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Effects of 17β-estradiol on the expression of interstitial collagenases-8 and -13 (MMP-8 and MMP-13) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in ovariectomized rat osteoblastic cells

Jian Li[1], Er-Yuan Liao[2,*], Ru-Chun Dai[2], Qi-You Wei[1] & Xiang-Hang Luo[2]
[1]*Department of Pathology*
[2]*Department of Endocrinology, Second Xiangya Hospital, Central South University, Changsha 410001, Hunan, P. R. China*

*Author for correspondence (e-mail: eyliao@21cn.com)

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Summary

Estrogen plays an important role in maintaining normal bone metabolism *via* the direct or indirect regulation of bone cells. Osteoblastic cells, as the target cells of estrogen, can secrete multiple matrix metalloproteinases (MMPs) that participate in bone remodeling. It has been demonstrated that bone loss induced by estrogen deficiency is closely related to the abnormal expression of multiple MMPs in osteoblastic cells. However, the regulating action of estrogen on the expression of interstitial collagenases MMP-8 and MMP-13 in osteoblastic cells *in vivo* remains unclear. We used an ovariectomized osteoporotic rat model to analyze the changes in the histomorphometric parameters of bone after and without treatment with 17β-estradiol (E_2); We also used immunohistochemistry and *in situ* hybridization to observe changes in the expression of mRNA and the proteins MMP-8, MMP-13 and TIMP-1 in osteoblastic cells in rat proximal tibia. In this study, we found that in the ovariectomized rat the expression of MMP-13 mRNA and protein increased markedly, whereas the expression of MMP-8 and TIMP-1 mRNA and protein did not change significantly. Our analysis showed that the expression of MMP-13 protein was correlated positively to bone trabecular separation, osteoid surface area, and negatively to trabecular numbers and the percentage of trabecula bone volume/total tissue volume. Our results suggest that MMP-13 plays an important role in estrogen deficiency-induced bone loss, while estrogen can inhibit bone resorption and reduce bone turnover rate by down-regulating the expression of MMP-13 in osteoblastic cells.

Introduction

Postmenopausal osteoporosis is a common disease characterized by loss of bone mass, deterioration in bone ultrastructure and an increase in bone fragility (Barondess *et al.* 2002). Estrogen deficiency results in high turnover bone loss, which can be prevented by estrogen replacement therapy (Notelovitz *et al.* 2002, Levine *et al.* 2003, Menon *et al.* 2003). However, the exact mechanism by which estrogen functions is as yet unclear.

Recent research has revealed that MMPs secreted by osteoblastic cells can degrade the osteoid that covers the surface of bone trabeculae and can initiate or activate bone remodeling in mice, rats and humans (Panagakos & Kumar 1994, McClelland *et al.* 1998). MMPs constitute a highly conservative proteinase family that participates mainly in the degradation of the extracellular matrix (ECM) (Bodey *et al.* 2000, Okada *et al.* 2000). MMPs can be classified into at least four groups (Hurst & Palmay 1999, Pustovrh *et al.* 2000, Massi *et al.* 2003): (1) collagenases (MMP-1, MMP-8 and MMP-13), which mainly degrade types I, II and III collagens; (2) gelatinases (MMP-2 and MMP-9), which degrade type IV collagen and various denatured collagen proteins; (3) stromelysins (MMP-3, MMP-10 and MMP-11) that exhibit broad substrate specificity by cleaving basement membrane components such as fibronectin and laminectin; and (4) other MMPs such as membrane type MMP (MT-MMP) (MMP-14, MMP-15 and MMP-16), acid MMP (MMP-6), and matrilysin (MMP-7). TIMPs are specific inhibitors of MMPs and include TIMP-1, TIMP-2, TIMP-3 and TIMP-4, which can bind with MMPs to form stable complexs that either disturb proenzyme activation or directly inhibit proenzyme activity. It has been found that the MMPs and TIMPs secreted by osteoblastic cells have a broad substrate specificity. They include collagenase, gelatinase, MT-MMP, TIMP-1 and TIMP-2 (Mizutani *et al.* 2001).

Many hormones or local cytokines of bone tissue can regulate the secretion of MMPs in osteoblastic cells. Factors such as PTH, IL-1, IL-6 and TNF accelerate the secretion of MMPs (Lin *et al*. 2003, Tsuzaki *et al*. 2003), while others as IGF-1 and TGF-1 inhibit their expression (Hui *et al*. 2001, Pattison *et al*. 2001). In an earlier study, we showed that estrogen can dose-dependently block the expression of MMP-1 mRNA and protein in MG-63 osteoblastic-like cells and human osteoblastic cells (HBO) cultured *in vitro* (Liao & Luo 2001). It can also enhance the MT1-MMP protein synthesis in MG63 cells but does not affect TIMP-1 expression (Luo & Liao 2001). The down-regulation of collagenase MMP-1 inhibits the decomposition of type I collagen and the rate of bone remodeling. In contrast, MT1-MMP can accelerate the decomposition of TNF-α and inhibit bone resorption. The reverse regulation of estrogen on MMP-1 and MT1-MMP in osteoblastic cells would impede bone remodeling. Breckon *et al*. (1999) observed that in the osteoblast of an estrogen-deficient mouse the expression of stromelysin MMP-3 is up-regulated which leads to intensified bone resorption. However, it is unclear whether or not estrogen regulates MMP-8 and MMP-13 in osteoblastic cells *in vivo*. Therefore, we used the ovariectomized rat model to investigate the changes in expression of MMP-8, MMP-13 and TIMP-1 in bone osteoblastic cells with or without E_2 treatment.

Materials and methods

Experimental animals

Eighteen female Sprague–Dawley rats, aged 7 months, weighting 230–280 g, were used. They were divided randomly into three groups: ovariectomized group (OVX), 17β-estradiol-treated group (ERT) and sham-operated group (SHAM). The rats in the ERT group were treated with a daily oral administration of 100 μg 17β-estradiol/kg body weight/day (Beijing Sihuan Pharmaceuticals, Beijing, China). The rats were fed continuously for 35 weeks. Calcein (0.5%) was injected into the abdominal cavity (0.1 ml/100 g body weight) on the fourteenth and fourth day, respectively, before they were sacrificed. After anesthesia with 3% pentobarbital (0.1 ml/100 g body weight), they were sacrificed by bloodletting from the ventral aorta. The above procedure was performed according to animal protocols institutionally approved by the Xiangya Medical College of Central South University.

Bone plastic embedding

One-third proximal right tibiae of rats were used. Surrounding muscular conjunctive tissues were carefully removed. Bone tissues were fixed for 24 h with 4% paraformaldehyde (pH 7.4) containing 0.1% DEPC, followed by a wash for 12 h with 0.1 M phosphate-buffered saline (PBS) pH 7.4 containing 10% sucrose. They were then dehydrated with 70% ethanol for 2 days, 95% ethanol 2 days, 100% ethanol 2 days, and finally dimethylbenzene 2 days. Fixation and dehydration were performed at 4 °C. Specimens were successivley soaked in plastic polymerization solutions I, II and III for 3 days each (Erben 1997). The formulae of the polymerization solutions were as follows. Solution I: 100 ml methylmethacrylate (MMA) (TianJin BoDie Chemical Factory) + 35 ml butyl methacrylate + 5 ml methylbenzoate + 1.2 ml polyethylene 400 (Sigma, USA). Solution II: solution I + 0.4 g dry benzoylperoxide. Solution III: solution I + 0.8 g dry benzoylperoxide, plus 400 μl *N*,*N*-dimethyl-*p*-toluidine (Sigma, USA.) added at 4 °C. The solutions were stirred on a magnetic mixer for a few minutes. The mixture was quickly added to 20 ml embedding glass vials. Bone specimens were then placed into the vials. CO_2 was used to evacuate the oxygen in the embedding medium. The vial caps were screwed tightly. The glass vials were allowed to polymerise at −20 °C for 3 days.

Deplasticinization of sections

The mill was used to trim. The resultant plastic blocks were trimmed to $3 \times 2 \times 2$ cm. Sections, 4 μm- and 10 μm-thick, were cut with a HM 360 microtome (Microm;Walldorf, Germany) non-continually along the long axis of the tibiae. The sections were transferred to slides coated with chromalum-gelatin and dried for 36 h at 37 °C. They were deplastinized by placing them in three changes of 2-methoxyethylacetate (Shanghai Chemical Reagents Factory) for 20 min each, two changes of acetone for 5 min each, and one change of deionized water for 5 min. The deplastinized sections were hydrated in 0.1 M Tris–HCl buffer, pH 7.4, for 5 min.

Goldner's Masson trichrome staining method and fluorescence observations

Goldner's Masson trichrome method was slightly modified according to the method specified by Gruber (1992). The deplastinized, hydrated 4 μm-thick sections were treated as follows: (i) stained with 50:50 Harris's Hematoxylin-ferric chloride for 7 min, and rinsed twice with tap water at 25 °C; (ii) stained in 1% Ponceau-Acid Fuchsin for 2 min, and rinsed with 1% acetic acid; (iii) stained in 1% phosphomolybdic acid for 6 min, and washed by 1% acetic acid; (iv) stained in 0.1% Light Green SF for 5 min, and washed in 1% acetic acid; (v) dried at room temperature and mounted in gum.

The 10 μm-thick plasticized sections were examined directly by laser scanning confocal microscopy (Bio-Rad 1024, USA) to observe calcein fluorescent markers.

Bone histomorphometry

Static and dynamic parameters of bone formation and resorption were measured using the BIOQUANT 98 image software (R&M Biometrics, Inc. USA) .The observation fields were 1 mm under the epiphysial plate of tibiae and visualized at 100 × magnification for 10 fields of vision. The histomorphometric parameters included: trabecular thickness (Tb.Th), the percentage of trabecular bone volume within the total tissue volume (TBV/TTV), trabecular separation (Tb.Sp), trabecular number (Tb.N), mineral apposition rate (MAR), osteoid thickness (O.Th) and osteoid surface (OS/TBA). The above parameters comply with the guidelines of the nomenclature committee of the American Society of Bone and Mineral Research (Parfitt *et al*. 1987).

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin–biotin–peroxidase complex (SABC) method slightly modified from that specified by Chen *et al*. (1992). The procedure was as follows.

(1) Deplastinized slides were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, followed by a wash with 0.1 M PBS.
(2) The sections were placed in 10 mM citrate buffer, pH 6.0, and heated in a microwave oven at 800 W for 10 min.
(3) Next, the sections were incubated with normal goat serum for 15 min to reduce non-specific binding. Thirty microliters of diluted rabbit anti-MMP-13, MMP-8 or TIMP-1 polyclonal antibodies (Boster Company, WuHan, China) in PBS was placed on each section and incubated overnight for 18 h at 4 °C. The dilutions of the antibodies were 1:25, 1:25 and 1:30, respectively.
(4) After a wash in 0.1 M PBS buffer, biotinylated goat anti-rabbit IgG secondary antibody, diluted 1:80, was pipetted onto each section and incubated for 30 min at room temperature, followed by a wash in 0.1 M PBS.
(5) 3-Amino-9-ethylcarbazole (AEC) was used as the chromogen for 5 min. After extensive washing, the sections were lightly counterstained with Hematoxylin, and mounted in Aqueous Mounting Medium.

Normal placenta tissue was used for positive controls, and 0.1 M PBS was substituted for primary antibodies for negative controls.

Osteoblastic cells were considered immunopositive if their cytoplasm was coloured red. The total number of osteoblastic cells and the number of immunopositive osteoblastic cells in each section were recorded, and the percentage of labelled osteoblastic cells calculated. This method of counting was slightly modified from that specified by Langub *et al*. (2001). To assess the reproducibility of the method , two sets of sections at 1 mm intervals (Group A and Group B) from the same embedded samples subjected to two different immunohistochemistry applications (1 week apart) were quantified for comparison. The interval was larger than the sum of an average trabecular thickness (0.1 mm), a spacing between the marrow cavities (0.25 mm) and a bone remodeling unit (0.4 mm). Thus it seemed reasonable to sample two regions of the samples separated by at least 0.75 mm, and composed of different trabeculae and different remodeling sites.

In situ hybridization

The digoxigenin (DIG)-labelled oligonucleotide probes of MMP-8, MMP-13 and TIMP-1 were provided by the Boster Company (Boster Company, WuHan, China). The sequences of the probes are shown in Table 1. The *in situ* hybridization protocol was as follows:

(1) Deplastinized slides were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, and washed in 0.1 M PBS.
(2) The sections were then digested for 30 min at 37 °C with 3% citric acid-diluted pepsin, followed by a wash in 0.5 M PBS.
(3) Twenty microliters of pre-hybridization solution was placed on the sections for 2 h at 42 °C, followed by 20 μl of the hybridization probe for 12 h at 42 °C.
(4) After hybridization, the slides were washed successively in 2×standard saline-citrate (SSC), 0.5 × SSC and 0.2 × SSC for 15 min each, and then treated with the biotin–digoxin antibody for 60 min at 37 °C.
(5) After a wash in 0.5 M PBS for 5 min, the sections were incubated with streptavidin–biotin–peroxidase complex (SABC) for 60 min at 37 °C.
(6) Following a wash in 0.5 M PBS, the sections were subjected to the AEC chromogenic reaction for 5–10 min.

Table 1. The sequences of MMP-8, MMP-13 and TIMP-1 oligonucleotide probes.

Index	Probes sequences
MMP-8	5′-CCTCGCTGTGGAGTGCCTG ACAGTGGTGGT-3′
	5′-ACTCCTCTGACCCTGGTGCC TTGATGTATC-3′
	5′-CAGGTACTTCTGGAGAAGGCATCCTCAGCT-3′
MMP-13	5′-AGGCTCCGAGAAATGCAGTCTTTCTTCGGC-3′
	5′-TGATCTTTAAAGACAGATTCTTCTGGCGCC-3′
	5′-CATACAGTTTGAATACAGCATCTGGAGTAA-3′
TIMP-1	5′-ACCACCTTATACCAGCGTTATGAG ATCAAGATGAC-3′
	5′-CACAGGTCCCACAACCGCAGCGA GGAGTTTCTCAT-3′

(7) Lastly, the sections were lightly counterstained with hematoxylin and mounted in Aqueous Mounting Medium.

Normal liver tissue was used as a positive control and pre-hybridization solution was substituted for the hybridization solution for negative controls. Osteoblastic cells were considered positive if their cytoplasm presented a red colour. The method for calculating the percentage of positive osteoblastic cells was the same as that used in immunohistochemistry.

Statistics

Data analysis was performed using SPSS10.0 analyzing software. Multiple group comparisons were determined using one-way ANOVA with statistical significance at $p < 0.05$. Pearson correlation coefficients were calculated to assess the relationships between MMP-13 protein expression and the bone histomorphometric parameters.

Results

Bone histomorphometric parameters

Figure 1a–c shows that in the OVX group the trabecular number clearly decreased and the space between trabecula widened, compared to the ERT group. In the OVX group, TBV/TTV fell to 13.55% from 34.39% ($p < 0.05$) compared to the ERT group. Tb.N decreased to 4.21/mm from 7.94/mm. Tb.Sp widened to 211.93 μm from 84.90 μm. Bone metabolic kinetic indices OS/TBA and MAR rose 7.72 μm and 0.25 μm/day, respectively, and showed significant difference ($p < 0.05$). There was no evident change in trabecula thickness (Tb.Th) and osteoid thickness (O.Th) (Table 2). These results suggest that estrogen deficiency led to a large loss of bone in trabecular bones, while bone turnover rate rose significantly. Estrogen replacement therapy can effectively reverse the above changes.

Table 2. Comparison of histomorphometric parameters of proximal tibiae in three experimental groups. Data are means ± SEM.

Parameters	*n*	SHAM	OVX	ERT
Tb.Sp (μm))	6	101.53 ± 45.88	211.93 ± 35.61*	84.90 ± 24.85
Tb.N (/mm)	6	7.78 ± 2.44	4.21 ± 0.78*	7.94 ± 1.19
TBV/TTV (%)	6	29.54 ± 5.99	13.55 ± 5.26*	34.39 ± 12.43
Tb.Th (μm)	6	43.22 ± 24.03	33.27 ± 11.35	43.26 ± 13.52
OS/TBA (%)	6	16.77 ± 5.29	24.79 ± 7.75*	17.07 ± 3.61
O.Th (μm)	6	3.22 ± 1.85	4.59 ± 1.72	4.65 ± 2.69
MAR (μm/day)	6	0.67 ± 0.24	1.09 ± 0.30*	0.84 ± 0.29

*$p < 0.05$ *versus* SHAM group; $p < 0.05$ *versus* ERT group.

Immunohistochemistry and in situ *hybridization*

Figure 2a–d shows that in the OVX group compared to the ERT group, there was an evident increase in the number of osteoblastic cells which express MMP-13 mRNA and protein. Statistical analysis indicated that, compared to the ERT group, the positive expression of MMP-13 mRNA and protein in the OVX group rose 9.34% and 11.83%, respectively, which is significantly different ($p < 0.05$) (Tables 3 and 4). The positive rate of osteoblastic cells which express MMP-8 (Figure 3a-d) and TIMP-1 (Figure 4a–d) did not change significantly ($p > 0.05$). In two sets of sections, which represented different bone space sites (Group A and Group B), the expression of MMP-13, MMP-8 and TIMP-1 mRNA and proteins did not alter significantly (Tables 3 and 4). In addition to osteoblastic cells, positive expression of MMP-13, MMP-8 and TIMP-1 was also observed in part of the lining cells (Figure 5) and some sporadic monocytes in bone marrow cavities.

The relationship between MMP-13 protein expression and bone histomorphometric parameters

Analysis of the relationships showed (Table 5) MMP-13 protein expression is positively correlated to Tb.Sp and OS/TBA ($p < 0.05$), and negatively correlated to Tb.N and TBV/TTV ($p < 0.05$). These results indicated MMP-13 expression is closely related to estrogen deficiency-induced bone loss.

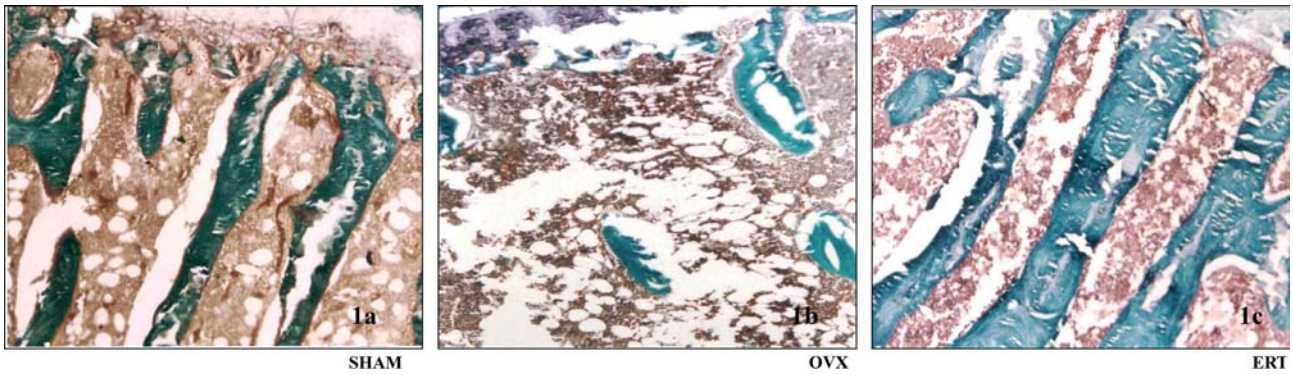

Figure 1. Goldner's Masson trichrome. In the OVX group (b) the trabecular number clearly decreased and the space between trabecula widened, compared to SHAM (a) and ERT group (c). (a)–(c), 100 ×.

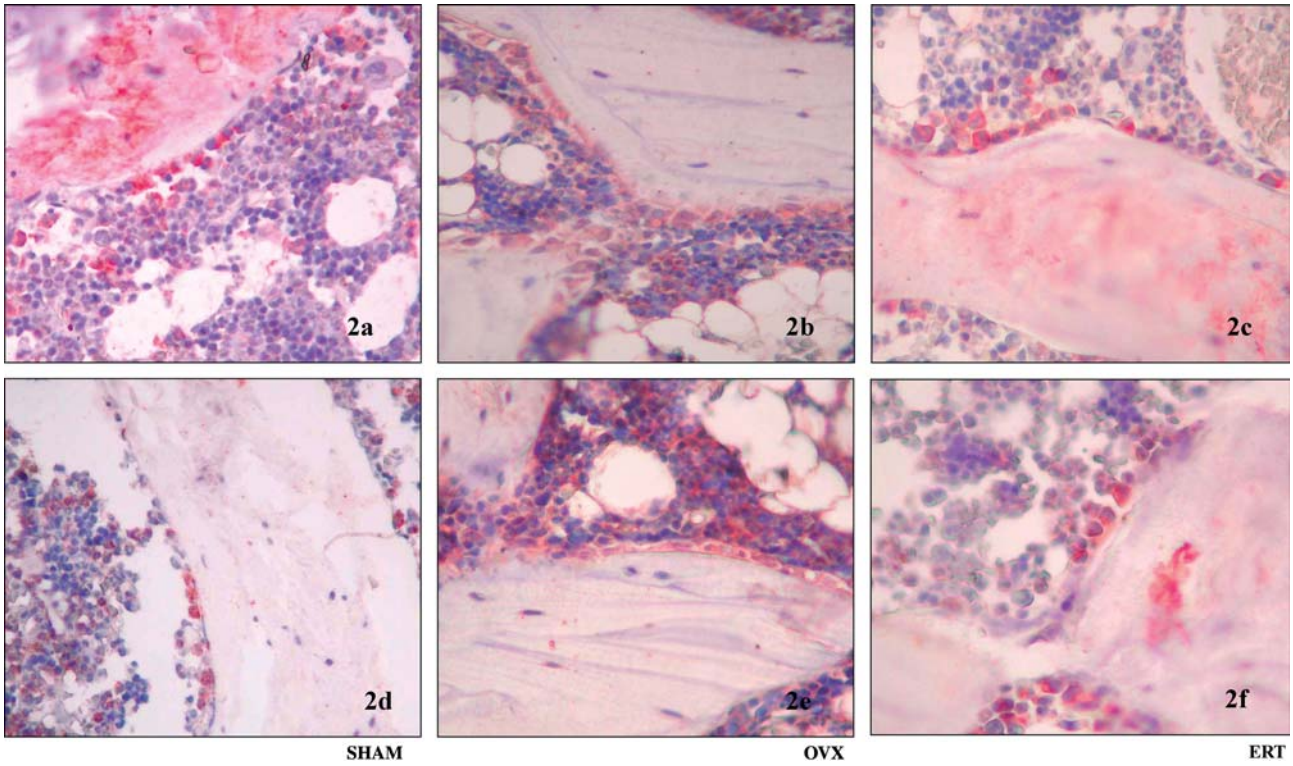

Figure 2. Positive expression of MMP-13 mRNA (d,e,f) and protein (a,b,c) in osteoblasts. In the OVX group there was an evident increase in the number of osteoblastic cells which expressed MMP-13 mRNA and protein compared to the ERT group or SHAM group. AEC staining (a–f), 200 ×.

Table 3. Comparison of the percentages of positive osteoblasts of MMP-13, MMP-8 and TIMP-1 protein in three experimental groups. Data are means ± SEM.

Rat groups	*n*	MMP-13		MMP-8		TIMP-1	
		Group A	Group B	Group A	Group B	Group A	Group B
SHAM	6	24.50 ± 4.04	25.50 ± 3.08	16.83 ± 3.76	17.17 ± 4.36	22.67 ± 3.32	22.83 ± 3.43
OVX	6	38.66 ± 3.3*	36.17 ± 3.1*	19.18 ± 3.16	16.67 ± 5.57	25.50 ± 5.00	23.67 ± 2.94
ERT	6	26.83 ± 4.17	24.33 ± 3.89	17.33 ± 3.92	14.67 ± 4.08	23.67 ± 3.74	24.00 ± 3.09

* $p < 0.05$ *versus* SHAM group; $p < 0.05$ *versus* ERT group.

Table 4. Comparison of the percentages of osteblasts positive for MMP-13, MMP-8 and TIMP-1 mRNA in three experimental groups. Data are means ± SEM.

Rat groups	*n*	MMP-13		MMP-8		TIMP-1	
		Group A	Group B	Group A	Group B	Group A	Group B
SHAM	6	22.67 ± 3.78	23.00 ± 3.34	19.00 ± 50.6	19.33 ± 5.46	21.83 ± 4.17	23.00 ± 3.03
OVX	6	34.67 ± 3.17*	35.00 ± 4.74*	19.33 ± 4.16	17.67 ± 6.50	26.33 ± 4.84	25.50 ± 4.72
ERT	6	25.33 ± 4.89	22.67 ± 3.23	18.17 ± 4.00	16.00 ± 3.74	23.50 ± 4.51	24.00 ± 4.98

* $p < 0.05$ *versus* SHAM group; $p < 0.05$ *versus* ERT group.

Discussion

The pathogenesis of osteoporosis is closely related to the declining estrogen level in body. In order to investigate the mechanisms of estrogen-induced bone loss, we studied the 7-month-old ovariectomized osteoporotic rats for 35 weeks. The analysis of bone histomorphometry demonstrated (Table 2) that in the OVX group compared to the ERT group, the static indices of bone metabolism, such as bone trabecular number and TBV percentage, declined 3.73/mm and 20.84%, respectively. Bone trabecular septa rose to 211.93 μm from 84.90 μm, which meant the width of septa increased by 127.03 μm (150%). This result

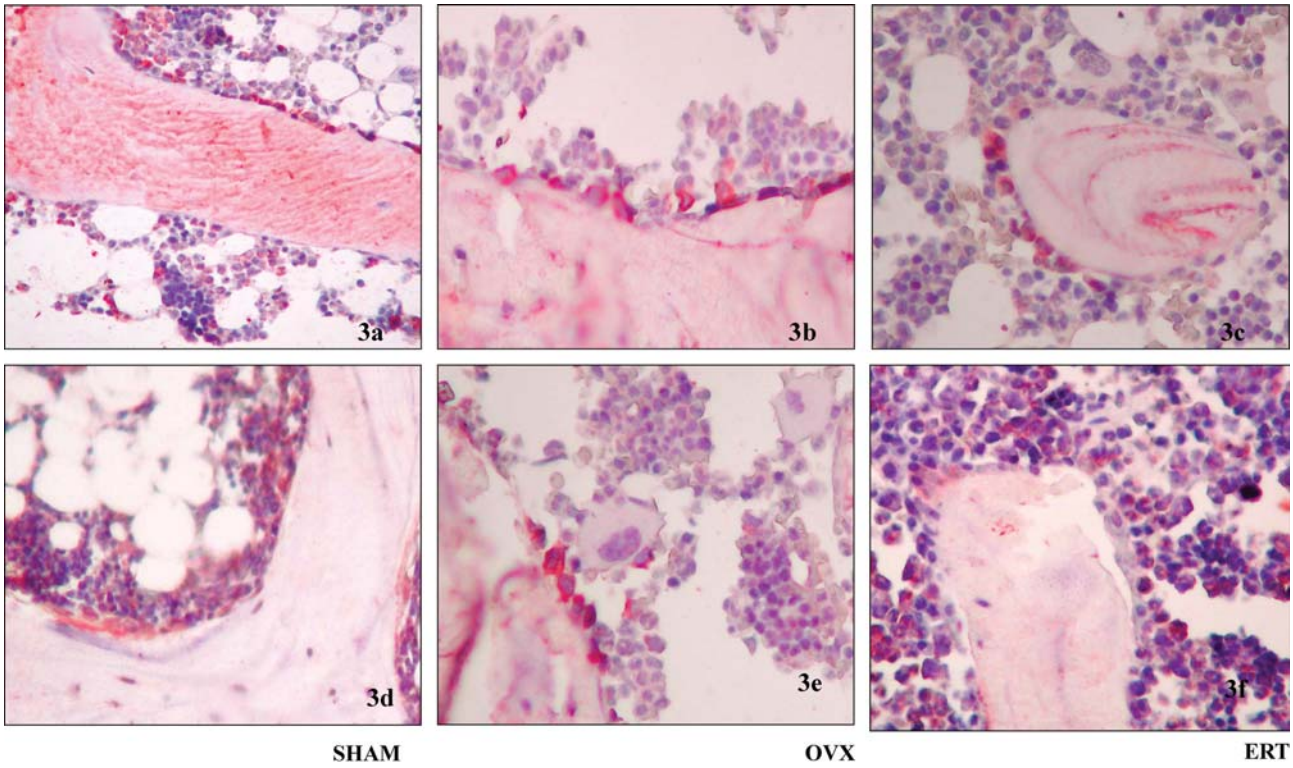

Figure 3. Positive expression of MMP-8 mRNA (d,e,f) and protein (a,b,c) in osteoblasts. In the OVX group, compared to the ERT group or SHAM group, the positive rate of osteoblastic cells which expressed MMP-8 did not change significantly. AEC staining (a–f), 200 ×.

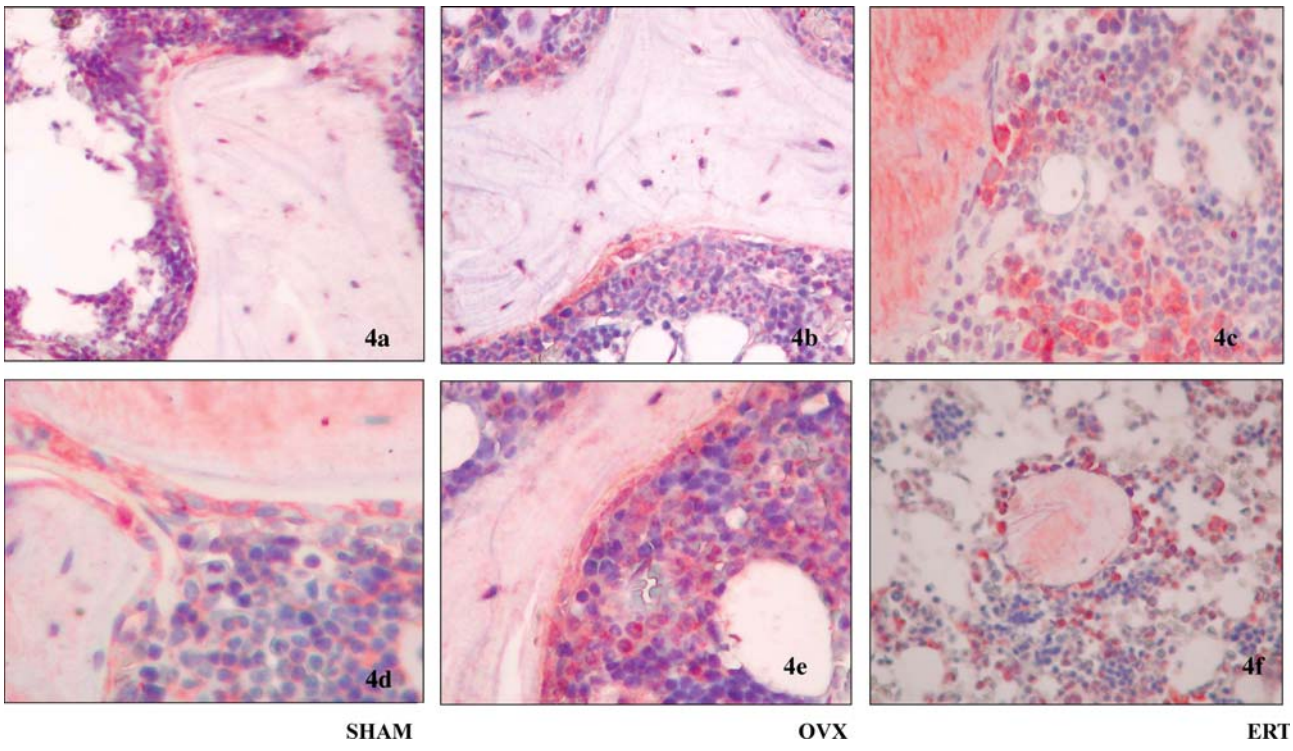

Figure 4. Positive expression of TIMP-1 mRNA (d,e,f) and protein (a,b,c) in osteoblasts. In the OVX group, compared to the ERT group or SHAM group, the positive rate of osteoblastic cells that express TIMP-1 did not change significantly. AEC staining (a–f), 200 ×.

indicated that bone loss in the proximal tibiae was large. Moreover, the osteoid percentage and the bone mineral deposition rate rose 7.72 μm and 0.25 μm/day. This showed that in the OVX rats group the bone metabolism was active and in a state of high bone turnover rate.

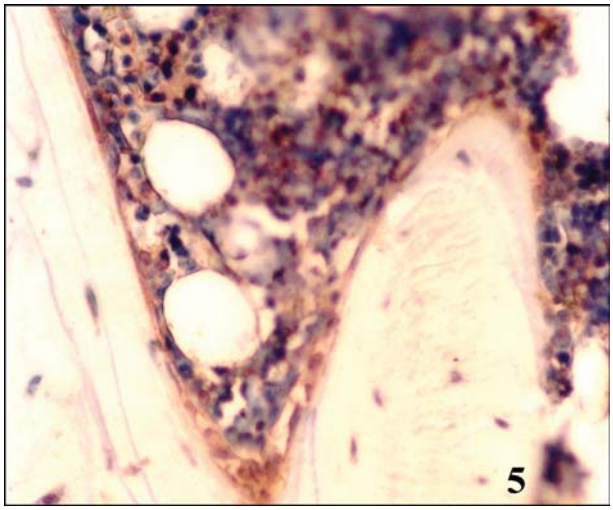

Figure 5. Positive expression of MMP-13 protein in lining cells . AEC staining 100 ×.

Table 5. Correlation between MMP-13 protein expression and bone histomorphometric parameters.

Parameters	*n*	Mean ± SEM	Correlation coefficient
Tb.Sp (μm)	18	132.81 ± 67.34	0.72*
Tb.N (/mm)	18	6.66 ± 2.35	−0.63*
TBV/TTV (%)	18	25.83 ± 12.17	−0.58*
Tb.Th (μm)	18	38.95 ± 18.86	−0.15
OS/TBA (%)	18	19.56 ± 6.65	0.61*
O.Th (μm)	18	4.09 ± 1.74	0.495
MAR (μm/day)	18	0.86 ± 0.32	0.40

* $p < 0.05$.

The surface of bone is covered by a layer of non-mineralized osteoid, mainly composed of type I collagen to prevent osteoclastic cells from coming into contact with mineralized bone matrix. Holliday *et al*. (1997) thought that if osteoid was degraded to expose the RGD (arg–gly–asp) sequence of $\alpha_v\beta_3$ integrin binding sites, it could then combine with the $\alpha_v\beta_3$ integrin in osteoclastic cells, and thus stimulate its activation. Collagenase, as an important member of the MMPs family, mainly degrades type I collagen in bone matrix and causes the loss of the osteoid barrier, and thus plays an important role in the initiation and activation of bone remodeling as well as bone resorption (Krane 1995, Ma *et al*. 2000, Uusitalo *et al*. 2000). Overexpression of collagenase will cause pathological bone loss. The expression of collagenase in osteoblastic cells has species differences. HOB can secrete three types of collagenase (MMP-1, MMP-8 and MMP-13), but rat osteoblastic cells only secrete MMP-8 and MMP-13 but not MMP-1 (Sasano *et al*. 2002). Our previous study has showed that estrogen can down-regulate MMP-1 protein expression of osteoblastic cells *in vitro* (Liao & Luo 2001). However, the regulation of estrogen on collagenase MMP-8 and MMP-13 in osteoblastic cells is still unclear.

Our present study, showed that in the OVX group, compared to the ERT group, the positive expression rate of MMP-13 mRNA and protein in osteoblastic cells increased by 9.34 and 11.83%, respectively, (Tables 3-4). The difference between the two groups was significant. The results are consistent with the study of Golub *et al*. (1999), who found the protein expression of MMP-8 and MMP-13 and collagenase activity in gingival tissues elevated significantly in the OVX adult female rat which also exhibited large amounts of alveolar bone loss. CMT-8, the MMPs inhibitor, suppressed this effect. It demonstrated that the overexpression of MMP-8 and MMP-13 protein may also be the important cause of alveolar bone loss. However, our research did not reveal any expression changes of MMP-8 mRNA and protein. Perhaps the regulatory effects of estrogen on osteoblastic cells differ from those of fibroblasts in gingival tissues.

TIMP-1 can inhibit the activity of MMPs including collagenase. In our experiments, we found that TIMP-1 expression displayed no obvious change in osteoblastic cells in the OVX group compared to the ERT group. This result was consistent with our earlier study on osteoblast-like cell cultures *in vitro* (Liao & Luo 2001) that indicated estrogen might have no effect on TIMP-1.

A previous study showed MMP-13 can be regulated by many osteotrophic hormones and cytokines. Uchida *et al*. (2001) reported hypercalcemia induced by PTH (1–34) was dependent positively to the expression level of MMP13 mRNA and protein in cranial bones. It indicated that PTH-stimulated bone resorption might be mediated by MMP-13. Delany *et al*. (1995) discovered cortisol could up-regulate the expression of MMP-13 protein in rat osteoblastic cells by prolonging the half-life of MMP-13 mRNA. Our study revealed the expressions of both MMP-13 mRNA and protein were up-regulated in estrogen-deficient OVX rats, suggesting estrogen could inhibit the expression of MMP-13. However, further study is required to determine whether the up-regulation mechanism of MMP-13 protein expression proceeded by increasing either the MMP-13 mRNA transcription or improving the mRNA stability, or both. The relationship between the MMP-13 protein and bone histomorphometric parameters (Table 5) showed that the expression of MMP-13 protein is positively correlated to Tb.N and OS/TBA ($p < 0.05$), and negatively correlated to Tb.N and TBV/TTV ($p < 0.05$). We also found that the correlation coefficient between MMP-13 mRNA and mineral apposition rate (MAR) was 0.53 and two of them were positively related ($p < 0.05$). However, MMP-13 protein was not correlated to MAR. The reason for the lack of the correlation is still unclear. Increasing the number of samples might yield a positive result. Correlation analyses demonstrated that the higher the MMP-13 protein expression, the greater is the bone loss, and is accompanied by an increase in the rate of bone turn-

over, which suggests that MMP-13 plays an important role in estrogen deficiency-induced bone loss.

We also noticed a positive expression of MMP-13, MMP-8 and TIMP-1 in part of the lining cells on the surface of bone trabeculae. This result confirms previous observations of Breckon *et al.* (1999). At present, most researchers in the field think lining cells and osteoblastic cells are homologous and osteoblastic cells can transform to lining cells when they are at the stationary phase (Dobnig & Turner 1995). We assumed that MMP-13, MMP-8 and TIMP-1 expression in lining cells may also participate in the degradation process of osteoid on the surface of bone trabeculae. However, whether the expression of MMPs in lining cells can be regulated by estrogen-like osteoblastic cells is not clear at present and needs further investigation. In addition, the reason for the positive expression of MMP-13, MMP-8 and TIMP-1 observed in a small number of monocytes in bone marrow is also unclear. They might be precursor cells or ancestral cells in cellular differentiation from multipotential mesenchymal cells to osteoblastic cells and possess phenotypic characteristics of osteoblastic cells.

Our research has shown that 17-β-estradiol can inhibit the expression of MMP-13 in osteoblastic cells. In estrogen-deficient states, without the effective antagonism of TIMP-1, the overexpression of MMP-13 would lead to excessive activation of bone remodeling, enhancing bone resorption and increasing bone loss. This pathway might be another important mechanism for estrogen deficiency-induced osteoporosis.

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