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ORIGINAL ARTICLE

Suppressive effect of dexamethasone on TIMP-1 production involves murine osteoblastic MC3T3-E1 cell apoptosis

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Abstract High dose glucocorticoid (GC) treatment induces osteoporosis partly via increasing osteoblast apoptosis. However, the mechanism of GC-induced apoptosis has not been fully elucidated. Osteoblast-derived tissue inhibitor of metalloproteinase-1 (TIMP-1) was recently reported to be involved in bone metabolism. Our previous study demonstrated that TIMP-1 suppressed apoptosis of the mouse bone marrow stromal cell line MBA-1 (pre-osteoblast) induced by serum deprivation. Therefore, we tested the effect of the GC dexamethasone (Dex) on TIMP-1 production in murine osteoblastic MC3T3-E1 cells and further determined whether this action is associated with Dex-induced osteoblast apoptosis. Dex decreased TIMP-1 production in MC3T3-E1 cells, and this effect was blocked by the glucocorticoid receptor (GR) antagonists, RU486 and RU40555. Recombinant TIMP-1 protein reduced caspase-3 activation and apoptosis induced by Dex in MC3T3-E1 cells. In addition, the pro-apoptotic effect of the Dex was augmented by suppression of TIMP-1 with siRNA. Furthermore, mutant TIMP-1, which has no inhibitory effects on MMPs, yet protects MC3T3-E1 cells against Dex-induced apoptosis. Our study demonstrates that Dex suppresses TIMP-1 production in osteoblasts through GR, and this effect is associated with its induction of osteoblast apoptosis. The anti-apoptotic action of TIMP-1 is independent of its inhibitory effects on MMPs activities. The decrease in TIMP-1 production caused by Dex may contribute to the mechanisms of Dex-induced bone loss.

Keywords Dexamethasone · Tissue inhibitor of metalloproteinase-1 · Apoptosis · Osteoblast

Introduction

Glucocorticoids (GCs) are widely used as anti-inflammatory and immuno-suppressive drugs in the treatment of autoimmune, pulmonary and gastrointestinal disorders, as well as in transplantation (Bjelakovic et al. 2006; Heitzer et al. 2007). A frequent side effect of long-term GC therapy is reduction in bone density. It is the third most prevalent form of osteoporosis after postmenopausal and senile osteoporosis (Migliaccio et al. 2007; Weinstein et al. 1998). High-dose GC treatment induces osteoporosis partly via increasing osteoblast apoptosis (Weinstein et al. 1998; Zalavras et al. 2003). However, the mechanism of GC-induced apoptosis of osteoblast has not been fully elucidated.

The effects of GCs on osteoblasts occur via glucocorticoid receptor (GR) (Espina et al. 2008; Gu et al. 2005; Suarez et al. 1993). In its inactivated state, the GR associates with heat-shock proteins, such as hsp90 and hsp70, and with immunophilins to form a complex in the cytoplasm (Beato 1989). Without a ligand, the complex resides predominantly in the cytoplasm and prevents the interaction of GR with DNA. When GC hormone enters the cell, it

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binds to GR and induces a conformational change of the receptor causing its "activation" and dissociation from the protein complex. The activated GR is transferred to the nucleus, where it dimerizes and binds to specific DNA sequences (Beato 1989).

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 (Krane and Inada 2008; Liao and Luo 2001). Over the past decade, evidence has accumulated for an active participation of osteoblast-derived MMPs in the initiation of bone resorption by degrading the unmineralized osteoid layer of the bone surface to allow osteoclasts to attach to the mineralized matrix (Krane and Inada 2008). In the TIMP family, TIMP-1 is the predominant form expressed by bone cells (Liao and Luo 2001). Recently, it was shown that TIMP-1 over-expression increased bone density in mice (Geoffroy et al. 2004). Merciris et al. (2007) reported that over-expression of TIMP-1 in osteoblasts increases the anabolic response to parathyroid hormone (PTH). The ability of TIMP-1 to inhibit MMP activation could partially account for the increased bone density. Furthermore, studies have shown that the anti-apoptotic activity of TIMP-1 appeared to be a direct cellular effect and was independent of its function as an MMP inhibitor (Guedez et al. 1998a, b; Guo et al. 2006; Lambert et al. 2003; Liu et al. 2005).

The present study was undertaken to investigate the action of dexamethasone (Dex) on TIMP-1 production in osteoblastic MC3T3-E1 cell and to further determine whether this effect is associated with Dex-induced osteoblast apoptosis.

Materials and methods

Reagents

Anti-TIMP-1 and anti-rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). Substrates for caspase-3-like proteinase (Ac-DEVD-MCA), bovine serum albumin (BSA), Dex, RU40555 and RU486 were purchased from Sigma Chemical Company (St Louis, MO, USA).

MC3T3-E1 cell culture

The murine osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in α-MEM (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbic acid. Cells were maintained in a humidified, 95% air, 5% CO_2 atmosphere at 37°C. The medium was changed twice a week and the cells were subcultured using 0.05% trypsin with 0.01% EDTA.

Quantification of TIMP-1 mRNA expression by real-time quantitative RT-PCR

Cells were plated at a density of 1×10^5 cells/well in six-well plates in α-MEM containing 10% FBS for 1 day followed by culture in α-MEM containing 1% BSA for 24 h in the presence of vehicle or 10^{-9} to 10^{-6} M Dex. To study the effects of GR inhibitors, the cells were pretreated with 10^{-5} M RU486 or 10^{-5} M RU40555 for 2 h prior to 10^{-6} M Dex treatment for 24 h. Total RNA from cultured MC3T3-E1 cells was isolated using Trizol reagent (GIBCO-BRL) according to the manufacturer's recommended protocol. Reverse transcription was performed using 2 μg total RNA and the Reverse Transcription System (Promega Corporation). The resulting cDNA was used for real-time quantitative PCR analysis. The PCR primers were as follows: TIMP-1 sense, 5′ CCAGAACCGCAGTGAA GAGT 3′; TIMP-1 antisense, 5′ GGGATAGATAAACA GGGAAACA 3′. Real-time PCR was performed using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) and the SYBR Premix Ex TaqTM Kit (TaKaRa, Kyoto, Japan). The *β*-actin gene was used as a reference housekeeping gene. Two microliters of template cDNA from each sample was mixed with the ready-to-use primers and SYBR Premix Ex *Taq* (including TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+} and SYBR Green I) in a final volume of 20 μl. The preincubation and denaturation of the template DNA were performed at 95°C for 20 s. This was followed by amplification of the target cDNA through 40 cycles of denaturation at 95°C for 5 s and annealing and elongation at 60°C for 20 s. The fluorescence resulting from SYBR Green I incorporation into double-stranded DNA was repeatedly detected at the end of the elongation phase of each PCR cycle. At the completion of cycling, melting curve analysis was carried out by increasing the temperature from 65 to 95°C in increments of 0.1°C/s to establish the specificity of the amplified product. The samples were then cooled to 40°C. The expression level of each mRNA and the estimated crossing point in each sample were determined relative to the standard preparation using the LightCycler computer software. A ratio of TIMP-1 mRNA/*β*-actin amplification was then calculated to correct for any differences in RT efficiency.

Western blot analysis

The cells were plated at a density of 1×10^5 cells/well in six-well plates in α-MEM containing 10% FBS for 1 day followed by culture in α-MEM containing 1% BSA for

24 h in the presence of vehicle or 10^{-9} to 10^{-6} M Dex. To study the effects of GR inhibitors, the cells were pretreated with 10^{-5} M RU486 or 10^{-5} M RU40555 for 2 h prior to 10^{-6} M Dex treatment for 24 h. In addition, the cells were cultured in α-MEM containing 1% BSA and treated with 10^{-6} M Dex for 0, 6, 12 or 24 h. The cell layers were homogenated with Triton lysis buffer (50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, and 1 μg/ml aminoethylbenzenesulfonyl fluoride). The lysates were centrifuged for 15 min at 3,000*g* to remove debris. Protein concentrations were determined using the Bradford protein assay. Forty micrograms of protein from each cell layer homogenate was loaded onto a 10% polyacrylamide gel and transferred to a PVDF membrane. After blocking with 5% nonfat milk, the membranes were incubated with anti-TIMP-1 or anti-β-actin antibodies. After extensive washing, the membranes were re-probed with peroxidase-conjugated secondary antibody. Blots were processed using an ECL kit and exposed to film, then analyzed by densitometry.

ELISA

The cells were plated at a density of 1×10^5 cells/well in six-well plates in α-MEM containing 10% FBS for 1 day, followed by culture in α-MEM containing 1% BSA for 24 h in the presence of vehicle or 10^{-9} to 10^{-6} M Dex. To study the effects of GR inhibitors, the cells were pretreated with 10^{-5} M RU486 or 10^{-5} M RU40555 for 2 h prior to 10^{-6} M Dex treatment for 24 h. In addition, the cells were cultured in α-MEM containing 1% BSA and treated with 10^{-6} M Dex for 0, 6, 12 or 24 h. TIMP-1 protein levels in various conditioned media were detected by performing ELISA using a commercial kit that was obtained from ADL (San Antonio, TX, USA). The absorbance was read and the concentration was calculated by a μQuant^{TM} Microplate spectrophotometer (Bio-tec) and its software. Protein concentrations of extracts were estimated by conducting a Bradford protein assay using BSA as a standard.

Cell apoptosis measurement

Apoptosis was assessed directly by measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histones in the cytoplasm) using a Cell Death Detection ELISA Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) according to the kit protocol. The Cell Death Detection ELISA Kit allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates. Briefly, the cells were plated at a density of 2.5×10^4 cells/well in 24-well plates in α-MEM containing 10% FBS for 1 day, followed by culture in α-MEM containing 1% BSA for 6 h in the presence of vehicle or TIMP-1 (200–1,000 ng/ml) and 10^{-6} M Dex. The cell layers were rinsed with phosphate buffered saline (PBS) and extracted with 0.5 ml of lysis buffer after 30 min incubation at 4°C. The cell lysates were then centrifuged for 10 min at 15,000 rpm, and the aliquots of aqueous supernatant were tested for apoptosis using the Cell Death Detection Kit.

Assays for caspase-3

MC3T3-E1 cells were plated at a density of 2.5×10^4 cells/well in 24-well plates in α-MEM containing 10% FBS for 1 day, followed by culture in α-MEM containing 1% BSA for 1.5 h in the presence of vehicle or TIMP-1 (200–1,000 ng/ml) and 10^{-6} M Dex. The cells were lysed with 400 μl of lysis buffer (10 mM HEPES, pH 7.5 containing 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 2 mM phenylmethylsulfonylfluoride). Aliquots (50 μl) of the extracts were incubated for 1 h at 37°C with 50 μM enzyme substrate (Ac-DEVD-MCA for caspase-3-like proteinase) in 10 mM HEPES, pH 7.5, containing 50 mM NaCl, 2.5 mM dithiothreitol in a 100-μl reaction mixture. The fluorescence of the released AMC was measured by a spectrofluorometer, with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Protein concentrations of extracts were estimated by conducting a Bradford protein assay.

RNA interference for TIMP-1

Small-interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology Inc., Waltham, MA, USA (Catalog No: sc-37274). For gene knockdown experiments, the cells were plated at a density of 1×10^5 cells/well in six-well plates and cultured for 24 h in a medium without antibiotics. The cells were transfected with siRNAs (1 nmol per well) using Lipofectamine 2000 (Invitrogen Inc. Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h of culture, the cells were retransfected with siRNAs and then recultured for another 24 h and then treated with vehicle or 10^{-6} M Dex for 6 h. The level of TIMP-1 protein in medium was measured using an ELISA Kit. Cell apoptosis was assayed by ELISA as above.

Plasmid constructs and transfection

Mouse wild-type full-length TIMP-1 cDNA (WT TIMP-1) and mutant TIMP-1 cDNA (Mut TIMP-1) were constructed and transfected into MC3T3-E1 cells as previously described (Guo et al. 2006).

Cultured WT TIMP-1 and Mut TIMP-1 over-expression clones were treated with vehicle or 10^{-6} M Dex for 6 h. The levels of WT TIMP-1 and Mut TIMP-1 in media were measured using the ELISA Kit (ADL San Antonio, TX, USA). MMP activity in media was evaluated using the MMP-2 and MMP-9 activity assay systems (Amersham, Piscataway, NJ, USA). Cell apoptosis was assayed by ELISA as above.

Statistical analyses

SPSS 13.0 was used for the statistical analyses. Data are presented as the mean ± SD. Statistical comparison between groups and treatments was performed using Student's *t* test. $P < 0.05$ was considered to be significant. All experiments were repeated at least three times, and representative experiments are shown.

Results

Effects of Dex on mRNA levels of TIMP-1

Real-time quantitative RT-PCR analysis revealed that Dex inhibited the levels of TIMP-1 mRNA in a dose-dependent manner (Fig. 1). At 24 h of MC3T3-E1 cell culture, the levels of TIMP-1 at 10^{-8} M Dex concentration were slightly decreased compared to controls (77 ± 6% of control; $P < 0.05$). At 10^{-7} M Dex concentration, the levels of TIMP-1 decreased dramatically compared to controls (50 ± 7% of control; $P < 0.05$). At 10^{-6} M Dex concentration, the levels of TIMP-1 decreased more obviously compared to controls (26 ± 7% of control; $P < 0.05$).

Effects of Dex on TIMP-1 protein expression

To determine the effect of Dex on intracellular TIMP-1 protein expression in cultures of MC3T3-E1 cell, Western blot was performed. The intensity of the 28 kDa TIMP-1 bands at a 10^{-8} M Dex concentration was less than that of controls (41 ± 16% of control; $P < 0.05$); at a 10^{-7} M Dex concentration, the intensity of the 28-kDa TIMP-1 bands decreased dramatically (19 ± 10% of control; $P < 0.05$), and at a 10^{-6} M Dex concentration, the bands decreased much more (8 ± 6% of control; $P < 0.05$) (Fig. 2a). Figure 2b shows that treatment with 10^{-6} M Dex for 6, 12 and 24 h, the TIMP-1 protein levels were inhibited dramatically compared to the control. These results demonstrated that Dex inhibited TIMP-1 protein expression by MC3T3-E1 cells in a dose- and time-dependent manner.

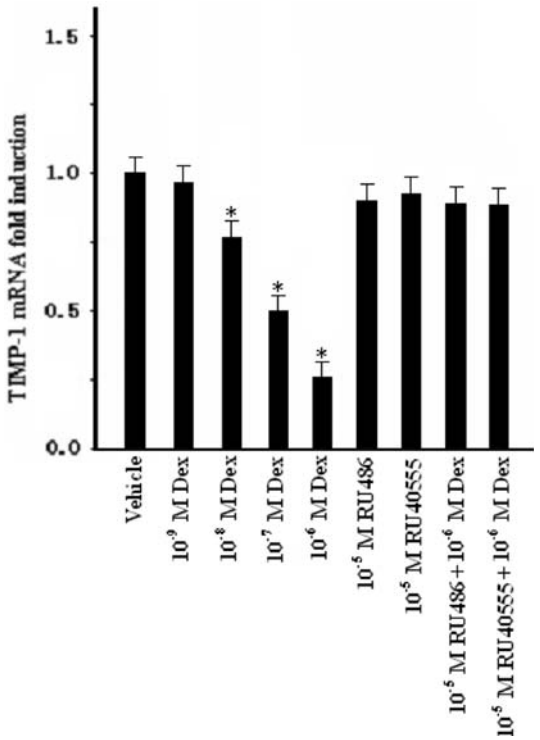


Fig. 1 Effects of dexamethasone (Dex) on TIMP-1 mRNA expression in MC3T3-E1 cells. mRNA expression is given as fold induction relative to control. *Bars* represent mean ± SD ($n = 4$). *$P < 0.05$ versus vehicle-treated cell cultures. 10^{-8} to 10^{-6} M concentrations of Dex significantly inhibit TIMP-1 mRNA expression. GR antagonists, RU486 or RU40555, respectively, oppose Dex-induced decreases in TIMP-1 mRNA expression, but these compounds alone do not have any obvious effect on TIMP-1 mRNA expression

Effects of Dex on extracellular TIMP-1 protein accumulation

To determine the effect of Dex on extracellular TIMP-1 protein accumulation in conditioned media of MC3T3-E1 cell cultures, ELISA was performed. The amounts of TIMP-1 in conditioned media of MC3T3-E1 cell cultures decreased with increasing doses of Dex (Fig. 3a). The levels of TIMP-1 at 10^{-6} M Dex concentration in MC3T3-E1 cell cultures were nearly three times lower than the control (Fig. 3a). A significant decrease of TIMP-1 was observed after 6 h of exposure to 10^{-6} M Dex in MC3T3-E1 cell cultures, whereas treatment for 24 h caused a maximal inhibition of TIMP-1 production (Fig. 3b).

GR mediates the suppressive effects of Dex on TIMP-1 production in MC3T3-E1 cells

Figure 1 shows that GR inhibitor RU486 (10^{-5} M) or RU40555 (10^{-5} M), respectively, by itself did not affect the levels of TIMP-1 mRNA in MC3T3-E1 cells, but it was

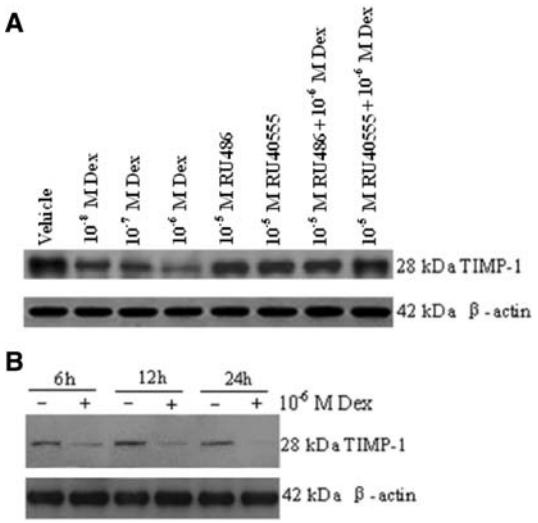


Fig. 2 Effects of Dex on TIMP-1 protein expression in MC3T3-E1 cells determined by Western blot. **a** Dose-dependent TIMP-1 protein expression in response to Dex in MC3T3-E1 cell cultures. The anti-TIMP-1 antibody identified a band at 28 kDa. The anti-β-actin antibody identified a band at 42 kDa. **b** Time course analysis for TIMP-1 protein expression in response to Dex in MC3T3-E1 cell cultures. Dex inhibits TIMP-1 protein expression. GR antagonists, RU486 and RU40555 oppose Dex-induced decreases in TIMP-1 protein expression, but, these compounds alone do not have any obvious effect on TIMP-1 production

able to abrogate the inhibitory action of Dex (10^{-6} M) on TIMP-1 mRNA expression.

Figures 2a and 3a show that 10^{-5} M RU486 or 10^{-5} M RU40555 by itself did not affect the intracellular and extracellular TIMP-1 protein production in MC3T3-E1 cells, but abolished the inhibitory effects of 10^{-6} M Dex on TIMP-1 protein accumulation.

These results support the conclusion that Dex suppresses TIMP-1 production in MC3T3-E1 cells by a GR-dependent mechanism.

TIMP-1 suppressed apoptosis induced by Dex in MC3T3-E1 cells

Figure 4 shows that Dex induced apoptosis of MC3T3-E1 cells. After 6 h of incubation, apoptotic cells at 10^{-7} M (1.32 ± 0.18 ELISA absorbance units) and 10^{-6} M (2.23 ± 0.16 ELISA absorbance units) Dex were more than that of controls (0.29 ± 0.11 ELISA absorbance units, all $P < 0.05$).

Figure 5 shows that 200 ~ 1,000 ng/ml TIMP-1 dose-dependently protected MC3T3-E1 cells against Dex-induced apoptosis. After 6 h of incubation, apoptotic cells at 200 ng/ml (1.50 ± 0.11 ELISA absorbance units), 400 ng/ml (1.24 ± 0.09 ELISA absorbance units), 600 ng/ml (1.02 ± 0.10 ELISA absorbance units), 800 ng/ml

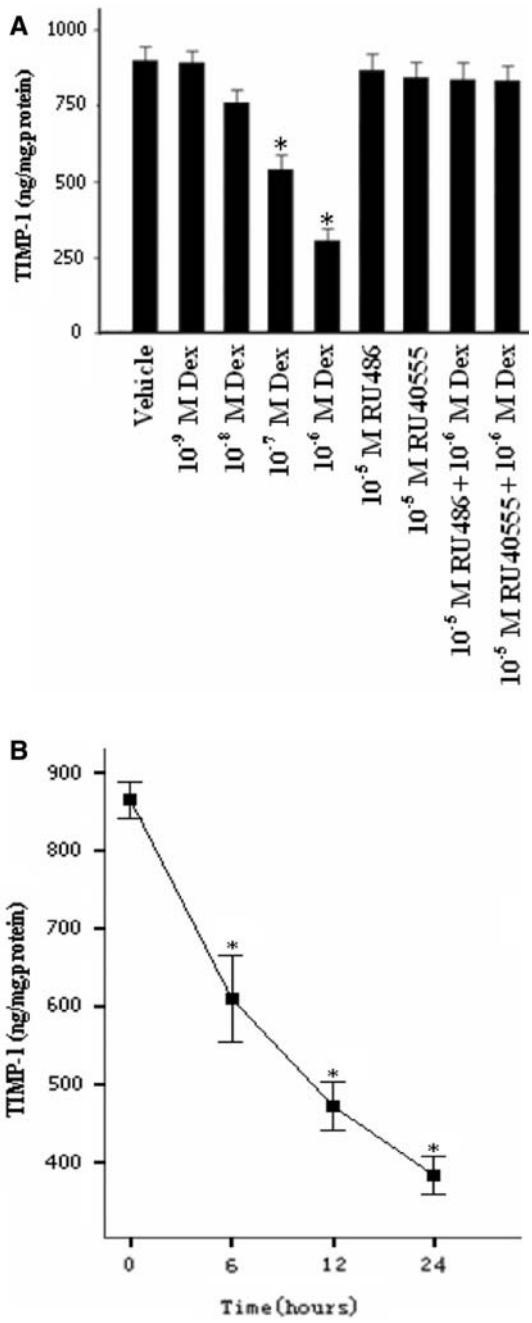


Fig. 3 Effects of Dex on TIMP-1 protein levels in conditioned media of MC3T3-E1 cells determined by ELISA. **a** Dose-dependent TIMP-1 protein secretion in response to Dex in cultured MC3T3-E1 cells. *Bars* represent mean ± SD ($n = 6$). *$P < 0.05$ versus control. **b** Time course analysis for TIMP-1 protein secretion in response to Dex in cultured MC3T3-E1 cells. *Bars* represent mean ± SD ($n = 6$). *$P < 0.05$, versus control. Dex decreases TIMP-1 protein levels in conditioned media of MC3T3-E1 cells. GR antagonists, RU486 or RU40555 oppose Dex-induced decreases in TIMP-1 protein secretion, but these compounds alone do not have any obvious effect on TIMP-1 production

(0.69 ± 0.08 ELISA absorbance units) and 1,000 ng/ml (0.80 ± 0.12 ELISA absorbance units) TIMP-1 were less than that of the group treated with Dex alone (2.19 ± 0.17

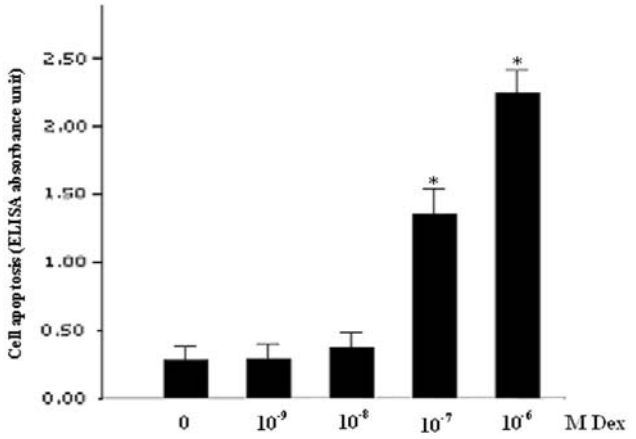


Fig. 4 Effect of Dex on MC3T3-E1 cells apoptosis by Cell Death ELISA. The *bars* represent the mean ± SD ($n = 6$). $^{*}P < 0.05$ versus control. 10^{-7} or 10^{-6} M Dex significantly promotes cell apoptosis

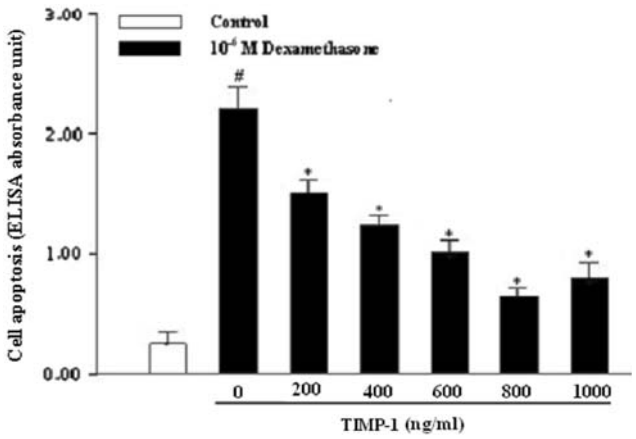


Fig. 5 Effect of TIMP-1 on apoptosis induced by Dex in MC3T3-E1 cells as determined by Cell Death ELISA. The *bars* represent the mean ± SD ($n = 6$). $^{\#}P < 0.05$, versus control. $^{*}P < 0.05$ versus 10^{-6} M Dex alone. 200 ~ 1,000 ng/ml TIMP-1 significantly inhibits cell apoptosis induced by Dex

ELISA absorbance units, all $P < 0.05$), showing a maximal antiapoptotic effect at 800 ng/ml.

Dex-induced activation of caspase-3 was also markedly suppressed in cells treated with 200 ~ 1,000 ng/ml TIMP-1 (Fig. 6).

Reduction of the TIMP-1 expression in the pro-apoptotic effect of Dex

Suppression of TIMP-1 with siRNA significantly increased Dex-induced cell apoptosis (Fig. 7). Our data indicate that reduction of the TIMP-1 expression is at least partly responsible for Dex-induced osteoblast apoptosis.

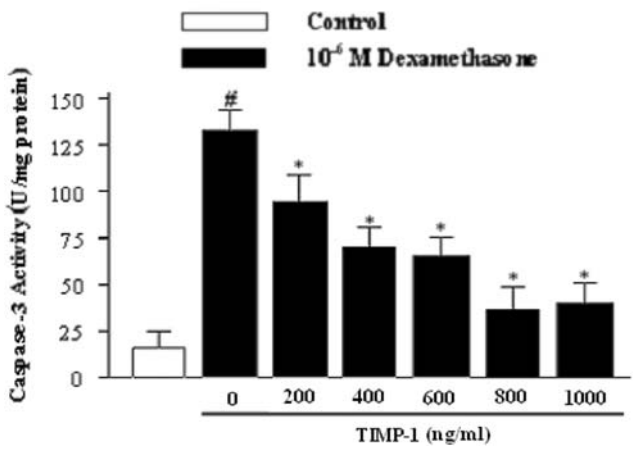


Fig. 6 Fluoro-substrate assays of the effects of TIMP-1 on Dex-induced caspase-3 activation in MC3T3-E1 cells. Data (caspase activity: U/mg protein) are expressed as the mean ± SD ($n = 6$). One unit was defined as the amount of the enzyme required to release 1 μM AMC per hour at 37°C. $^{\#}P < 0.05$ versus control. $^{*}P < 0.05$ versus 10^{-6} M Dex alone. 200 ~ 1,000 ng/ml TIMP-1 significantly inhibits caspase-3 activity induced by Dex

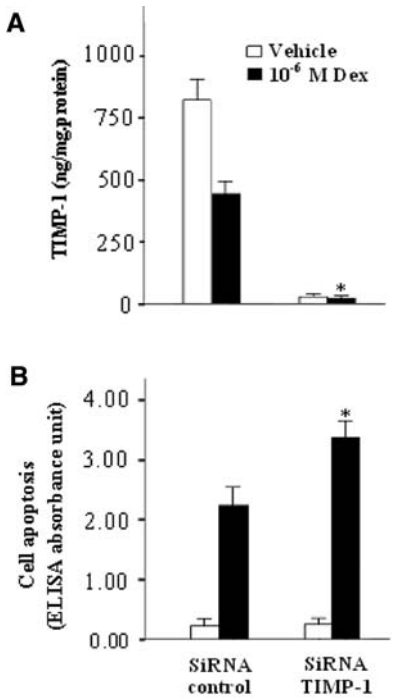
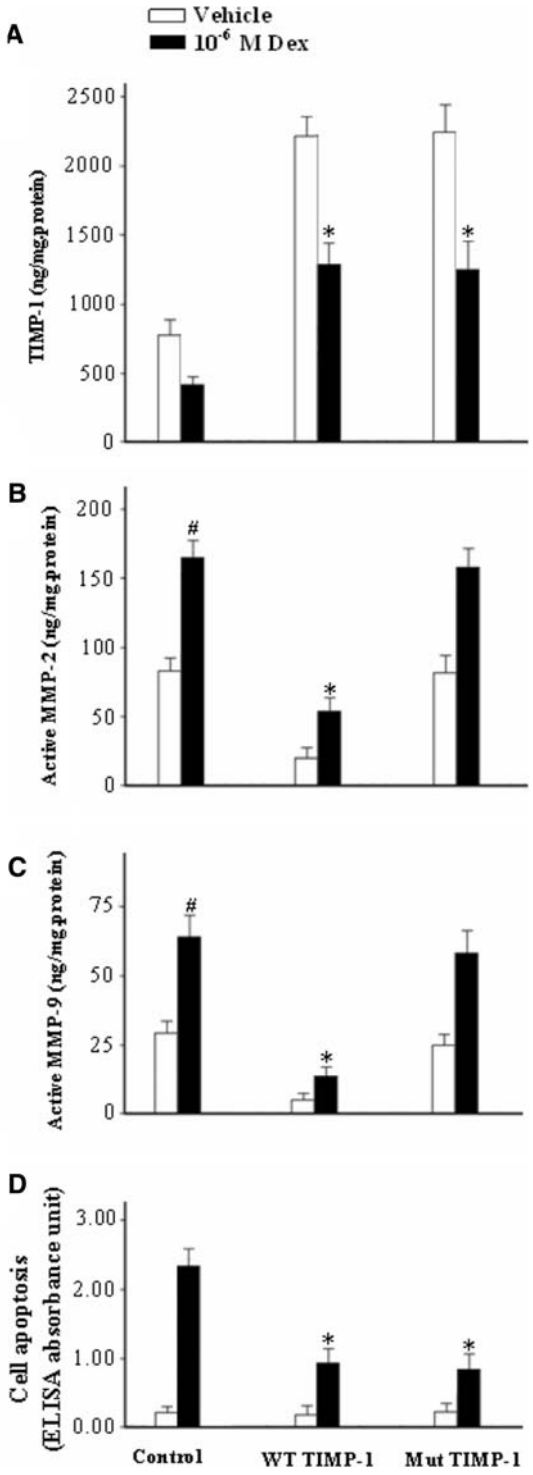


Fig. 7 Reduction of the TIMP-1 expression in the pro-apoptotic effect of the Dex. **a** Expression of TIMP-1 in the media of cultured siRNA-transfected cells by ELISA. *Bars* represent mean ± SD ($n = 4$). $^{*}P < 0.05$ versus Dex-treated control cells. **b** SiRNA control or siRNA TIMP-1 on the pro-apoptotic effect of the Dex. *Bars* represent mean ± SD ($n = 4$). $^{*}P < 0.05$ versus Dex-treated control cells. Reduction of the TIMP-1 expression increases Dex-induced osteoblast apoptosis



Effects of WT TIMP-1 and Mut TIMP-1 transfection on MMP activity and cell apoptosis

WT TIMP-1 and Mut TIMP-1 were over-expressed in the medium compared to control (Fig. 8a). Over-expression of WT TIMP-1, but not of Mut TIMP-1, dramatically decreased the levels of active MMP-2 and MMP-9 (Fig. 8b, c). This demonstrated that Mut TIMP-1, constructed by side-directed mutagenesis through deletion from Cys1 to Ala4, has no inhibitory activity on MMPs. Similarly, compared with control, over-expression of WT TIMP-1 decreased Dex-induced MC3T3-E1 cells apoptosis. However, importantly, over-expression of Mut TIMP-1 also protected MC3T3-E1 cells from Dex-induced apoptosis (Fig. 8d).

◀ **Fig. 8** Expression of WT TIMP-1 and Mut TIMP-1 in MC3T3-E1 cells, as well as WT TIMP-1 and Mut TIMP-1 effects on MMP activity and on apoptosis of MC3T3-E1 cells induced by Dex for 6 h. **a** Expression of TIMP-1 in the media of cultured, stably transfected cells by ELISA. *Bars* represent mean ± SD ($n = 4$). $^{*}P < 0.05$ versus Dex-treated control cells. **b** Effects of WT TIMP-1 and Mut TIMP-1 expression on MMP-2 activity assayed by ELISA. *Bars* represent mean ± SD ($n = 4$). $^{*}P < 0.05$ versus Dex-treated control cells. $^{\#}P < 0.05$ versus vehicle-treated control cells. **c** Effects of WT TIMP-1 and Mut TIMP-1 expression on MMP-9 activity assayed by ELISA. *Bars* represent mean ± SD ($n = 4$). $^{*}P < 0.05$ versus Dex-treated control cells. $^{\#}P < 0.05$ versus vehicle-treated control cells. **d** Apoptosis was determined using the Cell Death Detection Kit. Apoptosis was expressed as ELISA absorbance units. *Bars* represent mean ± SD ($n = 5$). $^{*}P < 0.05$ versus Dex-treated control cells. Mut TIMP-1 has no inhibitory activity on MMPs, yet protects MC3T3-E1 cells from Dex-induced apoptosis

Discussion

Our studies demonstrated that the transcription, synthesis and secretion of TIMP-1 in MC3T3-E1 cells were down-regulated by Dex. To our knowledge, this is the first report to reveal reduced levels of TIMP-1 in osteoblastic cells with Dex treatment. We also found that Dex inhibited TIMP-1 production through a GR-dependent mechanism, and this effect was associated with its induction of osteoblast apoptosis. Furthermore, we demonstrated that the anti-apoptotic action of TIMP-1 was independent of its inhibitory effects on MMPs activities.

Our present results show that Dex represses TIMP-1 production in osteoblastic MC3T3-E1 cells. Considering that TIMP-1 over-expression can increase bone density in mice (Geoffroy et al. 2004) and over-expression of TIMP-1 in osteoblasts increases the anabolic response to PTH (Merciris et al. 2007), we can hypothesize that suppression of TIMP-1 by GC in osteoblasts might be responsible for the deleterious effects of GCs on bone.

The physiological effects of GCs are mediated by the interaction of these hormones with the GR. GR expression has been identified in MC3T3-E1 cells (Masuyama et al. 1992). In the present study, we investigated whether Dex down-regulated TIMP-1 production through a GR-dependent mechanism. RU486 has both anti-GC and anti-progestin activity (Spitz and Bardin 1993). An important point is that higher doses of RU486 are needed to obtain an

anti-GC effect than an anti-progestin effect (Shoupe et al. 1987). Therefore, we applied a high concentration of RU486 (10^{-5} M) in our experiments. We also used another GR antagonist, RU40555, in our experiments. RU486 or RU40555, respectively, opposed Dex-induced decreases in TIMP-1 production in MC3T3-E1 cells, but, importantly, these compounds alone did not have any obvious effect on TIMP-1 production. These data support the conclusion that Dex suppresses TIMP-1 production in osteoblasts by a GR-dependent mechanism.

The rate of bone formation and resorption is largely determined by the numbers of bone-forming (osteoblast) and bone-resorbing (osteoclast) cells present in the basic multicellular units, responsible for the regeneration of the adult skeleton (Manolagas 2000; Manolagas and Jilka 1995; Tang et al. 2007; Xie et al. 2007). Similarly to other regenerating tissues, the number of bone cells is controlled by changes not only in the production of mature cells, but also in their survival. The majority of osteoblasts die by apoptosis. The frequency of osteoblast apoptosis could have a significant impact on the number of osteoblasts present at the site of bone formation (Manolagas 2000; Jilka et al. 1999).

Supra-physiological doses of GCs dramatically decrease OB number and bone formation rate and this may be explained in part by an increase in OB apoptosis, which has been reported in vivo and in vitro (Gohel et al. 1999; O'Brien et al. 2004; Plotkin et al. 1999; Weinstein et al. 1998; Xie et al. 2007). However, apoptosis following Dex treatment in our model is characterized by the specific determination of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates. After Dex treatment, MC3T3-E1 cells also displayed caspase-3 activation, which is a hallmark of apoptosis that has been previously reported (Chua et al. 2003; Xie et al. 2008; Wang et al. 2009).

Recently, TIMP-1 has been shown to display anti-apoptotic activity in B cells, Burkitt's lymphoma cell lines, a breast cell line and erythroid cells (Guedez et al. 1998a, b; Lambert et al. 2003; Liu et al. 2005). 10^{-8} M Dex approximates to a physiological amount of GC and 10^{-7} M Dex approximates to a supra-physiological amount of GC (Walsh et al. 2001). The concentration of TIMP-1 in human plasma was determined to be 100 ng to 1 μg/ml (Chevalier et al. 2001; Ishikawa et al. 2008; Maxwell et al. 2001). The present data showed that physiological doses (200 ~ 1,000 ng/ml) of TIMP-1 suppressed supra-physiological amount (10^{-6} M) of Dex-induced apoptosis in osteoblasts. However, considering that our previous study showed suppressive effect of TIMP-1 on the apoptosis of the mouse bone marrow stromal cell line MBA-1 (pre-osteoblast) induced by serum deprivation (Guo et al. 2006), we could hypothesize that TIMP-1 may be part of a more general mechanism involved in apoptosis suppression in osteoblasts and not only related to GCs-induced apoptosis. From our results, we can suggest that the inhibitory effect of TIMP-1 on Dex-induced apoptosis in osteoblasts may benefit bone formation.

Furthermore, we found that the anti-apoptotic action of TIMP-1 was independent of its inhibitory action on MMP activities. TIMP-1 is a classical inhibitor of MMPs; thus, some of its biological effects are likely mediated by inhibition of enzymatic activity (Brew et al. 2000; Westermarck and Kahari 1999). 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 (Baker et al. 2002; Haviernik et al. 2004). We constructed Mut TIMP-1 by side-directed mutagenesis, which abolished the inhibitory activity of MMPs by deletion of Cys1 to Ala4. Yet, it protected the MC3T3-E1 cells against Dex-induced apoptosis. This is consistent with our previous report (Guo et al. 2006), which showed that the anti-apoptotic actions of TIMP-1 in MBA-1 cells were independent of its inhibitory effects on MMP activities.

In summary, in osteoblasts, GC-induced down-regulation of TIMP-1 requires GR. We have confirmed a functional correlation between the levels of TIMP-1 and the degree of apoptosis induced by Dex. We further demonstrated that the anti-apoptotic action of TIMP-1 is independent of its inhibitory action on MMP activities. We therefore suggest that TIMP-1 is a novel regulator of osteoblast apoptosis in response to high-dose GCs and may be a target for therapeutic approaches.

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