·论著·

Effect of estrogen on MMPs mRNA expression of osteoclasts

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Abstract: Objective To observe the effect of estrogen on the expression of matrix metalloproteinases (MMPs) mRNA in rabbit osteoclasts (OCs). **Methods** OCs were obtained from the long bone of newborn New Zealand rabbit directly and cultivated in M-199 medium supplemented with 15% fetal bovine serum. Cells were incubated in absence or presence of various concentrations of estrogen (0, 10^{-5} ~ 10^{-13} mol·L^{-1}), or treated with estrogen for different period according to the experimental design. Total cellular RNA was isolated with Trizol. Semiquantitative reverse transcription PCR method was used to determine the expression level of MMP-9 mRNA and MMP-8 mRNA. **Results** Estrogen dose and time-dependently inhibited the activities of osteoclasts. Treatment with estrogen at different concentrations down regulated the expression of MMP-9 mRNA level, especially when the concentrations of estrogen were 10^{-5} mol·L^{-1}, 10^{-6} mol·L^{-1} and 10^{-7} mol·L^{-1} ($P < 0.05$). There was no expression of MMP-8 mRNA in osteoclasts despite three repeated experiments. **Conclusion** Estrogen inhibits osteoclasts and then suppresses the bone resorption activities by decreasing the MMP-9 expression of OCs at mRNA level.

Key words: Estrogen; Osteoclast; Matrix metalloproteinases; Osteoporosis

雌激素对破骨细胞基质金属蛋白酶 mRNA 表达的影响

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摘要: 目的 本实验通过体外分离培养兔破骨细胞,观察不同浓度雌激素对兔破骨细胞基质金属蛋白酶 MMP mRNA 表达的影响。**方法** 体外分离培养出生 24 h 内的新西兰兔破骨细胞,用含有不同浓度 17β-雌二醇(0、10^{-5} ~ 10^{-13} mol·L^{-1})的 M199 培养液分别作用于破骨细胞,观察不同浓度雌激素及相同浓度雌激素不同时间对破骨细胞活性的影响,采用半定量逆转录-聚合酶链反应(RT-PCR)方法观察兔破骨细胞 MMP-9 mRNA、MMP-8 mRNA 表达状况。**结果** 不同浓度 17β-雌二醇对破骨细胞的活性有不同程度的抑制,同时对 MMP-9 mRNA 的表达有明显抑制作用,以 10^{-5}、10^{-6}、10^{-7} mol·L^{-1} ($P < 0.05$) 最为显著。所有破骨细胞均未表达 MMP-8 mRNA。**结论** 不同浓度的雌激素呈时间和剂量依赖性地抑制破骨细胞的活性,对兔破骨细胞基质金属蛋白酶表达的调控作用随其浓度变化而不同。

关键词: 雌激素; 破骨细胞; 基质金属蛋白酶; 骨质疏松

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Estrogen deficiency in both postmenopausal women and ovariectomized animals causes a substantial decrease in bone mass, leading to osteopenia and increased fracture risk. The efficiency of E_2 replacement therapy in preventing bone loss has been known for a long time. However, the exact cellular and molecular mechanisms behind the effects of E_2 on osteoclasts have remained questionable. Osteoclasts resorb bone by secreting acid and proteolytic enzymes into an extracellular resorption lacuna. Acid solubilizes the inorganic matrix, thus making the organic matrix available for the proteases. Matrix metalloproteinases (MMPs) have been suggested to play major roles in degradation of the organic matrix, which is composed mainly of type I collagen. To understand the molecular mechanism of pathogenesis of osteoporosis we investigated the expression and localization of MMPs transcription in osteoclasts regulated by E_2.

1 Material and methods

1.1 Animals and reagents

24-h-old New Zealand white rabbits were obtained from Nanjing Animal Company. M199 culture medium, HEPES, 17β-E_2, Amphotercin B and TRAP kit (387-A) were purchased from Sigma. Fetal bovine serum was purchased from Hangzhou Sijiqing Bioscience Company. RNAPCR kit was purchased from Takara Company.

1.2 Osteoclasts culture

A mixed rabbit bone cell population was cultured on glass coverslices as described in detail previously[1]. Briefly, osteoclasts were mechanically harvested from the long bones of 24-h-old rabbits and allowed to attach to glass coverslices for 30 min, after which non-attached cells were washed away with ice-cold phosphate buffer saline PBS. All cells on slices were cultured in M199 buffered with 20 mm HEPES containing 100 IU penicillin, 100μg streptomycin · mL^{-1}, and 15% heat-inactivated fetal bovine serum at a seeding density of 4×10^6 cells · mL^{-1}. All individual experiments were performed with osteoclasts isolated from bones of the same animal pool. After 24 hours the glass coverslices with cells were fixed with 5% glutaraldehyde in s-collidin-HCl buffer (pH 7.4, 0.16 M) for 45 minutes. Cells were stained with tartrate-resistant acid phosphatase (TRAP) to detect osteoclasts and observed by light microscope (100 ×, 200 ×).

1.3 Resorption lacunae formation assay

The bone slices were obtained by cutting perpendicularly to the long axis of the bovine long limb cortical bone. There were no living bovine cells in the slices because the bones were frozen during storage and the slices underwent ultrasonication prior to use. After 30 minutes, non-attached cells were washed away and the bone slices were transferred to 24-well plates. Cells were cultured at 37℃ (5% CO_2) for 48 hours to create resorption lacunae. Then bone slices were fixed with 5% glutaraldehyde in s-collidin-HCl buffer (pH 7.4, 0.16 M) for 45 minutes. Subsequently, samples were stained with diaminobenzidine solution and observed by light microscope and counted. The area resorbed was quantitated using an image analysis system (SIMPLE PCI). Finally, bone slices were dehydrated in an ascending ethanol series, processed for critical point drying. The pits were analyzed by laser scanning confocal microscope (Nanjing Normal University). The bone slices were then stored in PBS at 4℃ until used. The average size of bone slices was about 5 × 5 × 0.1 mm.

1.4 17β-E_2 treatment

To further specify the possible effects of E_2 on MMP enzyme activity, after washing with PBS osteoclasts suspension was seeded into 24-well plates (1 mL/well) with small bone slices at 37℃ in 5% CO_2 atmosphere. They were divided into 10 groups (6 wells/group) and treated with different concentrations of 17β-E_2 from 0, 10^{-5} ~ 10^{-13} mol · L^{-1} 1mL for 24 h. For osteoclasts activity observation, another three groups (6 wells/group) were treated with 0, 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} mol · L^{-1} 1mL for different periods. Culture medium was replaced after every 2 days of culture. In the following 1, 3 and 6 days, bone slices were taken out and stained. Resorption lacunae were observed and the areas of pits were analyzed using the methods mentioned above.

1.5 RT-PCR

Total RNA was extracted using the Trizol RNA extraction. One microgram of RNA was reverse transcribed using RNA PCR kit (Takara). PCR primer pairs used are listed in Table 1. For quantitative analysis, primers for the housekeeping gene α-tubulin

were used along with primers for the gene being analyzed annealing at 65℃. PCR-amplified products were analyzed on 1.5% agarose gel containing ethidium bromide.

Table 1 Primer pairs

Gene	Primers	bp	Reference
MMP-9	Fw 5′-AgTTTggTgTCgCggAgCAC-3′ Rv 5′-CCtgtCaCaaaagCCagctg-3′	450	GenBank
MMP-8	Fw 5′-ggAcccAATggAATccTTgt-3′ Rv 5′-ccTgAAAgCATAgTTgggATACAT-3′	420	GenBank
α-tubulin	Fw 5′-TCCTTCAACACCTTCTTCAG-3′ Rv 5′-TGGCCTCATTGTCTACCATG-3′	484	GenBank

bp, base pairs; Fw, forward; Rv, reverse.

1.6 Statistics

Data are expressed as the mean $\bar{x} \pm s$ of at least three independent experiments. By using SPSS 11.0 statistical analysis was performed by the one-way ANOVA. A P value < 0.05 was conventionally considered statistically significant.

2 Results

2.1 Identification of osteoclasts

2.1.1 Observation by phase contrast microscopy

The size of newly isolated osteoclasts was larger than other bone marrow cells distinctively. The activity of TRAP, a marker enzyme indicating osteoclast differentiation, was assayed in the cells in each well. The nuclei of these TRAP-positive giant multinucleate cells could be observed clearly, most of which had more than 10 nuclei with prominent nucleoli. Osteoclasts attached bone slices after 30 min, with the increase of culture periods; they extended fully and grew bigger. After being cultured for 2 or 3 hours, osteoclasts exhibited continuous changes in their appearance with some being oval-shaped, some being long strips, some infundibular and irregular, as shown in Fig.1a. Smaller osteoclast-like cells had extensive ruffled membranes on their "apical" surfaces with extending pseudopods in all directions. Notably, the multinucleate TRAP positive cells not only increased in number, but also in size, with multiple nuclei and pseudopod-like projections (Fig.1b). They also exhibited irregular morphology. The intensity of pseudopod activity varied from cell to cell; even though continuous it was not maintained at a constant level. This was indicated by spontaneous fluctuations in the intensity of their motility in the individual cells.

2.1.2 Resorption pits assay

TRAP-positive giant multinucleate cells were able to produce numerous resorption pits on the bone slice after 24 hours. The formation of resorption lacunae could be observed after which diaminobenzidine solution was added to the bone slices. The number and size of resorption lacunae formed *in vitro* are a quantitative measure of osteoclastic activity. Resorption pits were analyzed by light microscope, and digital images were recorded. At the beginning resorption lacunae were round and elliptic in shape, the resorption area was small. As the culture period continued, more and more resorption pits were shown on bone slices, the morphology of individual resorption pits became distinct and the depth of pits varied widely, some of which were connected to each other. Further observation using scanning microscope identified that although the appearance of pits could widely vary even on the same bone slice (round, elliptic, petal-like or irregular in shape), they had a definite clear boundary (Fig.1, c and d).

2.2 Effect of estrogen on the osteoclasts activity

2.2.1 Estrogen potently suppressed the number of TRAP positive cells in a dose-dependent manner for 24 hours. Treatment with estrogen of 10^{-5} mol·L^{-1} or higher concentration decreased TRAP positive cells significantly compared to the other groups ($P < 0.05$) (Table 2).

Table 2 The effect of E_2 on osteoclasts activity for 24-hour culture($\bar{x} \pm s$)

Dose (mol·L^{-1})	TRAP positive cells		Number of resorption lacunae per slice	Average resorption area per slice (μm^2)
	N (well)	Positive cells per slice		
10^{-5}	6	74.5 ± 29.2*	9.20 ± 0.24*	458.49 ± 84.39*
10^{-6}	6	87.2 ± 18.3*	13.9 ± 0.52*	469.30 ± 29.40*
10^{-7}	6	95.6 ± 12.4*	18.2 ± 1.87*	489.35 ± 86.32*
10^{-8}	6	103.4 ± 24.3	21.5 ± 0.12	520.67 ± 102.67
10^{-9}	6	103.7 ± 53.8	23.2 ± 1.12	528.39 ± 98.68
10^{-10}	6	105.6 ± 28.9	24.8 ± 0.74	536.38 ± 138.93
10^{-11}	6	105.4 ± 35.0	26.2 ± 1.38	550.25 ± 183.29
10^{-12}	6	108.7 ± 27.5	27.5 ± 1.58	573.48 ± 198.34
10^{-13}	6	111.5 ± 34.8	27.4 ± 1.36	586.25 ± 138.37
0	6	110.2 ± 32.0	28.3 ± 1.45	593.24 ± 143.50

* $P < 0.05$, the average size of bone slices was about 5 × 5 mm.

2.2.2 A well established osteoclast resorption assay was used to investigate whether E_2 directly affects bone resorption. The osteoclasts-induced formation of resorption pits was changed by estrogen. The number and area of resorption pits were decreased by estrogen dose-

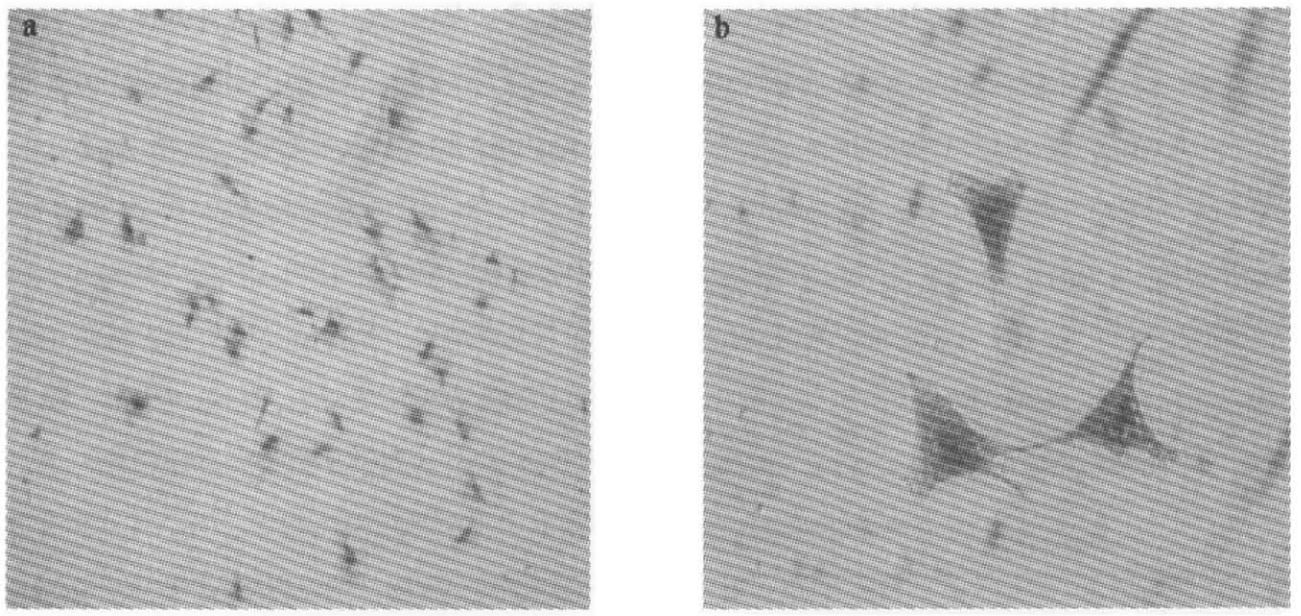

Fig 1 TRAP-positive cells (a (100 ×) and b (200 ×)); Osteoclasts resorption lacunae scanning microscope assay (c and d)

dependently. All experiments shown are representative of at least three similar experiments (Table 2).

2.2.3 Time-lapse effects of estrogen

Isolated rabbit osteoclasts were cultured for on bovine bone slices in the presence of different concentrations of E_2 for 1, 3 and 6 days. Although the average number and area of resorption pits gradually increased daily, the trend was opposed effectively by estrogen especially when its concentration was at 10^{-6} mol·L^{-1} (Table 3, 4).

Table 3 The effect of E_2 on number of resorption lacunae for different culture days($\bar{x} \pm s$)

Dose (mol·L^{-1})	Number of resorption lacunae per bone slice		
	1(d)	3(d)	6(d)
10^{-6}	11.9 ± 0.52*	13.8 ± 0.32*	14.4 ± 0.73*
10^{-8}	18.5 ± 0.12	23.3 ± 0.24	26.3 ± 0.34
10^{-10}	21.8 ± 0.74	25.5 ± 0.52	29.4 ± 0.32
10^{-12}	26.5 ± 1.58	30.3 ± 0.39	33.2 ± 0.63
0	28.3 ± 1.45	33.4 ± 0.27	37.3 ± 0.37

* $P < 0.05$, the average size of bone slices was about 5 × 5 mm.

Table 4 The effect of E_2 on average resorption area for different days($\bar{x} \pm s$)

Number	Dose (mol·L^{-1})	Resorption area(μm^2)		
		1(d)	3(d)	6(d)
1	10^{-6}	469.30 ± 89.40*	481.24 ± 98.34*	503.37 ± 103.32*
2	10^{-8}	517.67 ± 102.67	573.28 ± 123.74	626.34 ± 154.75
3	10^{-10}	535.38 ± 138.93	594.34 ± 98.46	656.53 ± 128.38
4	10^{-12}	573.48 ± 198.34	647.57 ± 172.85	727.43 ± 174.48
5	0	593.24 ± 143.50	704.85 ± 164.96	764.57 ± 189.37

* $P < 0.05$, the average size of bone slices was about 5 × 5 mm.

Table 5 The effect of E_2 on the expression of MMP-9 mRNA for 24-hour culture($\bar{x} \pm s$)

Number	Dose(mol·L^{-1})	MMP-9/α-tubulin
1	10^{-5}	0.189 ± 0.046*
2	10^{-6}	0.235 ± 0.031*
3	10^{-7}	0.203 ± 0.037*
4	10^{-8}	0.304 ± 0.059
5	10^{-9}	0.333 ± 0.071
6	10^{-10}	0.339 ± 0.012
7	10^{-11}	0.542 ± 0.038
8	10^{-12}	0.529 ± 0.078
9	10^{-13}	0.706 ± 0.077
10	0	1.000 ± 0.056

* $P < 0.05$, the average size of bone slices was about 5 × 5 mm.

2.3 Effect of estrogen on the mRNA level of MMP-9 and MMP-8

2.3.1 RT-PCR analyses revealed that estrogen decreased mRNA levels of MMP-9 (Table 5, Figure 2).

2.3.2 The expression of MMP-8 was not detected in all treatment groups by RT-PCR analysis.

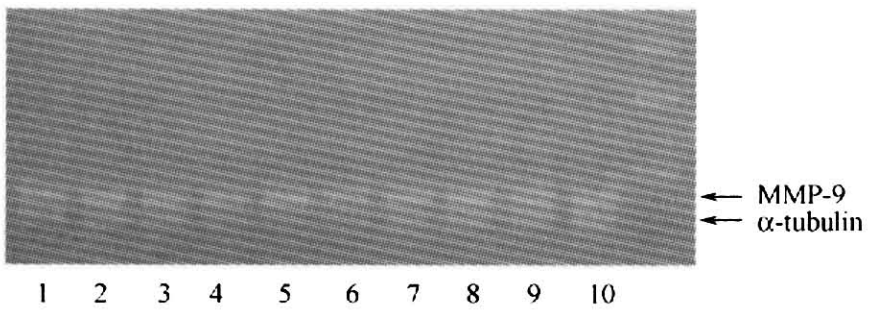

Fig 2 The effect of E_2 on the expression of MMP-9 mRNA for 24-hour

3 Discussion

Osteoclasts were identified and some morphological features were described in 1873 by Albert Kölliker in Germany. It took almost a hundred years to develop suitable *in vitro* models to study bone resorption and its regulation in detail. Development of tissue culture methods for isolated neonatal rat/mouse bones opened new possibilities to study especially the effects of various hormones and other physiological and pharmacological substances on bone resorption[2]. In this study, we isolated osteoclasts from long bone mechanically. This method was simple and stable and can be applied to most bio-functional and pharmacodynamics research. According to time-lapse observation, osteoclasts underwent several growth phases: from attached growth to quick proliferation, then to mature stage and finally apoptosis. The appearance of osteoclasts varied considerably and the size of osteoclasts was always bigger than other marrow cells. Osteoclasts, multinucleated giant cells, known to be derived from hemopoietic cells of the monocyte/macrophage lineage, resorb bone, and play an important role in calcium homeostasis. Bone resorption is known to be associated with pit formation on dentine slices *in vitro*. In tissue cultures it was not possible to identify, if compounds expressed their effects directly on osteoclasts or if they modified them via other cell types. However, bone tissue culture methods opened a new possibility to screen limited amount of compounds for their possible effects on bone resorption. This method has had a big impact on the development of new bone resorption inhibitors[3]. The maturation of osteoclasts is dependent on two factors: monocyte macrophage-colony-stimulating factor (M-CSF) and a receptor activator of nuclear factor kappa B-ligand (RANKL). Osteoclasts express high levels of osteoclast-associated genes, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and calcitonin receptor (CTR)[4].

The functionally active osteoclast is highly polarized and the cellular morphology reflects bone-resorbing capacity. Polarization is achieved by generation of the functional ruffled membrane. Ruffled border membrane is the actual resorbing organ of the osteoclast. Ultrastructurally, the normal osteoclasts exhibited multiple nuclei, a distinct ruffled membrane, and a well-defined sealing zone[5]. Tartrate resistant acid phosphatase (TRAP) is an enzyme highly expressed in osteoclasts and widely used as a phenotypic marker of osteoclasts. In our study, cells were identified as osteoclasts because they were TRAP positive and the formation of resporption launae on bone slices.

We investigated whether estrogen affects osteoclastic differentiation. Thus, we examined the formation of TRAP-positive multinucleated cell and pit formation in order to understand the effect on bone resorption by estrogen. Estrogen inhibited TRAP-positive giant multinucleate cells and oteoclasts-induced formation of resporption pits in time and dose-dependent manner. The number and area of pits were significantly opposed by estrogen especially when its concentrations were 10^{-5} $mol \cdot L^{-1}$、10^{-6} $mol \cdot L^{-1}$、10^{-7} $mol \cdot L^{-1}$ ($P < 0.05$). It has been reported that the multinuclearity of osteoclasts plays a pivotal role in resorptive activity of osteoclasts[6]. It is probable that estrogen inhibited resorptive activity in osteoclasts cells in a dose-dependent manner; therefore the resorptive areas were decreased by estrogen dependent on the decrease in resorptive activity.

Previous histomorphometric studies of bone biopsies on hormone replacement therapy in patients have indicated that decrease of activation frequency for remodeling being markedly decreased. This suggests that estrogen may inhibit osteoclast formation and this is further supported by direct experiments focusing on osteoclast differentiation from bone marrow cells[3]. It is now recognized that E_2

prevents bone loss via multiple and complex effects on bone marrow and bone cells, which results in decreased OCs formation, increased OCs apoptosis, and decreased capacity of mature OCs to resorb bone. E_2 modulates OCs apoptosis and OCs activity both directly and indirectly via regulation of growth factors and prostaglandins. Conversely, inhibition of OCs formation *in vivo* results, in part, from the ability of E_2 to block the production of the pro-osteoclastogenic cytokines IL-1, IL-6 and TNF. Another relevant effect of E_2 *in vivo* is its capacity to block the production of soluble M-CSF via an IL-1- and TNF-dependent mechanism. *In vitro* studies have also revealed that E_2 directly block the differentiation of OC precursors into mature OCs, although the relevance of these phenomenon *in vivo* remains to be determined[7].

Over the past decade, evidence has accumulated for a role of osteoblast-derived matrix metalloproteinases (MMPs) in the initial stage of osteoid removal prior to osteoclast attachment, using both murine and human *in vitro* model systems[8]. MMPs are a family of zinc-dependent endopeptidases, including collagenases, gelatinases and stromelysins, which have the combined ability to degrade the organic components of connective-tissue matrices[9]. Several MMPs have been suggested to be present in cells of the osteoclast lineage, although only MMP-9 has been shown to be highly and predominantly expressed in osteoclasts. The cysteine proteinase cathepsin K has been found to be abundantly and selectively expressed in osteoclasts, and the lack or inhibition of cathepsin K is known to reduce the resorption activity[10]. Studies show that the levels of an MMP in osteoclasts are influenced by a series of factors. An important role to consider for osteoclastic MMPs is modulation of extracellular signals since MMPs cleave cytokines, growth factors, cell surface molecules, and matrix molecules, all known to influence deeply cell activities. They include the destruction of cytokines and growth factors, the bioactivation of cytokines and growth factors, or the generation of new factors from quiescent extracellular molecules. The osteoclast MMPs may thus affect osteoclast differentiation and resorption through their effects on RANKL, TNF-α, VEGF, kit-ligand, IL-1β, or substance P; osteoclast motility through their effects on αvβ3, osteopontin, or bone sialoprotein; coupling between resorption and formation through their effects on TGF-β or IGF; coordination of endothelial cell and osteoclastic events through their effects on galectin, endostatin, angiostatin, FGF, or VEGF. As a matter of fact, knockout approaches have shown that the lack of MMP-9 impairs normal osteoclast recruitment and motility as well as angiogenesis[11].

In the present study, we showed by RT-PCR analysis that estrogen inhibited the expression of MMP-9 mRNA in osteoclasts using a concentration manner. The level of MMP-9 mRNA varied according to the osteoclastic resorption activity. These results suggested that physical concentration ($10^{-9} \sim 10^{-10}$ mol·L^{-1}) was best suited for the metabolism of osteoclasts, while the pharmacological concentration (10^{-6} mol·L^{-1}) or higher concentrations both opposed the expression of MMP-9 mRNA. As presently demonstrated, isolated osteoclasts were found to express a significant level of MMP-9. More importantly, significant expression of MMP-9 was also detected *in vivo* osteoclasts by in situ hybridization. Therefore, MMP-9 seems to be one of the major proteases constitutively produced by osteoclasts under physiological conditions, and may be involved in the degradation of bone matrix proteins during osteoclastic bone resorption. This is in line with the fact that MMP-9 is the best established MMP in osteoclasts[5]. Together these results suggested that the inhibitory effect of E_2 on the mature osteoclast is not related to the early phase of the resorption cycle, but mainly affects those later events allowing deeper penetration of cells into the mineralized matrix[10].

Although we carried out research three times, there was no expression of MMP-8 mRNA in all the TRAP positive cells isolated from our culture.

Our results support the idea that estrogen has an inhibitory effect on mature osteoclasts. The major effect of E_2 is to decrease the resorption depth via a receptor-mediated mechanism. This is, as far as we know, the first study to show the effect of E_2 on the expression of MMP-9 mRNA in rabbits' long bone primary osteoclasts *in vitro* directly, thus providing information to study the mechanism of E_2 action. It is still obscure whether collagen molecules solubilized by cathepsins can be degraded by MMP-9; however, these facts suggest the possibility that, initially, bone type I collagen is

solubilized by lysosomal cysteine proteinases and subsequently degraded by MMP-9 into small peptides by its gelatinase activity. This hypothesis may explain the cooperative action of cysteine proteinases and metalloproteinases in osteoclastic bone resorption. Until now, although the importance of metalloproteinases in osteoclastic bone resorption has been apparent, production of MMPs by osteoclasts has not been well investigated.

E_2 deficiency after ovariectomy is known to result in severe osteopenia. Penetrative resorption, observed especially in post- and perimenopausal women, leads to instability of trabecular bone architecture increasing the risk of fracture. However, early postmenopausal women respond to E_2 replacement therapy showing an inhibition in the development of pronounced increases in osteoclastic erosion depth that is otherwise seen in this population. Although the bone-saving effects of E_2 at organ and tissue levels *in vivo* are quite evident, the cellular and molecular details and possible direct effect of E_2 on osteoclasts are poorly understood. At present there is no solid evidence that any other cells other than osteoclasts are able to resorb fully mineralised bone. In the course of evolution osteoclasts have developed an effective machinery to destroy mineralised tissues. These cells can thus offer an interesting model to study not only bone resorption but also to detect molecular mechanisms that are important in normal tissue invasion. After all, malignant cells can also use these mechanisms for invasion that have already been coded in our genome during normal evolution. Thus osteoclasts may offer some clues to study also other pathological processes like tissue invasion mechanisms of malignant cells also.

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