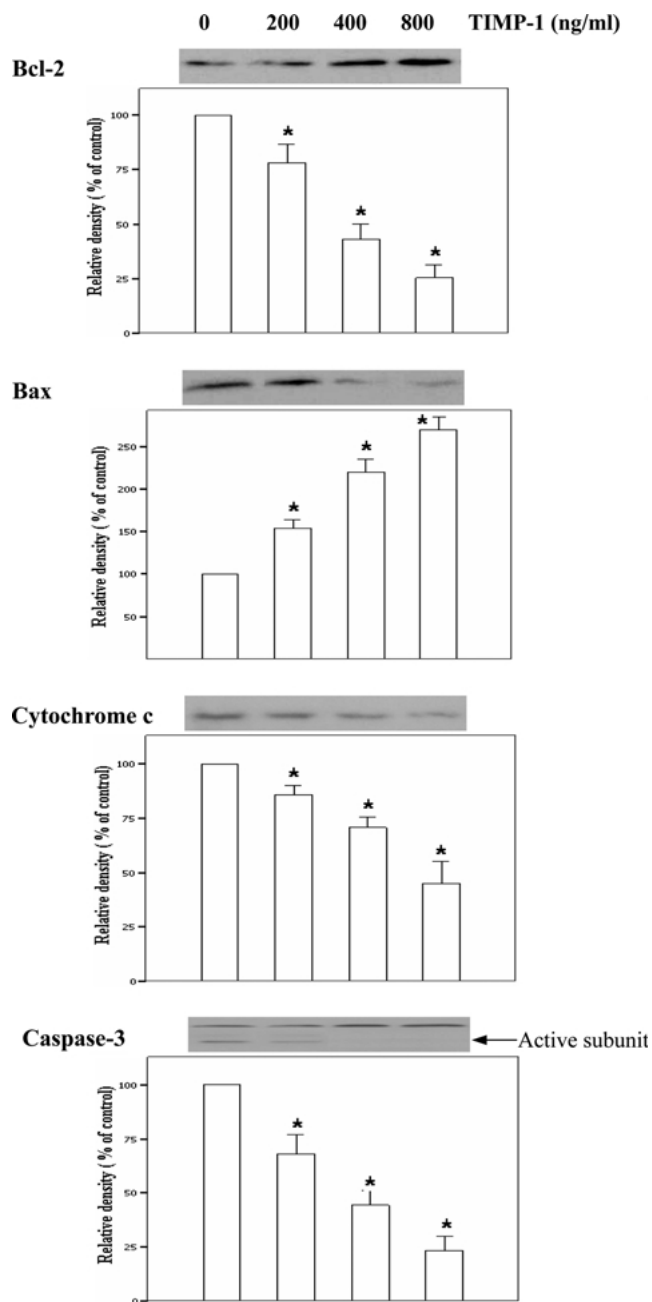


Fig. 2. Effects of TIMP-1 on Bcl-2 and Bax protein expression, cytochrome *c* release, and caspase-3 activity in MBA-1 cells. Cells were exposed to 200–800 ng/mL TIMP-1 for 48 hours. Western blot analysis was performed using anti-Bcl-2, -Bax, -cytochrome *c*, and -caspase-3 antibodies. The band intensity of Bcl-2, Bax, cytochrome *c*, and active caspase-3 subunit was quantified by densitometry and is shown in the corresponding bar graph as percentage of control (mean \pm SD, $n = 3$). * $P < 0.05$ compared with control.

cells. WT TIMP-1 and Mut TIMP-1 were overexpressed in the medium compared to control (Fig. 5A).

Conditioned media were collected from the following: vector-transfected MBA-1 cells (control), WT TIMP-1-transfected MBA-1 cell clones 1 and 6 (WT TIMP-1 #1

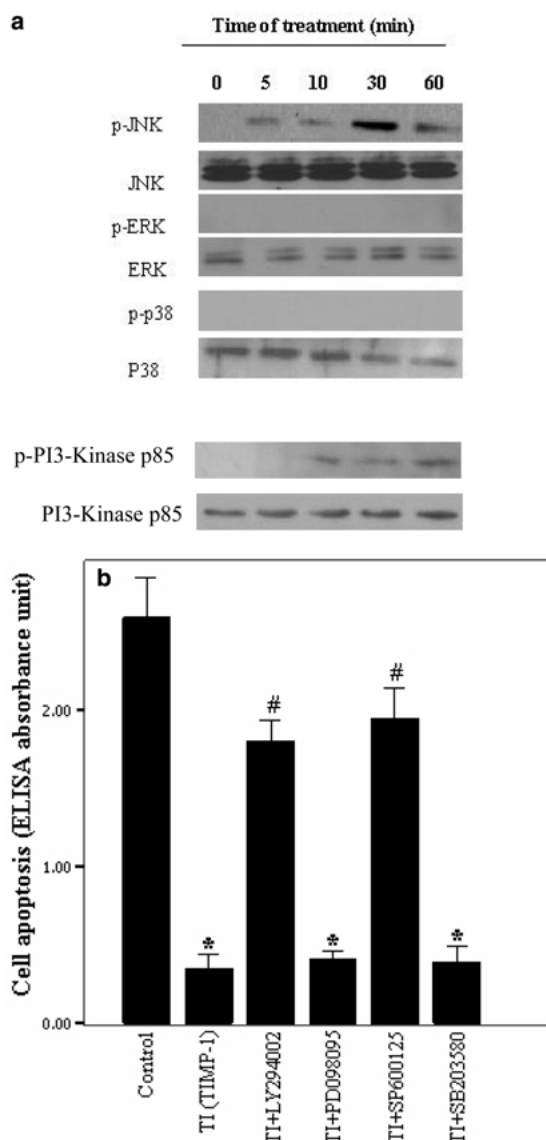


Fig. 3. Effects of TIMP-1 on MAPK (p38, ERK1/2, and JNK) and PI3-kinase activation in MBA-1 cells and effects of various inhibitors of signal transduction on TIMP-1-induced MBA-1 apoptosis. **(A)** Effects of TIMP-1 on MAPK (p38, ERK1/2, and JNK) and PI3-kinase activities in MBA-1 cells. MBA-1 cells serum-starved for 48 hours were treated with or without 800 ng/mL recombinant TIMP-1 proteins for 0, 5, 10, 30, and 60 minutes. Cell lysates (80 μ g/lane) were subjected to Western blot with anti-active ERK, JNK1/2, and p38 and anti-ERK, -JNK1/2, and -p38 antibodies. Active p85 was immunoprecipitated from equal amounts of infected whole-cell lysate (500 μ g) using anti-p-PI3-kinase p85 α . The amount of immunoprecipitated protein was determined by Western blot analysis using a primary antibody that recognizes PI3-kinase p85 α . **(B)** Effects of various inhibitors of signal transduction on TIMP-1-induced MBA-1 apoptosis. MBA-1 cells were cultured in serum-free medium as control (DMSO) or with 800 ng/mL recombinant TIMP-1 (T1), T1 + 50 nM LY294002, T1 + 10 μ M PD98059, T1 + 10 μ M SB203580, or T1 + 20 μ M SP600125. After 48 hours of culture, cell apoptosis was determined by Cell Death Detection assay. Shown are the means \pm SD of triplicate experiments. * P < 0.05 vs. control, # P < 0.05 vs. T1.

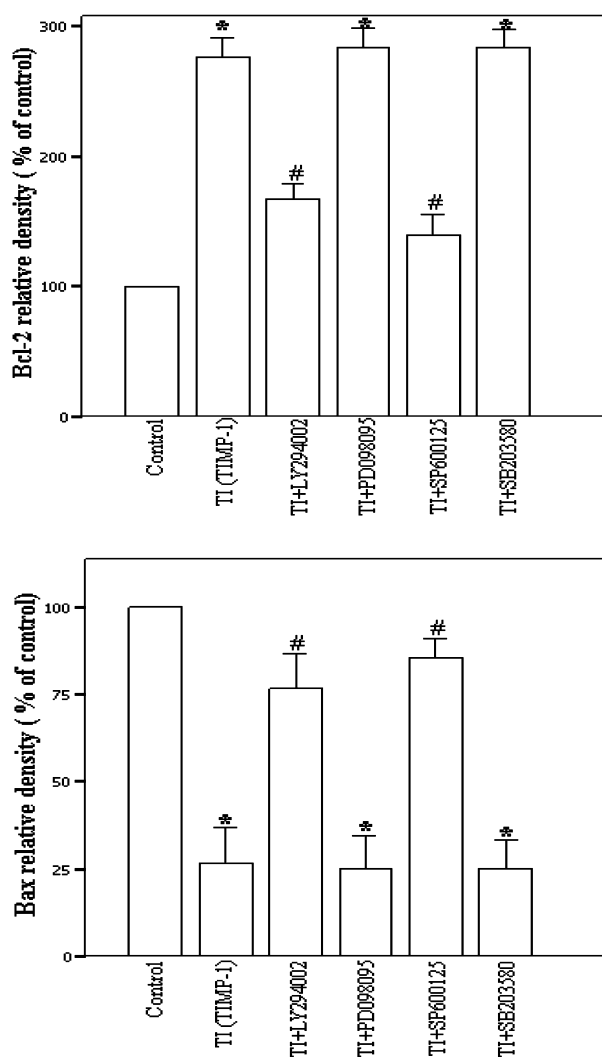


Fig. 4. Effects of various inhibitors of signal transduction on Bcl-2 and Bax expression by TIMP-1. MBA-1 cells were cultured in serum-free medium as control (DMSO) or with 800 ng/mL recombinant TIMP-1 (T1), T1 + 50 nM LY294002, T1 + 10 μ M PD98059, T1 + 10 μ M SB203580, or T1 + 20 μ M SP600125. After 48 hours of culture, Western blot analysis was performed using anti-Bcl-2/-Bax antibodies. The band intensity of Bcl-2 and Bax was quantified by densitometry and is shown in the corresponding bar graph as percentage of control (mean \pm SD, n = 3). * P < 0.05 vs. Control, # P < 0.05 vs. T1.

and #6), and Mut TIMP-1-transfected MBA-1 cell clones 2 and 8 (Mut TIMP-1 #2 and #8). These samples were subjected to MMP-2 and MMP-9 activity via ELISA. Overexpression of WT TIMP-1, but not of Mut TIMP-1, dramatically decreased the levels of active MMP-2 and MMP-9 (Fig. 5B). This demonstrated that Mut TIMP-1, constructed by side-directed mutagenesis through deletion from Cys1 to Ala4, could abolish the inhibitory activity of MMPs.

Similarly, compared with control, overexpression of WT TIMP-1 enhanced MBA-1 cell viability. Mut TIMP-1 overexpression, which abolished the inhibitory

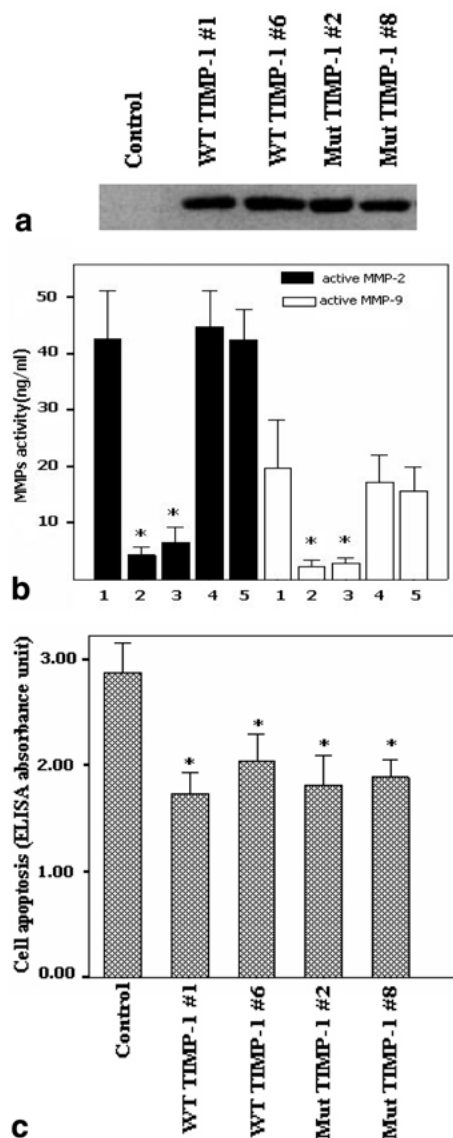


Fig. 5. Expression of WT TIMP-1 and Mut TIMP-1 in MBA-1 cells, as well as WT TIMP-1 and Mut TIMP-1 effects on MMP activity and on MBA-1 cell apoptosis. (A) Expression of TIMP-1 in the medium of cultured stably transfected cells by Western blot and ELISA. Bands were control, WT TIMP-1 #1, WT TIMP-1 #6, Mut TIMP-1 #2, and Mut TIMP-1 #8. (B) Effects of WT TIMP-1 and Mut TIMP-1 expression on MMP activity. Conditioned medium was collected and subjected to MMP-2 and MMP-9 activity ELISA analysis. 1, control; 2, WT TIMP-1 #1; 3, WT TIMP-1 #6; 4, Mut TIMP-1 #2; 5, Mut TIMP-1 #8. (C) Apoptosis was determined using the Cell Death Detection kit in control, WT TIMP-1 overexpression cell clones 1 and 6 (WT TIMP 1 #1 and #6), and Mut TIMP-1 overexpression cell clones 2 and 8 (Mut TIMP-1 #2 and #8) by culturing in serum-free medium for 48 hours. Apoptosis was expressed as ELISA absorbance units. Bars were control, WT TIMP-1 #1, WT TIMP-1 #6, Mut TIMP-1 #2, and Mut TIMP-1 #8. Bars represent means \pm SD ($n = 5$). * $P < 0.05$ vs. control.

activity of MMP, still protected cells from apoptosis (Fig. 5C).

Table 2. Effects of TIMP-1 on ALP activity in cultured MBA-1 cells

	TIMP-1 (ng/mL)
Control	2.6 \pm 0.9
WT TIMP-1 #1	184.0 \pm 7.2
WT TIMP-1 #6	178.6 \pm 14.6

Discussion

We investigated the effects of TIMP-1 on osteoblastic differentiation in cultures of MBA-1 cells. TIMP-1 had no effects on ALP activity, suggesting that TIMP-1 had no effect on MBA-1 cell differentiation. This finding was consistent with previous data that the differentiation and function of osteoblasts from TIMP-1 transgenic mice were normal [5].

Recent studies demonstrated that TIMP-1 was involved in bone metabolism and that TIMP-1 overexpression increased mouse bone density [5]. We have shown that the mouse bone marrow stromal cell line MBA-1 could be one of the direct targets of TIMP-1. TIMP-1 protected MBA-1 cells against apoptosis via the PI3-kinase and JNK signaling pathways. Additionally, this action was independent of TIMP-1's inhibitory action on MMP activities.

Apoptosis is a tightly regulated physiological process [16, 17]. In the present study, TIMP-1 induced expression of Bcl-2 protein and downregulated production of Bax protein in MBA-1; it also blocked the release of cytochrome *c* and activation of caspase-3. This indicated that the change in Bcl2/Bax correlates with the change in apoptosis. It suggested that TIMP-1 inhibited MBA-1 cell apoptosis by regulating Bax/Bcl-2 expression, then blocked the release of cytochrome *c* and activation of caspase-3.

We found that the antiapoptotic action of TIMP-1 was mediated via MAPK and PI3-kinase signaling pathway activation. Here, we show that among the MAPK family members, JNK was effectively activated in MBA-1 cells following treatment with recombinant TIMP-1 proteins, whereas the same treatment showed no effect on p38 kinase and ERK. TIMP-1 can activate ERKs in human osteosarcoma MG-63 cells, MCF10A cells, and breast carcinoma T-47D cells [18–20]. JNK, p38, and ERK function cooperatively or independently in apoptosis and various other biological processes [21, 22]. Our data demonstrated that TIMP-1 mediated MBA-1 cell viability through the JNK, but not the p38 or ERK, signaling pathway.

In addition to activation of JNK signaling, we demonstrated that TIMP-1 protects MBA-1 cells against apoptosis via PI3-kinase stimulation. PI3-kinase activity plays an active role in mitogenic and antiapoptotic signaling pathways [23]. It was shown that TIMP-1 protected human breast epithelial cells and erythroid cells

against apoptosis via the FAK and PI3-kinase signaling pathways [9, 12]. Our study suggests that the PI3-kinase signaling pathway is important for the inhibition of apoptosis by TIMP-1 in MBA-1 cells.

Furthermore, we found that the antiapoptotic action of TIMP-1 was independent of its inhibitory action on MMP activities. TIMP-1 is a classical inhibitor of metalloproteinases; thus, some of its biological effects are likely mediated by inhibition of enzymatic activity [1, 4]. However, other data showed that some biological activity of TIMPs appeared to be a direct cellular effect mediated by cell-surface "receptors" or "binding sites" and was independent of their functions as MMP inhibitors [10, 11]. We constructed Mut TIMP-1 by site-directed mutagenesis, which abolished the inhibitory activity of MMPs by deletion of Cys1 to Ala4. It still protected the induced MBA-1 cell against apoptosis. This strongly suggests that the antiapoptotic activity of TIMP-1 in MBA-1 cells is independent of its inhibition of MMP activities.

The role of TIMP-1 in MBA-1 cell differentiation and apoptosis may contribute partly to understanding the role of TIMP-1 in bone metabolism. Recently, it was shown that TIMP-1 overexpression increased bone density in the mouse [5]. Although the ability of TIMP-1 to inhibit MMP activation could be partially accounted for by increased bone density, the mechanism remains unknown. Using primary osteoblast culture, the differentiation and function of osteoblasts from transgenic TIMP-1 overexpressing mice were normal. Our study also showed that TIMP-1 had no effects on ALP activity in cultures of MBA-1 bone marrow stromal cells. Our data demonstrated that TIMP-1 protected MBA-1 cells against apoptosis and may contribute partly to the increased bone density in the TIMP-1 overexpressing mouse. However, there are limitations to using the stromal cell line since primary stromal cell or osteoblast cultures were not performed. It would be worth looking at apoptosis in TIMP-1 overexpressing mice to see whether what is reported in this cell line occurs *in vivo*.

In conclusion, our study provides evidence that TIMP-1 protected MBA-1 cells against apoptosis and induced expression of Bcl-2 protein, downregulated production of Bax protein, and blocked release of cytochrome *c* and activation of caspase-3. These TIMP-1 antiapoptotic actions are mediated via the PI3-kinase and JNK signaling pathways and independent of TIMP-1 inhibitory action on MMP activities. These data may contribute partly to the recent finding that TIMP-1 overexpression increases bone density in the mouse.

Acknowledgments. This work was supported by grant 30200322 from the China National Natural Scientific Foundation and special grant 200259 from the National Excellent Doctorate Dissertation.

References

1. Brew K, Dinakarpanian D, Nagase H (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 477:267–283
2. Liao EY, Luo XH (2001) Effect of 17 β -estradiol on the expression of matrix metalloproteinase-1, -2 and tissue inhibitor of metalloproteinase-1 in human osteoblast-like cell cultures. *Endocrine* 15:291–295
3. Sobue T, Hakeda Y, Kobayashi Y, Hayakawa H, Yamashita K, Aoki T, Kumegawa M, Noguchi T, Hayakawa T (2001) Tissue inhibitor of metalloproteinases 1 and 2 directly stimulate the bone-resorbing activity of isolated mature osteoclasts. *J Bone Miner Res* 16:2205–2214
4. Westermarck J, Kahari V (1999) Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 13:781–792
5. Geoffroy V, Marty-Morieux C, Le Goupil N, Clement-Lacroix P, Terraz C, Fraix M, Roux S, Rossert J, de Vernejoul MC (2004) *In vivo* inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. *J Bone Miner Res* 19:811–822
6. Guede L, Stetler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M (1998) *In vitro* suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. *J Clin Invest* 102:2002–2010
7. Guede L, Courtemanch L, Stetler-Stevenson M (1998) Tissue inhibitor of metalloproteinase (TIMP)-1 induces differentiation and an antiapoptotic phenotype in germinal center B cells. *Blood* 92:1342–1349
8. Liu XW, Taube ME, Jung KK, Dong Z, Lee YJ, Roshy S, Sloane BF, Fridman R, Kim HR (2005) Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells from extrinsic cell death: a potential oncogenic activity of tissue inhibitor of metalloproteinase-1. *Cancer Res* 65:898–906
9. Lambert E, Boudot C, Kadri Z, Soula-Rothhut M, Sowa ML, Mayeux P, Hornebeck W, Haye B, Petitfrere E (2003) Tissue inhibitor of metalloproteinases-1 signalling pathway leading to erythroid cell survival. *Biochem J* 372:767–774
10. Haviernik P, Lahoda C, Bradley HL, Hawley TS, Ramezani A, Hawley RG, Stetler-Stevenson M, Stetler-Stevenson WG, Bunting KD (2004) Tissue inhibitor of matrix metalloproteinase-1 overexpression in M1 myeloblasts impairs IL-6-induced differentiation. *Oncogene* 23:9212–9219
11. Baker AH, Zaltsman AB, George SJ, Newby AC (1998) Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death *in vitro*. TIMP-3 promotes apoptosis. *J Clin Invest* 101:1478–1487
12. Liu XW, Bernardo MM, Fridman R, Kim HR (2003) Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells against intrinsic apoptotic cell death via the focal adhesion kinase/phosphatidylinositol 3-kinase and MAPK signaling pathway. *J Biol Chem* 278:40364–40372
13. Benayahu D, Gurevitz OA, Shamay A (1994) Bone-related matrix proteins expression *in vitro* and *in vivo* by marrow stromal cell line. *Tissue Cell* 26:661–666
14. Gomis-Ruth FX, Maskos K, Betz M, Bergner A, Huber R, Suzuki K, Yoshida N, Nagase H, Brew K, Bourenkov GP, Bartunik H, Bode W (1997) Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. *Nature* 389:77–81
15. Meng Q, Malinovskii V, Huang W, Hu Y, Chung L, Nagase H, Bode W, Maskos K, Brew K (1999) Residue 2 of TIMP-1 is a major determinant of affinity and specificity for matrix metalloproteinases but effects of substitutions do not correlate with those of the corresponding P1' residue of substrate. *J Biol Chem* 274:10184–10189

16. Oliver L, Tremblais K, Guriec N, Martin S, Meflah K, Menanteau J, Vallette FM (2000) Influence of bcl-2-related proteins on matrix metalloproteinase expression in a rat glioma cell line. *Biochem Biophys Res Commun* 273:411–416
17. Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* 258:302–304
18. Grey A, Chen Q, Xu X, Callon K, Cornish J (2003) Parallel phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways subserve the mitogenic and antiapoptotic actions of insulin-like growth factor I in osteoblastic cells. *Endocrinology* 144:4886–4893
19. Liu XW, Bernardo MM, Fridman R, Kim HR (2003) Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells against intrinsic apoptotic cell death via the focal adhesion kinase/phosphatidylinositol 3-kinase and MAPK signaling pathway. *J Biol Chem* 278:40364–40372
20. Yamashita K, Suzuki M, Iwata H, Koike T, Hamaguchi M, Shinagawa A, Noguchi T, Hayakawa T (1996) Tyrosine phosphorylation is crucial for growth signaling by tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). *FEBS Lett* 396:103–107
21. Kyriakis JM, Avruch J (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* 18:567–577
22. Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9:180–186
23. Lee SJ, Yoo HJ, Bae YS, Kim HJ, Lee ST (2003) TIMP-1 inhibits apoptosis in breast carcinoma cells via a pathway involving pertussis toxin-sensitive G protein and c-Src. *Biochem Biophys Res Commun* 312:1196–1201