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# Osteoprotegerin differentially regulates protease expression in osteoclast cultures

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**Abstract**

Cysteine proteases and matrix metalloproteinases (MMPs) are important factors in the degradation of organic matrix components of bone. Osteoprotegerin (OPG) is an osteoblast-secreted decoy receptor that inhibits osteoclast differentiation and activation. This study investigated the direct effects of human OPG on cathepsin K, MMP-9, MMP-2, and tissue inhibitors of metalloproteinases (TIMP1 and TIMP2) expressed by purified rabbit osteoclasts. The expression of two osteoclast markers, namely tartrate-resistant acid phosphatase (TRAP) and cathepsin K, was inhibited by 100 ng/mL hOPG, whereas MMP-9 expression was enhanced. Gelatinase activities were measured using a zymographic assay, and hOPG was shown to enhance both pro-MMP-9 and MMP-2 activities. Concomitantly, TIMP1 expression was greatly stimulated by hOPG, whereas TIMP2 mRNA levels were not modulated. Overall, these results show that hOPG regulates the proteases produced by purified osteoclasts differentially, producing a marked inhibitory effect on the expression of cathepsin K, the main enzyme involved in bone resorption. 

*Keywords:* Osteoclast; Bone resorption; Osteoprotegerin; Metalloproteinases; Tissue inhibitors of metalloproteinases; Cathepsin K

Osteoclasts are large multinucleated cells originating from bone marrow and are involved in bone demineralization and resorption. In normal bone physiology, resorption, matrix synthesis, and mineralization are associated processes that appear to be dysregulated in various metabolic bone diseases, resulting in excessive bone loss (osteoporosis, osteolytic bone tumours) or excessive bone formation (osteopetrosis, osteosclerosis).

The key substances regulating osteoclast differentiation and activation have recently been identified. Receptor activator of NF-κB ligand (RANKL), a member of the tumour necrosis factor (TNF) cytokine family, is a downstream regulator of osteoclast formation and activation, inducing many hormones and cytokines to produce osteoresorptive effects [1]. Within the bone system, RANKL is expressed on osteoblast lineage cells and exerts its biological effect by binding to the RANK receptor at the surface of osteoclasts [2]. The third protagonist, osteoprotegerin (OPG), is produced by osteoblastic/stromal cells and acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK on the osteoclast surface [3]. Thus, the biological effects of OPG on bone cells include the inhibition of terminal stages of osteoclast differentiation, suppression of the activation of mature osteoclasts, and induction of apoptosis.

Once activated, the osteoclasts secrete both protons and proteinases at their attachment site, resulting in dissolution of bone mineral and degradation of the matrix (mainly composed of type I collagen) [4]. Several studies indicate that the proteinases involved in solubilization of this collagenous matrix belong to the cysteine proteinase and matrix metalloproteinase (MMP) groups [5,6]. The identity of the specific MMPs responsible for collagen degradation in the resorption zone is unknown, although some MMPs have been immunolocalized in this resorption compartment [7]. It seems that MMP-9 at least, which is known to be the most abundant gelatinolytic MMP in osteoclasts, is not rate-limiting [8]. The zinc-dependent endopeptidase activities of the

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MMP-9 and -2 are inhibited specifically by their intrinsic inhibitors called tissue inhibitors of metalloproteinases (TIMP)-1 and -2, respectively [9].

In contrast, there is increasing evidence that the cysteine proteinase crucial for matrix solubilization in the resorption compartment is cathepsin K (Cat K) [10]. This enzyme has rate-limiting activity for bone resorption, as evidenced by the deficiency induced in patients bearing a mutation in the cathepsin K gene (pycnodysostosis) as well as in cathepsin K knockout mice [11] and tissue cultures were performed in the presence of cathepsin K anti-sense oligonucleotides [12]. The importance of cathepsin K in collagen degradation is related to its ability to cleave the native triple helix of collagen in multiple sites [13]. The triple helix rapidly unwinds and becomes susceptible to any proteinase showing gelatinolytic activity. Moreover, the gelatinolytic activity of cathepsin K is extremely high as compared to that of other cysteine proteinases [14].

These observations indicate that cathepsin K and MMPs are key proteinases in the resorption process, which led us to study the effects of OPG on the expression and activities of these enzymes in primary cultures of rabbit osteoclasts, together with TIMP-1 and -2 expression.

## Materials and methods

*Reagents.* Recombinant human osteoprotegerin/Fc chimera (rhOPG-Fc) was kindly provided by Amgen (Dr. C. Dunstan, USA). α-Minimal essential medium (α-MEM), foetal bovine serum, antibiotic mixture, phosphate-buffered saline (PBS), MMLV-RT, DNase I, and Trizol reagent were purchased from Invitrogen (Eragny, France), and Taq polymerase from Promega (Charbonnières, France). Pronase E was purchased from Sigma (St. Quentin Fallavier, France).

*Cell culture.* Neonatal rabbit bone cells were isolated using a previously reported method [15]. Briefly, 11-day-old rabbits were killed by cervical dislocation, and their long bones were isolated and freed from soft tissue, minced with scissors, and placed in vortex in 20 mL α-MEM medium for 30 s. After sedimentation for 2 min, the cell suspension was harvested (repeated twice). The cells were washed in α-MEM in a centrifuge (400 rpm for 3 min) and resuspended in α-MEM supplemented with 10% foetal bovine serum and antibiotic mixture (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were then seeded into 75 or 25 $cm^2$ flasks at a density of $2.5 \times 10^6$ cell/$cm^2$, and the cultures were maintained in a humidified 95% air, 5% $CO_2$ atmosphere at 37 °C for 3 days.

Purified osteoclasts (purity of 95%) were obtained after total rabbit bone cells (cultured three days in complete medium) were washed with serum-free α-MEM and treated with 0.001% pronase E and 0.02% EDTA in PBS for approximately 5 min. After purification, the osteoclasts were washed and cultured in serum-free α-MEM for 24 h in the absence or presence of hOPG (10, 50, and 100 ng/mL). The purity and cell morphology of isolated mature osteoclasts were characterized after May Grünwald Giemsa (MGG) staining and studied for Tartrate-Resistant Acid Phosphatase (TRAP) activity (data not shown).

*Measurement of matrix metalloproteinase activities.* Matrix metalloproteinase activities were determined by zymography as previously reported [16]. After the culture period (24 h in serum-free α-MEM in the presence or absence of OPG), MMP activities were detected in cell culture supernatants. Electrophoresis gel was composed of 10% polyacrylamide gel containing 0.2% gelatin. Proteinase activity was apparent as unstained regions. The stained polyacrylamide gels were observed with a video camera that allowed transfer to software for image processing (ImageQuant software program) and isolation of migration bands. Gelatinase activity was expressed as a percentage (shades of grey compared to the control). The same experiment was performed by adding 10 μM EDTA, a chelating agent of bivalent ions, to inhibit MMP activities. In addition, as protein levels were not modified by the different treatments, the same volume of supernatant was assayed in all experiments, allowing strict comparison of the results.

*RNA extraction.* Rabbit osteoclasts were lysed with Trizol reagent. Extraction was performed after addition of 0.1 vol chloroform followed by centrifugation. RNA was recovered from the aqueous phase by precipitation with 0.5 vol isopropyl alcohol. Total RNA was quantified by measuring the $OD_{260}$, and integrity was checked by 1% agarose/formaldehyde gel electrophoresis. RNA samples were then treated with DNase I (0.1 U/μL) before the reverse-transcription step, to exclude the possibility of interference with contaminating genomic DNA.

*Expression of MMPs, TRAP, Cat K, and TIMPs by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR).* First-strand cDNA was synthesized by incubating 5 μg of total RNA with 50 μL of RT mixture [containing 0.5 μg oligo-dT, 5× RT buffer (250 mM Tris–HCl, pH 8.3, 10 mM $MnCl_2$, 500 mM KCl, and 40 mM DTT), 5 μL of 0.1 M DTT, 2.5 μL of 10 mM dNTP mix, 400 U MMLV-RT, and 0.5 μL of 40 U/μL RNAsine] at 42 °C for 30 min, 5 min at 95 °C, and then ice-chilled for 5 min. Two microlitres of the RT reaction mixture were subjected to PCR using upstream and downstream primers (30 pmoles each, Table 1), 5 μL of 10× PCR buffer (100 mM Tris–HCl, pH 9, 500 mM KCl, 15 mM $MgCl_2$, and 1% Triton X-100), 3 μL of 25 mM $MgCl_2$, 2 μL of 10 mM dNTP, 31.7 μL of DTT (1.25 μL of DTT 0.1 M with 1.411 mL of RNAse-free water), and 0.25 μL of 5 U/μL Taq polymerase.

*Analysis of PCR products.* PCR products were analysed in 1% agarose gels, stained with ethidium bromide, and photographed. Band densities were measured using the ImageQuant computer software program. Relative expression of the different genes (TRAP, Cat K, MMP-2, MMP-9, TIMP-1, and TIMP-2) was calculated as the ratio to 18S signal. After the number of PCR cycles was increased, a plot was done for each sample, and the cycle values corresponding to the linear part of the amplification curve were determined (Table 1) and used to quantify the messages versus the 18S signal determined in the same way.

Three separate experiments (4 rabbits each) were performed for each gene studied. A representative experiment is shown in Results section.

## Results

A semi-quantitative RT-PCR method was used to compare the message levels of each gene of interest (TRAP, Cat K, MMP-2, MMP-9, TIMP1, and TIMP2) between OPG-treated osteoclasts and control cultures. Data were normalized versus the 18S mRNA level of each sample, on the assumption that the message of this conventionally accepted house-keeping gene was relatively constant on a per cell basis. It was also determined that the amplification efficiency for each message was the same in control versus OPG-treated cultures (data not shown). A serial number of cycles were performed to establish the amplification linear portion of the curve for each gene,

Table 1
Oligonucleotide primers used for RT-PCR. Primers are representated in a 5′ to 3′ orientation, with that for the coding strand (+) and the non-coding strand (−). The location of each primer within the cDNA sequence is indicated, together with the product size generated by reverse transcription and PCR amplification of the authentic mRNA

| Molecule | Primers | Strand | Location | Size (bp) | Conditions ($T_m$; cycle number) |
|---|---|---|---|---|---|
| 18S | TCAAGAACGAAAGTCGGAGGTTCG | + | 1025–1048 | 462 | 62 °C |
| | TTATTGCTCAATCTCGGGTGGCTG | − | 1487–1464 | | 26 cycles |
| TRAP | AAGGAGGACTACGTGCTCGTGGCCGGC | + | 621–647 | 189 | 61 °C |
| | TCCACTCAGCACGTAGCCCACGCCGTT | − | 808–786 | | 28 cycles |
| Cat K | AGCTGGGGAGAAAGCTGGGGAAACAAAG | + | 913–940 | 244 | 68 °C |
| | AGGCACAAACAAATGGGGAAACCAAACA | − | 1157–1130 | | 26 cycles |
| MMP-2 | AAGGCCAAGTGGTCCGTGTGAA | + | 603–624 | 370 | 49 °C |
| | AACAGTGGACATGGCGGTCTCAG | − | 973–951 | | 28 cycles |
| MMP-9 | TGGCCGGCCACTGTGCGCCCCTCCGAG | + | 1416–1442 | 662 | 49 °C |
| | CACTAGGTTCACCTCGTTCCGGGTACT | − | 2078–2052 | | 28 cycles |
| TIMP1 | GCAACTCCGACCTTGTCATC | + | 61–80 | 413 | 58 °C |
| | AGCGTAGGTCTTGGTGAAGC | − | 474–455 | | 28 cycles |
| TIMP2 | GATCCAGTATGAGATCAAGC | + | 78–97 | 126 | 61 °C |
| | ATACTCCTTCTTGCCTCCCAC | − | 204–184 | | 28 cycles |

and the levels of TRAP, TIMP1, TIMP2, MMP-2, and MMP-9 messages were quantitated for 28 cycles and Cat K for 26 cycles (Table 1), before calculation of the ratio to the 18S signal determined at 26 cycles.

*hOPG decreases TRAP and Cathepsin K mRNA expression in purified cultured rabbit osteoclasts*

Expressions of TRAP and Cat K mRNA were studied as markers of bone resorption, and comparisons were made between control and OPG-treated osteoclast cultures. Typical results are shown in Fig. 1 (one experiment out of four), which indicates that the levels of both TRAP and Cat K messages (studied respectively at 28 and 26 cycles) were down-regulated in a dose-dependent manner in the presence of OPG. In both cases, 10 ng/mL of OPG stimulated marker expression, while OPG 50 ng/mL had no effect, and 100 ng/mL decreased TRAP and Cat K message levels significantly (respectively: +43%, −14%, and −32% for TRAP and +27%, −9%, and −41% for Cat K expressions; Fig. 1B). This is the first evidence for a direct inhibitory effect of hOPG on cathepsin K mRNA expression in osteoclast cultures.

*MMP-9 expression and pro-MMP-9 activity are stimulated by hOPG in rabbit osteoclasts*

In addition to cathepsin K expression, which is highly involved in the control of bone resorption, our experiments investigated the effects of hOPG on MMP expression and activities. Concerning MMP expression, no significative signal could be detected for an MMP-2 message in purified osteoclast cultures, although MMP-9 was highly expressed at the mRNA level (Fig. 2A). These results correlate with data obtained for protease activity. Zymography experiments showed that conditioned media from purified rabbit osteoclasts contained gelatinase activities, i.e., a major band occurred at 92 kDa and a minor, barely detectable at 62 kDa. Data in the literature indicate that the main 92 kDa band corresponds to gelatinase B (known as MMP-9 or type IV collagenase in its latent form), while the 62 kDa band is associated with gelatinase A, corresponding to MMP-2 in its active form.

The results shown in Figs. 2A and B demonstrate a dose-dependent enhancing effect of hOPG on MMP-9 expression and pro-MMP-9 activity. The level of the MMP-9 signal in RT-PCR experiments was increased by 25% with the highest concentration used (Fig. 2A), whereas the corresponding protease activity evidenced by the 92 kDa band in zymography was also highly stimulated by 100 ng/mL hOPG (Fig. 2B). The zymogram also revealed the weak intensification of the 62 kDa band, corresponding to the active form of MMP-2, in the presence of 100 ng/mL of hOPG.

Our results clearly show a differential effect of hOPG on MMP-9 activities and expression, on the one hand, and on cathepsin K expression, on the other hand.

*hOPG regulates TIMP1 and TIMP2 expression differentially in rabbit purified osteoclasts*

In an attempt to compare the effect of hOPG on the expression of metalloproteinases and their respective inhibitors in osteoclast cultures, our studies were extended to TIMP1 and TIMP2 expression. The results

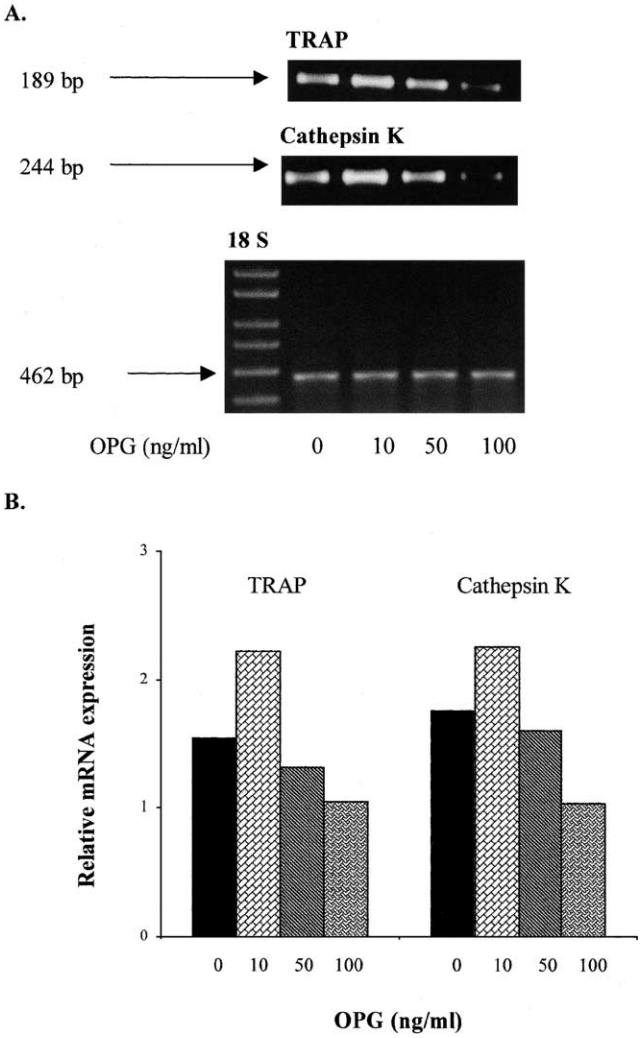


Fig. 1. hOPG dose-dependently inhibits cathepsin K and TRAP mRNA expression in rabbit purified osteoclasts. (A) A representative RT-PCR analysis of cathepsin K and TRAP message levels in the presence or not of 10, 50, and 100 ng/mL hOPG during the last 24 h of osteoclast culture. The relative mRNA levels of cathepsin K and TRAP were determined at 26 and 28 cycles, which correspond to the linear part of the respective amplification curve, and normalized to the 18S message (26 cycles). (B) The relative amounts of each signal corresponding to one representative study (out of three) were determined by scanning the bands and normalized to the 18S message level.

shown in Fig. 3 indicate that hOPG induced differential regulation of TIMP1 and TIMP2 message levels (both studied at 28 cycles). TIMP1 mRNA expression was enhanced with the highest hOPG concentration studied (100 ng/mL, +69%), whereas no significant effect was detected on a TIMP2 message level, regardless of the hOPG concentration used.

## Discussion

Proteinases act not only as solubilizers of bone matrix, but also as regulators of the initiation of bone resorption and associated bone formation. This has been demonstrated through the use of specific inhibitors that prevent bone resorption by inducing inhibition of extracellular matrix degradation in resorption lacunae. The key proteinases involved in the bone resorption process are cathepsin K and MMPs [15]. The data presented here provide the first evidence that hOPG exerts a differential effect on MMP and cathepsin K expression in purified cultured osteoclasts.

The conditioned media of purified osteoclast cultures showed a major metalloproteinase activity, which was identified in zymography experiments as a single band at 92 kDa corresponding to the latent form of MMP-9. No detectable activity was evidenced for MMP-2, although this enzyme can be clearly observed in total bone cell cultures [16]. In these circumstances, it is tempting to suppose that the MMP-2 activity is associated with the osteoblast/stromal cells. The MMP-9 activity in the supernatants of purified osteoclasts represents the latent form of the protease that may be activated in the physiological environment of cells, particularly by plasmin or stromelysin [17,18], which can be produced by bone cells [19,20].

The present study shows that hOPG induces a differential effect on proteases produced by purified osteoclasts through stimulation of MMP-9 activity and expression and inhibition of cathepsin K expression. Both enzymes play different roles relative to the mechanisms involved in bone resorption. MMP-9 and collagenase are essential for the initiation of bone resorption, whereas cathepsin K seems to be predominantly involved in matrix solubilization [15]. The decreased cathepsin K expression observed in our osteoclast cultures in the presence of hOPG may have been related to the strong inhibitory effect already described on osteoclastogenesis [21]. A previous report from Hakeda et al. [22] showed that OPG (30 ng/mL) reduced or disrupted the cytoskeletal structure of osteoclasts (which is correlated with bone resorption), but had no impact on mRNA cathepsin K levels. As the concentration of rhOPG used by Hakeda et al. was 30 ng/mL, our results are consistent with theirs. In our study, 50 ng/mL hOPG had no significant effect on mRNA cathepsin K levels, whereas 100 ng/mL increased those levels considerably. Therefore, our experiments indicate that hOPG decreases the in vitro expression of cathepsin K, the primary enzyme active in the osteoclastic bone resorption process. Although it would be quite interesting to measure the effect of hOPG on the cathepsin K activity released by osteoclasts, our culture conditions did not allow the quantification of cathepsin K activity in the presence of Z-Leu-Arg-NH-Mec as synthetic substrate.

Other than the effect of hOPG on cathepsin K expression, nothing is known about the regulation of MMP and TIMP mRNA expression by this factor. The stimulation of MMP-9 expression and activity and the

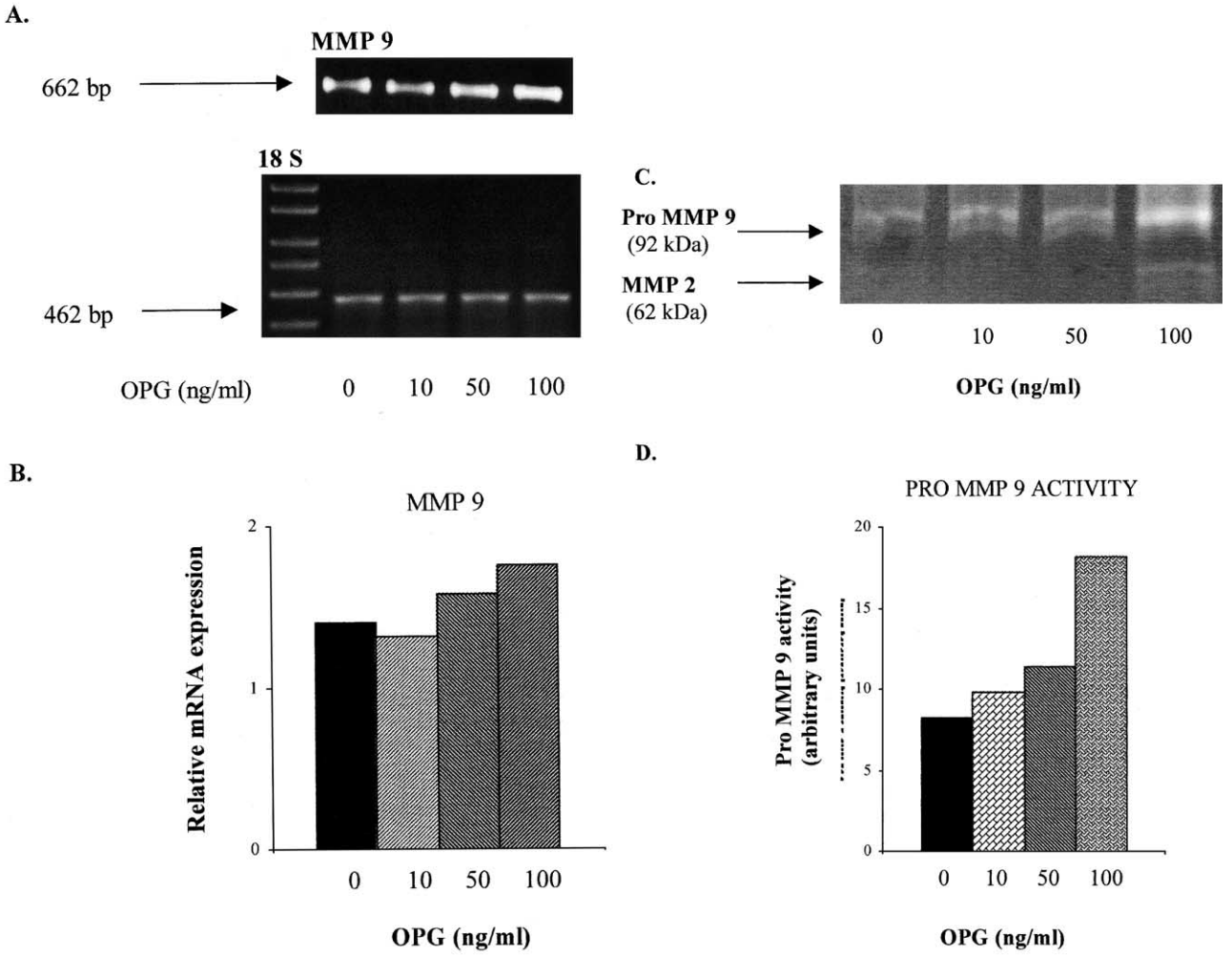


Fig. 2. hOPG Stimulates MMP-9 expression and MMP-2 and pro-MMP-9 activities in purified osteoclasts. (A) A representative RT-PCR analysis of MMP-9 message levels in the presence or not of 10, 50, and 100 ng/mL hOPG during the last 24 h of osteoclast culture. The relative mRNA levels of MMP-9 were determined at 28 cycles and normalized to the 18S message (26 cycles). The relative amounts of each signal corresponding to one representative study (out of three) were determined by scanning the bands and normalized to the 18S message level (B). (C) Metalloproteinase activities were determined using a zymographic assay. Same volumes of the culture supernatant were used for metalloproteinase activity measurements with an electrophoresis gel composed of 10% polyacrylamide gel containing 0.2% gelatin. Pro-MMP-9 and MMP-2 activities were detected, respectively, at 92 and 62 kDa. The bands were scanned and the intensity is represented in the histogram as arbitrary units (D).

induction of MMP-2 activity in the presence of 100 ng/mL hOPG are quite surprising, considering the known inhibitory effect of hOPG on bone resorption. MMPs are not only involved in different bone resorption steps, but are also required for the resorption of non-mineralized collagen. In fact, inhibition of MMP activity completely prevented the degradation of demineralized bone collagen left by osteoclasts [23]. Moreover, MMP-9 is involved in the invasive activity of osteoclasts [24]. The effects of MMP-9 deficiency on invasion occur without causing any apparent effect on osteoclast resorptive activity itself. The role of MMP-9 in invasion rather than in resorption is consistent with its early expression during differentiation of osteoclast-like cells [25]. It has been clearly established that MMPs expressed by osteoclasts themselves are responsible for the migratory and attachment activities of mature osteoclasts through the dissolution of osteoid matrix components, but are not responsible for the pit formation itself [26]. The upregulation of MMPs by hOPG observed in our study may therefore modulate the initiation of bone resorption.

However, the activity of MMPs measured in vitro does not reflect the in vivo situation in which regulation occurs through the presence of natural inhibitors. In vivo, the balance between MMP and TIMP activities is believed to determine the rate of matrix degradation. TIMP1 and TIMP2 have been detected in isolated osteoclasts [18,27], and they have been shown to inhibit osteoclastic bone resorption [28]. These observations led us to study the influence of hOPG on both MMP and TIMP mRNA expression. Our results indicate that

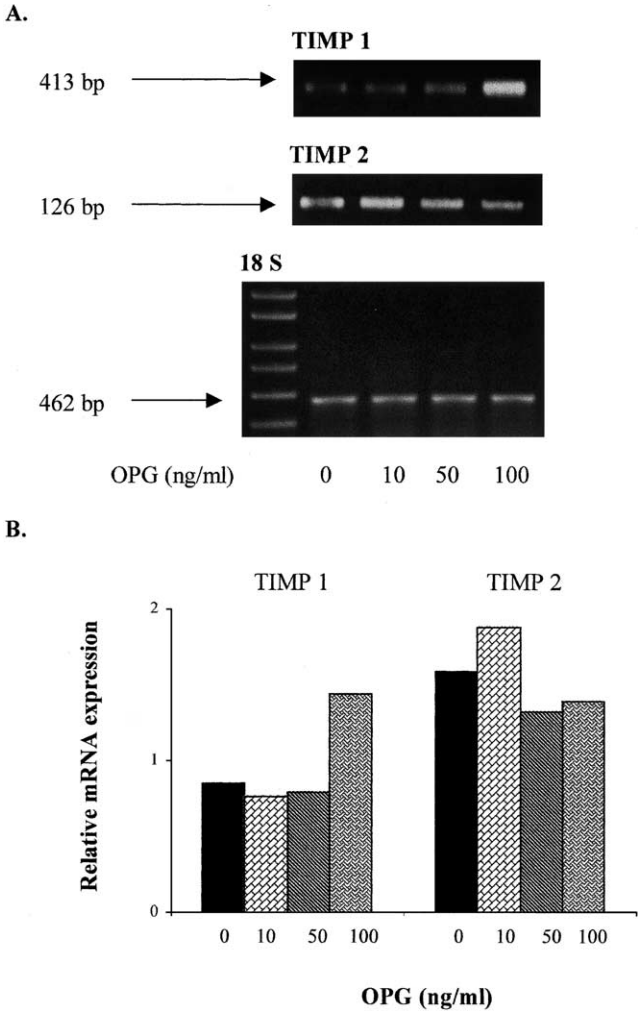


Fig. 3. hOPG increases TIMP1 expression in rabbit purified osteoclasts. (A) A representative RT-PCR analysis of TIMP1 and TIMP2 message levels in the presence or not of 10, 50, and 100 ng/mL hOPG during the last 24 h of osteoclast culture. The relative mRNA levels of TIMP1 and TIMP2 were determined at 28 cycles each, and normalized to the 18S message (26 cycles). (B) The relative amounts of each signal corresponding to one representative study (out of three) were determined by scanning the bands and normalized to the 18S message level.

hOPG stimulated TIMP1 mRNA expression highly, but had no significant effect on TIMP2 expression. Taken together, the effects of hOPG on MMP and TIMP expression suggest that the balance between MMPs and TIMPs could be equilibrated in the presence of this factor. Increased TIMP1 expression could counteract the stimulatory effect of hOPG on MMP expression and activity. As TIMP activities cannot be determined at the present time, it is difficult to determine the precise overall effect of hOPG on net proteolytic activity. The upregulation of TIMP1 expression by hOPG may also modulate pro-MMP-9 activation. TIMP-1 has been shown to form a specific complex with pro-MMP-9 [29], but the role of this complex remains unclear. By analogy with the TIMP2/MMP-2 complex, it is possible that TIMP1 plays a role in pro-MMP-9 activation [30].

Previous experiments have determined that RANKL has an enhancing effect on cathepsin K expression in osteoclasts [31], which suggests that RANKL enhances bone resorption, at least in part, by inducing cathepsin K gene expression. The present study showed that hOPG (100 ng/mL) exerts an inhibitory effect on cathepsin K expression in purified osteoclasts. A direct effect of hOPG on purified osteoclasts was also observed, which related not only to cathepsin K expression, as previously reported [22], but also to MMP and TIMP mRNA expression and MMP activity.

Earlier studies conducted in our laboratory found a stimulatory effect of RANKL on MMP-2 and -9 activities in the same cellular model (not shown). Together with the present results, this suggests that OPG and RANKL have a differential effect on cathepsin K expression directly reliable to bone resorption, whereas MMP activities seem to be stimulated similarly by both factors. Cathepsin K is clearly involved in bone resorption, whereas MMPs are likely to play different roles in bone cell metabolism. The regulation of these proteases by OPG and RANKL appears to be complex, and further experiments are needed to improve our understanding of the molecular mechanisms involved in osteoclast functions.

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