

Differential effects of interleukin-6 receptor activation on intracellular signaling and bone resorption by isolated rat osteoclasts

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Abstract

The effects of the related cytokines interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and oncostatin-M on bone resorption and cytosolic Ca^{2+} signaling were compared in isolated rat osteoclasts. In the traditional disaggregated osteoclast (pit) assay, IL-6 and LIF, but not oncostatin-M, conserved the bone resorption otherwise inhibited by high extracellular $[\text{Ca}^{2+}]$ (15 mM). It produced a paradoxical, concentration-dependent stimulation of resorption by elevated extracellular Ca^{2+} . In the micro-isolated single osteoclast resorption assay, IL-6, high $[\text{Ca}^{2+}]$ or IL-6 plus high $[\text{Ca}^{2+}]$ all increased pit formation. In contrast, the IL-6 receptor (IL-6R)-specific agonist antibody MT-18 inhibited bone resorption in a concentration-dependent manner (1:500 to 1:500 000). MT-18 triggered cytosolic Ca^{2+} signals in fura 2-loaded osteoclasts within ~10 min of application. Each cytosolic Ca^{2+} transient began with a peak deflection that persisted in Ca^{2+} -free, EGTA-containing extracellular medium, consistent with a

release of intracellularly stored Ca^{2+} . This was followed by a sustained elevation of cytosolic $[\text{Ca}^{2+}]$ that was abolished in Ca^{2+} -free medium, as expected from an entry of extracellular Ca^{2+} , and by the Ca^{2+} channel antagonist Ni^{2+} . The inclusion of either IL-6 or soluble human (sh) IL-6R specifically reversed both the above effects of MT-18, confirming that both effects were specific for the IL-6R. The findings suggest that IL-6R activation by IL-6 stimulates osteoclastic bone resorption either by reversing the inhibitory effect of high extracellular Ca^{2+} in stromal-containing systems or itself stimulating bone resorption along with Ca^{2+} by micro-isolated osteoclasts. In contrast, activation of the IL-6R by an agonist antibody produces an inhibition of bone resorption and an associated triggering of the cytosolic Ca^{2+} signals previously associated with regulation of bone resorptive function in other situations.

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Introduction

Activation of receptors for the interleukin-6 (IL-6)-like cytokine superfamily by their respective ligands, IL-6, IL-11, leukemia inhibitory factor (LIF) or oncostatin-M, induces dimerization of the common signaling glycoprotein gp130 which in turn triggers a cascade of phosphorylation signals (Ng & Martin 1998, Taga *et al.* 1989, Kishimoto *et al.* 1992). The phosphorylation substrate phospholipase $\text{C}\gamma$ then catalyzes inositol trisphosphate (IP_3) formation, which in turn releases intracellularly stored Ca^{2+} (Hughes *et al.* 1991, Rogers & Hammerman 1993, Yang *et al.* 1993, Berridge 1993).

There is strong evidence implicating IL-6, IL-11 and LIF in normal and disordered bone remodeling. Marrow

osteoblasts and stromal cells secrete cytokines following hormonal stimulation (Greenfield *et al.* 1996) that in turn enhance osteoclast formation and/or osteoblast differentiation (Bellido *et al.* 1996). Such actions suggest a role for IL-6 in the pathogenesis of postmenopausal osteopenia (Jilka *et al.* 1992, Tamura *et al.* 1993, Manolagas & Jilka 1995). Ovariectomy in mice leads to increased osteoclast formation and IL-6 expression. Furthermore, estrogen appears to regulate the IL-6 gene through the NF κ B site in the IL-6 gene promoter (Kurebayashi *et al.* 1997). Similarly, LIF enhances osteoclastogenesis *in vivo* although it is unclear whether the LIF gene is estrogen sensitive (Ware *et al.* 1995, Ng & Martin 1998). LIF nevertheless promotes an osteoblastic maturation that may underlie its *in vivo* osteogenic potential (Bellido *et al.* 1997).

However, the effects of these cytokines on the function of mature osteoclasts are less well characterized than their actions on osteoclast formation and apoptosis. IL-6 stimulates osteoclastic bone resorption by mature rat and human osteoclasts (Ohsaki *et al.* 1992, Ishimi *et al.* 1990, Adebajo *et al.* 1998). It reduces the sensitivity of mature osteoclasts to elevations in extracellular $[Ca^{2+}]$ (Adebajo *et al.* 1998). Conversely, extracellular Ca^{2+} elevation stimulates IL-6 synthesis and secretion. Taken together these findings are compatible with a positive feedback loop that involves IL-6 in the conservation of osteoclast activity (Adebajo *et al.* 1998). Finally, IL-6 inhibits osteoclast apoptosis, but again through a mechanism that is still unclear (Hughes *et al.* 1995).

The present experiments accordingly addressed the effects of IL-6 and its related cytokines on the function of mature osteoclasts in greater detail. First, they examined whether the related cytokines, LIF and oncostatin-M, resemble IL-6 in reversing the effect of a high extracellular Ca^{2+} . Secondly, they explored whether IL-6 acts directly on mature osteoclasts. This was unclear from our previous study that used the traditional disaggregated osteoclast resorption assay (Adebajo *et al.* 1998). This assay is invariably contaminated with bone marrow stromal cells and osteoblasts that possess both IL-6 and Ca^{2+} sensing receptors (Bellido *et al.* 1996, Pi *et al.* 2000). These cells might conceivably release soluble pro-resorptive signals in response to IL-6 or Ca^{2+} and indirectly modify osteoclast function. To provide more compelling evidence that IL-6 and Ca^{2+} interact directly with the osteoclast, we accordingly determined their effect, singly and in combination, on bone resorption by a single osteoclast using a micro-isolation technique (Wang *et al.* 1997). This technique allowed us to quantify resorption by a single osteoclast on a single dentine slice in the absence of any contaminating cells. Finally, the studies examined the effects of IL-6R activation by a specific agonist anti-IL-6R antibody, MT-18, on bone resorption. Use of MT-18 made it possible to relate the latter function to processes of cytosolic Ca^{2+} release and transmembrane Ca^{2+} influx in isolated osteoclasts.

A stimulatory effect of IL-6R activation by IL-6 upon bone resorption is demonstrated by the present experiments. This applied both in the presence of stromal cells in which IL-6 reversed the inhibitory effects of high extracellular $[Ca^{2+}]$, and micro-isolated osteoclasts in which both IL-6 and Ca^{2+} enhanced bone resorption. In contrast, activation of the same IL-6 receptor by an agonist antibody resulted in a paradoxical inhibition of bone resorption, and related this to intracellular Ca^{2+} release and transmembrane Ca^{2+} influx processes that have been implicated in osteoclast regulation in response to either local $[Ca^{2+}]$ or humoral agents on other occasions. The latter findings additionally represent the first demonstration of intracellular Ca^{2+} signaling following IL-6R activation in any cell.

Materials and Methods

Specificity of the anti-IL-6R antibody MT-18

The highly specific anti-IL-6R monoclonal antibody MT-18 (kindly provided by Dr Taga, Osaka, Japan) was raised to the human (h) IL-6R that had been transfected into the IL-6R-negative murine T cell line CTLL-2 (Hirata *et al.* 1989). MT-18 binds to hIL-6R in immunoprecipitation, immunoblot and immunocytochemical experiments, cell growth studies and ^{125}I -IL-6 binding assays (Hirata *et al.* 1989, Taga *et al.* 1989, Ohsaki *et al.* 1992, May *et al.* 1993, Narazaki *et al.* 1993). In addition, a binding of MT-18 specifically to rat osteoclast IL-6Rs is displaced by soluble hIL-6R (shIL-6R; Adebajo *et al.* 1998). Finally, MT-18 also detects known lower molecular weight (46 and 32 kDa) precursors in immunoblot studies with rat bone cell membranes. It is noteworthy that hIL-6R (GenBank, M20566) is ~55% homologous to rat or murine (m) IL-6R (X51975). The rat and murine receptors are ~95% homologous (Genebank CDS, PDB and Swissprot Databanks, Blast Search Software).

Bone resorption by disaggregated rat osteoclasts

Bone resorptive activity was measured using the pit assay first developed by Boyde *et al.* (1984) and Chambers *et al.* (1984), and subsequently modified by Dempster *et al.* (1987). Osteoclasts were obtained by curetting long bones obtained from neonatal Wistar rats killed by decapitation into 1 ml Hepes-buffered Medium 199 containing Hanks' salts (M199-H) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with heat-inactivated fetal bovine serum (FBS, 5% v/v, Sigma Chemical Co., St Louis, MO, USA). The resulting suspension (100 μ l) was agitated gently and dispersed onto devitalized cortical bone slices (4 mm \times 4 mm). Osteoclasts readily sedimented upon and attached to the respective substrate within 30 min (37 °C). Contaminating non-adherent cells were removed by rinsing with M199-H. The slices were then transferred to a multiwell dish containing Medium 199 with Earle's Salts (M199-E) and 10% v/v FBS. The following sets of experiments were performed. Set A: using 1.25 (control), 5, 10 or 15 mM Ca^{2+} with or without IL-6 (10 μ g/l). Set B: using 1.25 (control) or 15 mM Ca^{2+} with or without LIF (10 μ g/l). Set C: using 1.25 (control) or 15 mM Ca^{2+} with or without oncostatin-M (50 μ g/l). Set D: using MT-18 at dilutions of 1:500, 1:5000, 1:50 000 and 1:500 000. Set E: using MT-18 (1:5000) in the presence or absence of shIL-6R (500 μ g/l) or IL-6 (10 μ g/l) (to test for response specificity). All assays were conducted at pH 6.9 which ensured maximal osteoclast resorptive activity (Arnett & Dempster 1986). Human IL-6, LIF and oncostatin-M were full-length recombinant molecules (R&D Systems, Minneapolis, MN, USA).

After further incubation for 24 h in humidified CO_2 (5%), the slices were fixed with glutaraldehyde (10%, v/v)

and stained for the presence of tartrate-resistant acid phosphatase (TRAP) using Kit 386A (Sigma Chemical Co.). The number of osteoclasts with two or more nuclei was determined on each slice using a light microscope (Olympus, Tokyo, Japan). The cells were removed by treating the slices with NaOCl (5 min), the slices rinsed with distilled water followed by acetone, and then dried. They were stained subsequently with Toluidine Blue (1%, v/v, in 1% w/v borate) for 5 min. The number of resorption pits was determined on each slice by light microscopy.

Each experiment was performed with osteoclasts obtained from nine animals with nine replicate bone slices per treatment per experiment. Furthermore, every experiment was repeated between three and five times. The number of pits per bone slice was expressed as mean \pm S.E.M. and the dependence of number of pits per slice upon extracellular $[Ca^{2+}]$ was determined as positive or negative by simple linear regression and determination of regression coefficients. In other experiments, the number of pits per bone slice was expressed as mean \pm S.E.M. and repeated measures analysis of variance (ANOVA) was used to analyze the effect of treatment, which was considered significant at $P < 0.05$. Bonferroni's correction for inequality was used to maintain an overall error rate of 5%.

Bone resorption by a micro-isolated single osteoclast

The elements of this assay have been reported elsewhere (Wang *et al.* 1997). Osteoclasts were isolated from 48 h-old rat pups using the above technique and dispersed onto a 60 mm dish (Fisher, St Louis, MO, USA). Adherent cell cultures were then rinsed with phosphate-buffered saline (PBS) and incubated with collection buffer (130 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5 mM EDTA and 20 mM Hepes, pH 7.4). The buffer was decanted 5 min later and replaced with fresh collection buffer. Around 20 osteoclasts were collected each time using a micropipette held in a micromanipulator (Tong *et al.* 1994). The collected osteoclasts were then transferred onto the side of the dish where they were examined for quality and purity. Only ten of the best cells were then individually seeded on to pre-wetted sperm whale dentine slices (prepared specimens kindly provided by the National Marine Fisheries Service, Enforcements Division, Long Beach, CA, USA). Five such slices, each having a single osteoclast, were placed in a 35 mm culture dish (Fisher) with 2 ml α -minimal essential medium (MEM) supplemented with 10% FBS, penicillin G (100 kU/l) and streptomycin sulfate (100 mg/l) (pH 7.3). Ten slices (2 dishes) were assigned randomly to each of the following treatment groups: control ($[Ca^{2+}]$ 1.25 mM), high $[Ca^{2+}]$ (15 mM), IL-6 (10 μ g/l), and high $[Ca^{2+}]$ plus IL-6. Cultures were incubated for 24 h at 37 °C, 100% humidity, and 5% CO₂. This experiment was replicated three times ($n = 120$ slices).

After the 24 h incubation period, the cultures were rinsed with PBS, fixed for 15 min in formalin (10% v/v) with sucrose (2% w/v), rinsed again with PBS, and processed for TRAP histochemistry (Tong *et al.* 1994) to determine whether the seeded osteoclasts remained on the chip. The resorption pits were revealed using a staining method modified from that reported by Selander *et al.* (1994). Briefly, the chips were incubated in peroxidase-conjugated wheat germ agglutinin (WGA; 25 μ g/ml, w/v, in PBS) for 45 min, washed with PBS, and then developed in the dark for 10 min with 3-3'-diaminobenzidine (DAB; 10 g/l, w/v, in 0.3% v/v, H₂O₂). The stained slices were then washed with distilled water and the pit number was determined. Data from treatment groups were tested for homoscedasticity and significance was determined using repeated measures ANOVA where appropriate. Post hoc *t*-tests were conducted using the Bonferroni Correction for Inequality to adjust the calculated level of significance.

Microspectrofluorimetric measurements of cytosolic Ca²⁺

For measuring cytosolic Ca²⁺ in single cells, isolated osteoclasts were dispersed onto 22 mm, 0-grade, glass coverslips (Libro/ICN, Aurora, OH, USA). The coverslips were incubated for 30 min at 37 °C with 10 μ M fura 2/AM (Molecular Probes, San Diego, CA, USA) in serum-free M199-H. They were then washed in M199-H and transferred to a Perspex bath positioned on the stage of a microspectrofluorimeter. The latter was previously constructed from an inverted microscope (Diaphot, Nikon, Telford, UK). The cells were then exposed to pre-warmed test solutions of MT-18 (1:20 to 1:50 000) in M199-H ($[Ca^{2+}]$, 1.25 mM). In different experiments, osteoclasts were treated similarly, but were incubated in modified Ca²⁺-free, Krebs's solution (130 mM NaCl, 5 mM KCl, 10 mM Hepes, 10 mM glucose and 1.2 mM EGTA). Finally, osteoclasts were preincubated, for 3–4 min, with either shIL-6R (500 μ g/l) or IL-6 (10 μ g/l) before MT-18 application.

Osteoclasts were exposed alternately to excitation wavelengths of either 340 or 380 nm approximately every 1 s. For recording the emitted fluorescence, a single osteoclast was selected for each experiment by narrowing an optical diaphragm to approximate the cell's boundary. The cell was then removed and a background count was obtained for 10 s from the same field. The average background fluorescence (counts per second) was then subtracted automatically from subsequent counts obtained from the selected cell. This emitted fluorescence was collected by first deflecting the latter through a 400 nm dichroic mirror and subsequently filtering the transmitted light at 510 nm. The resulting beam was then directed to a photomultiplier tube (PM28B; Thorn EMI, London, UK), which converted the signal to 25 ns, 5 V transistor-transistor logic (TTL) pulses. These were counted by a

dual photon counter (Newcastle Photometrics, Newcastle, UK). Photon counts were recorded every second on a microcomputer to give the ratio of emitted intensities due to excitation at 340 and 380 nm, F_{340}/F_{380} .

The cytosolic Ca^{2+} measuring system was calibrated using an established protocol for intracellular calibration (Shankar *et al.* 1992). Briefly, fura 2-loaded osteoclasts were bathed in Ca^{2+} -free, EGTA-containing solution containing 130 mM NaCl, 5 mM KCl, 5 mM glucose, 0.8 mM $MgCl_2$, 10 mM-Hepes and 0.1 mM EGTA. 5 μ M ionomycin were first applied to obtain the minimum ratio due to lowest cytosolic Ca^{2+} (R_{min}) and the maximum fluorescence intensity at 380 nm (F_{max}). $CaCl_2$ (1 mM) was then applied with 5 μ M ionomycin to obtain values of the maximum ratio due to an elevated cytosolic Ca^{2+} (R_{max}) and the minimum fluorescence intensity at 380 nm (F_{min}). The dissociation constant K_d for Ca^{2+} and fura 2 at 20 °C, an ionic strength of 0.1 M, and pH 6.85, is 224 nM. The values were substituted into the equation: $[Ca^{2+}] = K_d \times [(R - R_{min}) / (R_{max} - R)] \times [(F_{max} / F_{min})]$.

Results

IL-6 converts the Ca^{2+} -induced inhibition of bone resorption to a stimulatory effect in the disaggregated osteoclast assay

We have previously shown that the inhibitory effects of 15 mM extracellular Ca^{2+} were abolished in the presence of IL-6 (Adebanjo *et al.* 1998). Here we examined this action in detail by using a range of extracellular Ca^{2+} concentrations in the traditional disaggregated rat osteoclast assay. In the absence of exogenous IL-6, elevations of extracellular $[Ca^{2+}]$ reduced the numbers of pits per slice in a concentration-dependent manner (linear regression analysis; $r = -0.776$, $n = 10$ slices per concentration; Fig. 1A). In contrast, the addition of IL-6 converted the latter negative sloping regression to a positive slope ($r = +0.7$) (Fig. 1B). Thus, elevated levels of extracellular Ca^{2+} in the presence of applied IL-6 now stimulated rather than inhibited bone resorption. The number of TRAP-positive multinucleated osteoclasts was also determined for each bone slice. This did not change with any of the applied treatments, thereby excluding possible cytotoxic effects.

LIF, but not oncostatin-M, mimics IL-6 effects in the disaggregated osteoclast resorption assay

We next examined whether the related cytokines LIF and oncostatin-M, whose actions also involve the common signal transducer gp130 (Kishimoto *et al.* 1992), mimicked IL-6 in its reversal of Ca^{2+} -induced resorption inhibition. Neither LIF (10 μ g/l) (Fig. 2) nor oncostatin-M (50 μ g/l) (Fig. 3), when applied by themselves, elevated pit number per slice (compared with control, $P = 0.789$ and $P = 0.679$ respectively, $n = 10$). In contrast, elevated levels (15 mM)

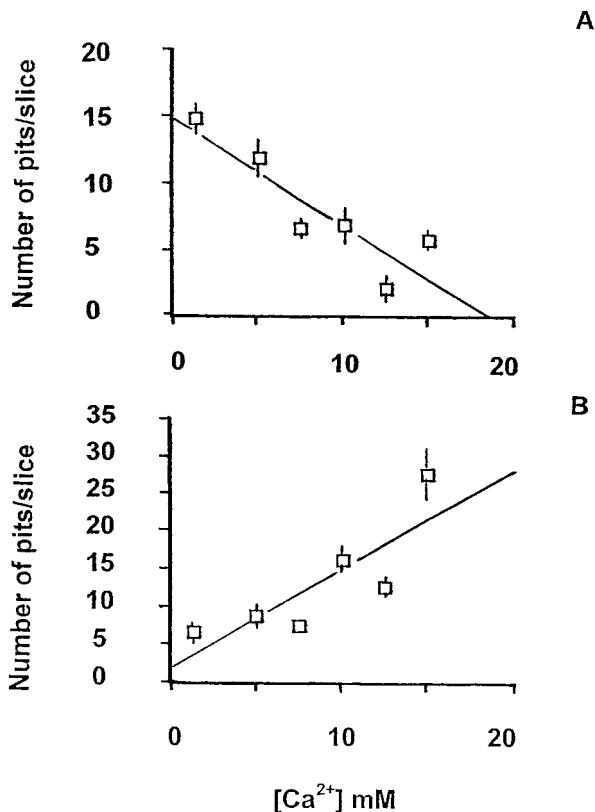


Figure 1 Bone resorption in the disaggregated rat osteoclast assay expressed as the pit number per bone slice (\pm S.E.M.) with different concentrations of $CaCl_2$ ($[Ca^{2+}]$ mM) (as shown) in the absence (A) or presence (B) of 10 μ g/l interleukin-6 (IL-6). Linear regression analysis was used to calculate slopes of the concentration-response curves in the absence or presence of IL-6 ($n = 10$ slices per concentration).

of extracellular Ca^{2+} expectedly inhibited resorption ($P < 0.05$) in the face of unaltered cell numbers (see Zaidi *et al.* 1991). However, LIF reversed the inhibitory effect of extracellular Ca^{2+} . Thus, pit number per slice in the treatment group, LIF plus elevated $[Ca^{2+}]$, did not differ significantly ($P = 0.735$) from that in control slices (Fig. 2). The number of osteoclasts per slice was not different between any of the treated groups and control excluding effects on osteoclast formation or demise. Taken together, these findings suggest that, like IL-6, LIF completely reverses the Ca^{2+} -induced inhibition of bone resorptive activity.

In contrast, Fig. 3 shows that treatment with oncostatin-M in combination with elevated $[Ca^{2+}]$ significantly reduced pit number per slice compared with control ($P = 0.006$). Oncostatin-M also caused a marginally significant decrease in the osteoclast number per slice (compared with control, $P = 0.089$); this became highly significant upon incubation in the presence of both oncostatin-M and 15 mM Ca^{2+} (compared with control, $P = 0.004$). Taken

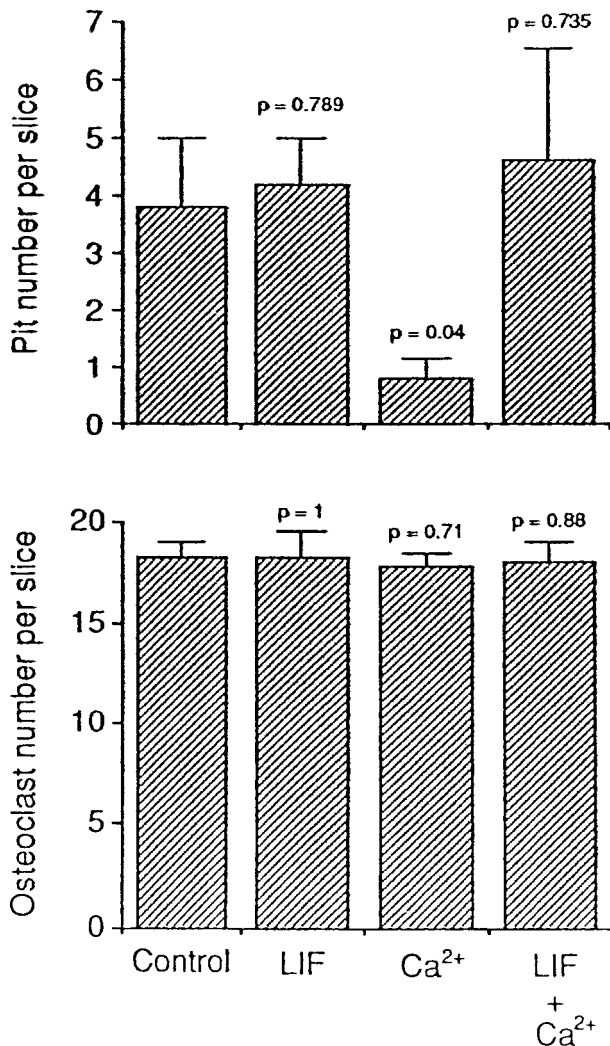


Figure 2 Pit number (upper panel) and osteoclast number (lower panel) per slice (mean \pm S.E.M.), in the presence of 15 mM CaCl₂ (Ca²⁺) and/or 10 μ g/l leukemia inhibitory factor (LIF). Each treatment was compared with control using analysis of variance with Bonferroni's correction for inequality (*P* values as shown; *n*=10 slices per variable).

together, these results indicate that oncostatin-M may reduce bone resorption by decreasing the number of viable osteoclasts either by enhancing osteoclast apoptosis or by reducing osteoclastogenesis.

IL-6 and high extracellular [Ca²⁺] together enhance bone resorption by a single osteoclast

We next assessed the effect of IL-6 and high extracellular [Ca²⁺] on the bone resorptive activity of a single micro-isolated osteoclast. Each osteoclast was picked using a micromanipulator and seeded onto a dentine slice. In each experiment, ten slices were assigned randomly to each of

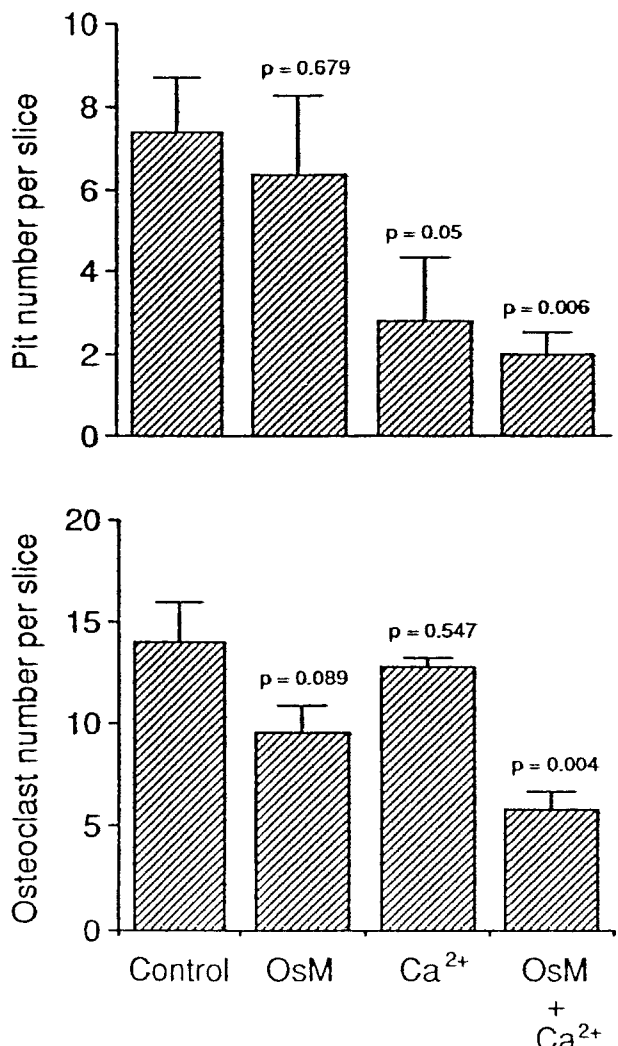


Figure 3 Pit number (upper panel) and osteoclast number (lower panel) per slice (mean \pm S.E.M.), in the presence of 15 mM CaCl₂ (Ca²⁺) and/or 50 μ g/l oncostatin-M (OsM). Each treatment was compared with control using analysis of variance with Bonferroni's correction for inequality (*P* values as shown; *n*=10 slices per variable).

four treatment groups and the experiment replicated three times. Resorptive activity was determined as the percentage of slices having pits, as illustrated in Fig. 4. Increases in extracellular [Ca²⁺] (15 mM) or the presence of IL-6 (10 μ g/l) either by themselves or in combination significantly increased the resorptive activity of a single osteoclast (compared with control, *P*<0.05) (Fig. 5).

The anti-IL-6R antibody MT-18 inhibits bone resorption

We have shown that the monoclonal anti-IL-6R antibody, MT-18, binds specifically to the surface of intact osteoclasts (Adebanjo *et al.* 1998). Here we show that MT-18

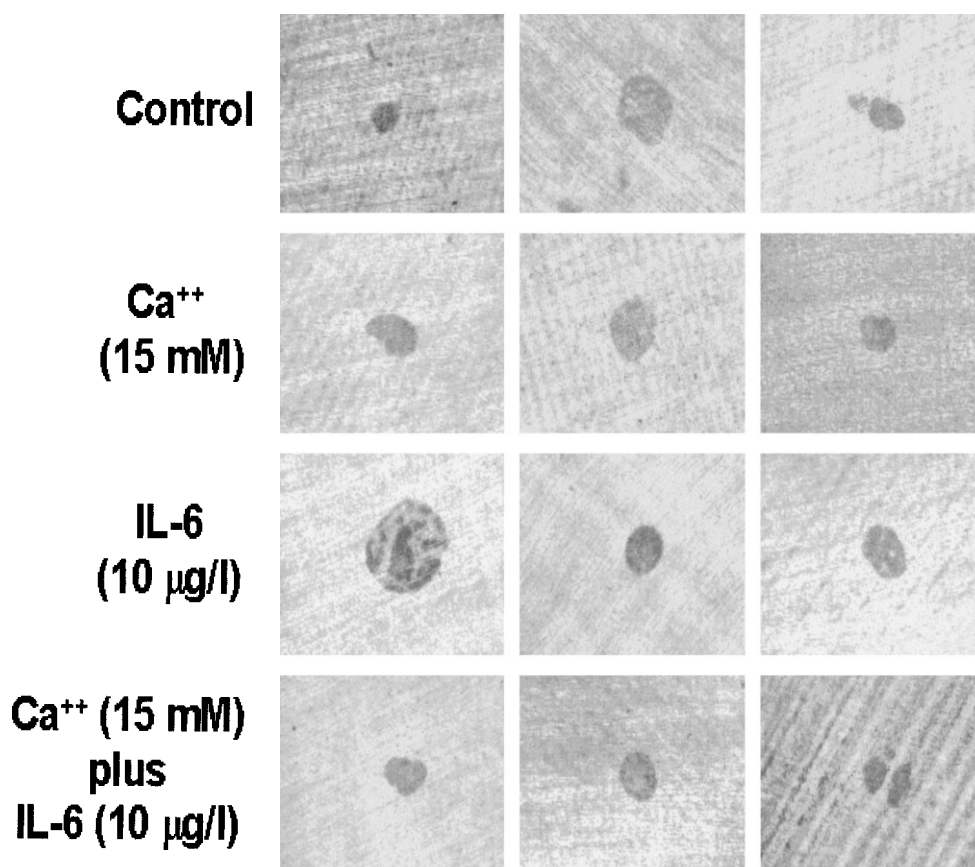


Figure 4 Pits formed on dentine by single isolated osteoclasts; each image frame covers $80 \times 62.5 \mu\text{m}$. Each column has images taken from three dentine chips from each of the treatment groups. The WGA-positive area represents a pit or pits formed by a single osteoclast.

inhibits osteoclastic bone resorption through IL-6R activation and that this effect is associated with, and probably mediated by, an elevated cytosolic $[\text{Ca}^{2+}]$. Figure 6 shows that MT-18 inhibits bone resorption in the disaggregated osteoclast assay in a concentration-dependent manner. Thus, pit numbers in the presence of various MT-18 dilutions were significantly different from control ($P=0.001$ and $P=0.061$ for 1:500 and 1:5000, respectively; $n=10$ slices).

This action resulted specifically from IL-6R activation. Either IL-6 (10 $\mu\text{g/l}$) or shIL-6R (500 $\mu\text{g/l}$) were applied together with the MT-18 (1:5000). Table 1 confirms that MT-18 by itself significantly inhibited bone resorption ($P=0.022$ versus control; $n=10$ slices). However, co-incubation of the cells with MT-18 and IL-6 at least partially reversed this anti-resorptive effect of MT-18 and reduced bone resorption to near-control levels ($P=0.769$, $n=10$ slices).

We next used shIL-6R which would compete with the binding of MT-18 with the osteoclastic IL-6R. Soluble hIL-6R partially reversed the inhibitory effects of MT-18

on bone resorption. Thus, there was no significant difference ($P=0.185$, $n=10$ slices) between resorption in controls versus MT-18 plus shIL-6R. This suggests once again that the MT-18 was interacting with IL-6R in inhibiting resorption.

Table 1 The effect of anti-interleukin-6 receptor (IL-6R) antibody (MT-18) on bone resorption (mean \pm S.E.M.) by disaggregated rat osteoclasts in the presence or absence of IL-6 (10 $\mu\text{g/l}$) or shIL-6R (500 $\mu\text{g/l}$). Each treatment is compared with control using analysis of variance with Bonferroni's correction for inequality (P values are as shown; $n=10$ slices per variable)

Treatments	Osteoclasts/slice	Pits/slice	P
Control	24.6 ± 0.51	19.8 ± 2.84	
MT-18	21.8 ± 1.36	$11.5 \pm 1.69^*$	$P=0.022$
IL-6	21.6 ± 1.17	15.4 ± 1.63	$P=0.209$
IL6+MT-18	23.6 ± 0.51	18.1 ± 4.93	$P=0.769$
shIL-6R	22.4 ± 0.75	24.6 ± 1.50	$P=0.278$
shIL-6R+MT-18	20.8 ± 1.16	14.0 ± 0.89	$P=0.185$

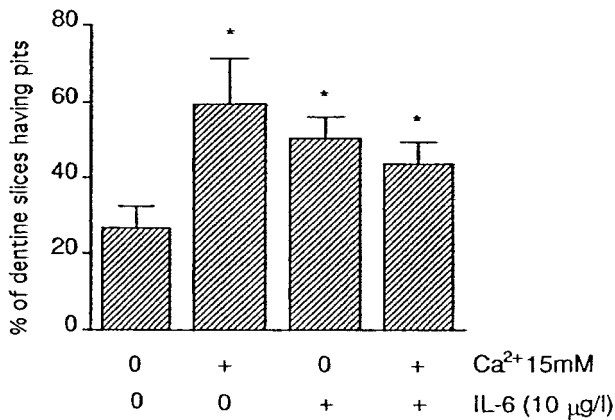


Figure 5 The effect of CaCl_2 (Ca^{2+} ; 15 mM) and interleukin-6 (IL-6; 10 $\mu\text{g/l}$) on resorption by single micro-isolated osteoclasts recorded as a percentage of dentine slices having pits. Three individual experiments were pooled, each having 40 slices (or osteoclasts) per treatment (total, $n=120$ slices). For statistics see Materials and Methods (* $P<0.05$).

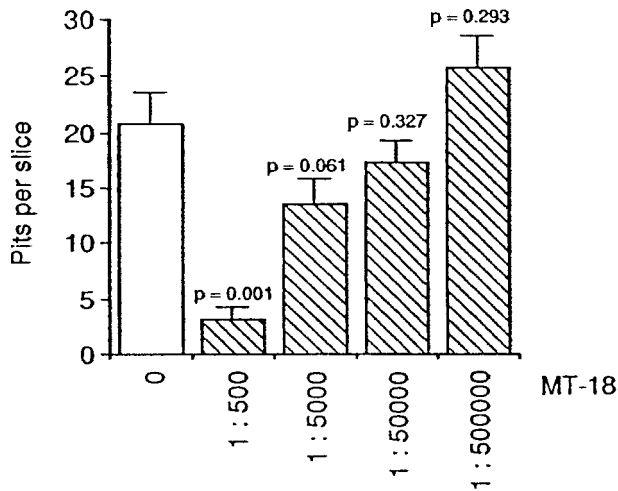


Figure 6 Pit number per slice (mean \pm S.E.M.), in the presence of different titres of the anti-interleukin-6 receptor antibody, MT-18 (1:500 to 1:500 000). Statistics by analysis of variance with Bonferroni's correction for inequality (P values as shown; $n=10$ slices per variable).

MT-18 triggers cytosolic $[\text{Ca}^{2+}]$ transients in isolated osteoclasts

Cytosolic $[\text{Ca}^{2+}]$ was measured in fura 2-loaded rat osteoclasts that were exposed to various dilutions of MT-18 (between 1:20 and 1:50 000). Figure 7 demonstrates that MT-18 triggered a ~ 10 -fold rise in cytosolic Ca^{2+} following a latency of between 400 and 1000 s. The cytosolic Ca^{2+} responses consisted of sharp transients to peak values (generally $>1 \mu\text{M}$; hence not quantifiable because fura 2 saturates above $1 \mu\text{M}$ Ca^{2+}) followed by slow declines either to baseline or first to a plateau level. These responses resembled hormone-induced Ca^{2+} release

via IP_3 in their prolonged latencies and time courses (see Discussion).

In common with the effects of MT-18 on bone resorption described above, MT-18-induced cytosolic Ca^{2+} signals were also mediated by IL-6R activation. Once again, either shIL-6R (500 $\mu\text{g/l}$) (Fig. 8b) or IL-6 (10 $\mu\text{g/l}$) (Fig. 8c) applied prior to MT-18 application (1:5000), abolished the Ca^{2+} signals induced by MT-18 (Fig. 8a). These findings associate the effects of MT-18 upon bone resorption with its effects upon intracellular $[\text{Ca}^{2+}]$.

MT-18 triggers both intracellular Ca^{2+} release and extracellular Ca^{2+} influx

We next explored whether the MT-18-induced cytosolic Ca^{2+} signals resulted from extracellular Ca^{2+} influx or from intracellular Ca^{2+} release. Figure 9a demonstrates a typical cytosolic Ca^{2+} transient triggered by MT-18 in 1.25 mM-extracellular $[\text{Ca}^{2+}]$. The response consisted of an initial transient that was followed by a sustained elevation of cytosolic $[\text{Ca}^{2+}]$. In contrast, Fig. 9b shows a typical response from a cell incubated in Ca^{2+} -free, EGTA-containing Krebs solution. The early transient peak was now followed instead by an early return to baseline. This suggested that the rapid cytosolic Ca^{2+} transient did not depend on extracellular Ca^{2+} and therefore most probably resulted from intracellularly released Ca^{2+} . In contrast, the secondary sustained elevation that was abolished in Ca^{2+} -free medium clearly depended upon extracellular Ca^{2+} .

Further experiments investigated the effects of the Ca^{2+} channel blocker Ni^{2+} . Its application during the plateau phase of the MT-18-induced Ca^{2+} signal elicited a sharp fall in cytosolic Ca^{2+} , suggesting a blockade of an extracellular Ca^{2+} influx (Figs 9c and 9d). Note that Ni^{2+} itself triggers a release of intracellularly stored Ca^{2+} (Fig. 9e; Shankar *et al.* 1993, Zaidi *et al.* 1991, 1992, 1993).

Discussion

We have shown recently that IL-6 conserves the osteoclastic bone resorptive activity that would otherwise become inhibited by a rising extracellular $[\text{Ca}^{2+}]$ (Shankar *et al.* 1993, Zaidi *et al.* 1991, 1992, 1993). This may reflect a reduced sensitivity of the resorbing cell to changes in ambient $[\text{Ca}^{2+}]$ (Zaidi *et al.* 1991, Adebajo *et al.* 1998, Zaidi *et al.* 1999). Ca^{2+} released during resorption conversely enhances IL-6 expression and secretion as well as IL-6R expression (Adebajo *et al.* 1998, Franchimont *et al.* 1997). Taken together, these phenomena are compatible with a feedback mechanism that would maintain resorption even in the face of a rising (inhibitory) extracellular Ca^{2+} concentration due to local hydroxyapatite dissolution (Adebajo *et al.* 1998).

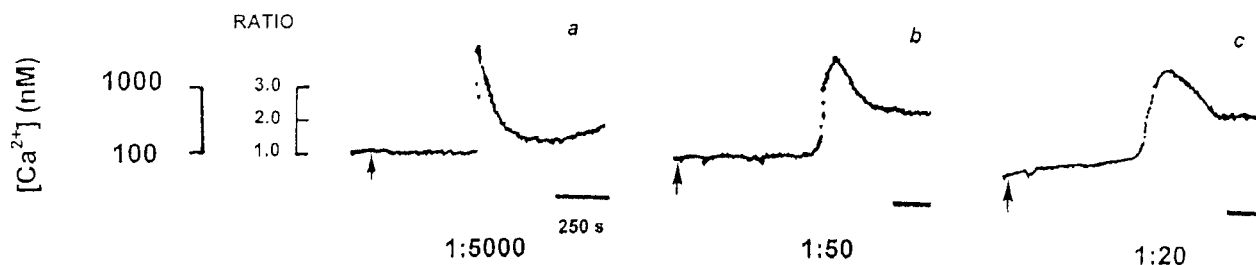


Figure 7 Representative traces showing the effect of the anti-interleukin-6 receptor antibody (MT-18) (1:20 to 1:5000) on cytosolic Ca^{2+} levels in fura 2-loaded single rat osteoclasts (n =up to 10 cells per treatment).

The present experiments (a) extend these initial findings to other IL-6-related cytokines, LIF and oncostatin-M, and (b) attribute these effects to an osteoclastic (as opposed to osteoblastic) IL-6R. The results also show that activation of this receptor by a specific agonist antibody (as opposed to IL-6 itself) results in a paradoxical inhibition of bone resorption, intracellular Ca^{2+} release and transmembrane Ca^{2+} influx. We have shown that IL-6 and LIF, but not oncostatin M, reversed this Ca^{2+} -induced resorption inhibition; that Ca^{2+} and IL-6 stimulate bone resorption in the micro-isolated single osteoclast assay; that IL-6R activation by a highly specific agonist antibody, MT-18, paradoxically inhibits bone

resorption; that the MT-18-induced resorption-inhibition is accompanied by mostly biphasic cytosolic Ca^{2+} signals; and that the latter results both from Ca^{2+} release and Ca^{2+} influx.

The experiments first investigated the effects of the IL-6 family cytokines, LIF and oncostatin-M, on bone resorption. LIF and oncostatin-M appear to have important osteoclastogenic actions, although their known effect is on osteoblastic differentiation (Ng & Martin 1998). Indeed, osteoclast number increases 6-fold in mice overexpressing the LIF receptor (LIFR) (Ware *et al.* 1995). Furthermore, both LIF and oncostatin-M act through low-affinity receptors that form heterodimeric complexes with gp130 (Kishimoto *et al.* 1992). The same holds true for IL-11, but the latter did not reverse the anti-resorptive effect of Ca^{2+} even at >10-fold higher molar concentrations than IL-6 (Adebanjo *et al.* 1998). In this study, LIF did in fact reverse the inhibitory effects of Ca^{2+} on osteoclastic resorption like IL-6, while oncostatin-M did not. Instead, oncostatin-M diminished osteoclast numbers over a timescale compatible with an apoptotic effect. Thus, it appears that this cytokine family triggers separate intracellular signaling pathways downstream of gp130 to produce distinct effects on the activity and survival of mature osteoclasts.

Secondly, the present experiments explored direct actions that IL-6 and Ca^{2+} could exert upon the osteoclast without a requirement for a mediation through other cells. This was achieved through the use of the micro-isolated osteoclast assay which estimates proportions of single osteoclasts, each individually deposited onto a devitalized dentine slice, such cells are active in pit formation. In contrast, measurements, whether of pit numbers or overall slice resorption in traditional pit assays (Adebanjo *et al.* 1998), are likely to be influenced by phenomena such as osteoclast formation and activation in addition to cellular resorptive activity. Such culture systems also invariably contain osteoblasts and bone marrow stromal cells that themselves may produce and respond to IL-6 (Manolagas & Jilka 1995). These additionally have been reported to possess Ca^{2+} sensing receptors that could potentially allow them also to respond to high extracellular Ca^{2+} (Pi *et al.* 2000).

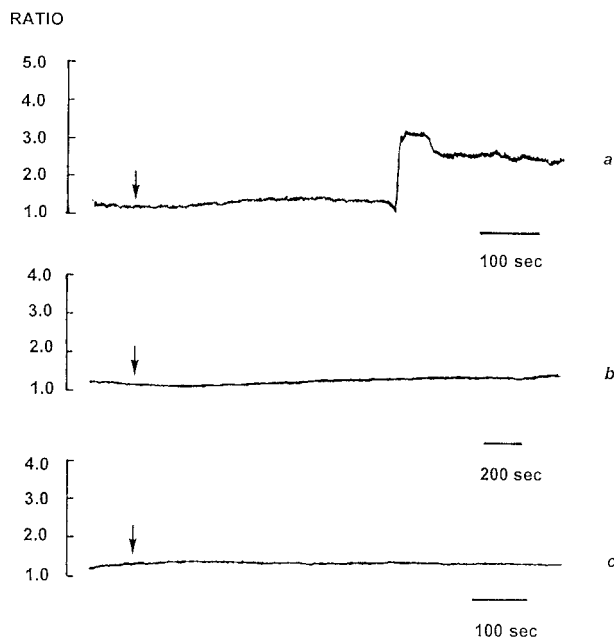


Figure 8 Representative traces showing the effect of the anti-interleukin-6 receptor (IL-6R) antibody (MT-18) (1:5000, a) on cytosolic Ca^{2+} levels in fura 2-loaded single rat osteoclasts and those that were additionally pre-incubated with either IL-6R (500 $\mu\text{g}/\text{l}$) (b) or IL-6 (10 $\mu\text{g}/\text{l}$) (c) (n =up to 10 cells per treatment).

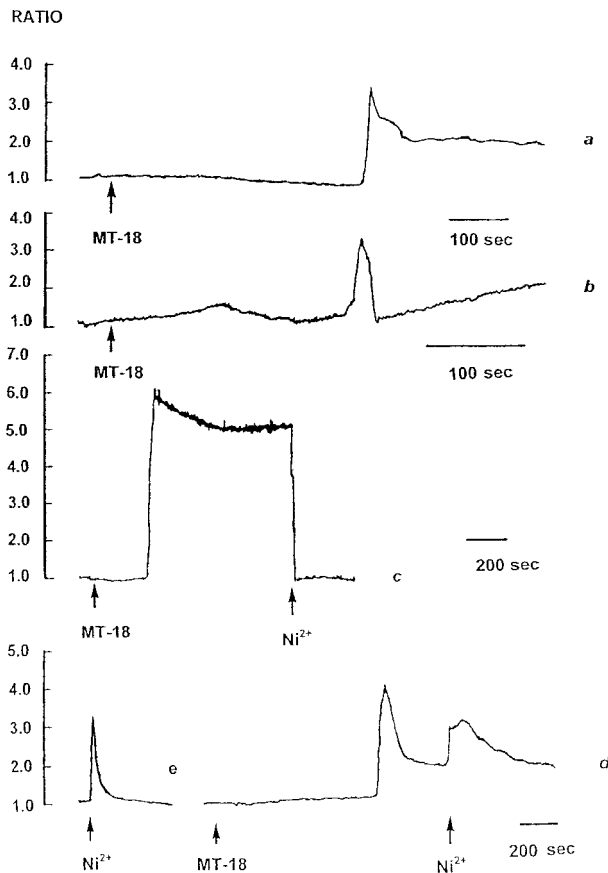


Figure 9 Representative traces showing the effect of the anti-interleukin-6 receptor (IL-6R) antibody (MT-18) (1:5000) on cytosolic Ca^{2+} levels in fura 2-loaded single rat osteoclasts that were incubated either in M199-H ($[\text{Ca}^{2+}]_i$, 1.25 mM) (a) or in Ca^{2+} -free, EGTA-containing medium ($[\text{Ca}^{2+}]_i < 5 \text{ nM}$, by fura measurements) (b). NiCl_2 (5 mM) was applied to intercept the plateau of the MT-18-induced cytosolic Ca^{2+} signals (c and d). (e) Control recording with 5 mM Ni^{2+} alone ($n=10$ cells per treatment).

The use of such a tightly controlled microenvironment made it possible to confirm that IL-6 and Ca^{2+} directly stimulate the unitary resorptive activity of each single osteoclast, whether applied alone or in combination, without the necessity for mediation by other cell types. This excludes the possibility, for example, that IL-6 regulates osteoclast Ca^{2+} sensing and bone resorption exclusively through its action on the osteoblast by modulating osteoprotegerin-L release (Lacey *et al.* 1998). The present experiments also demonstrated positive regulatory effects of IL-6 on bone resorption in two separate situations in which elevated $[\text{Ca}^{2+}]_i$ otherwise inhibits osteoclastic bone resorption in a mixed culture including stromal elements but where it enhances bone resorption by isolated osteoclasts. For example, Ca^{2+} itself stimulated, rather than inhibited, bone resorption, in contrast to earlier

results using the traditional pit assays. Such differences might well reflect the different conditions and parameters measured in the two kinds of assay. Besides being an important physiological phenomenon, this may have important pathophysiological implications in the IL-6-mediated bone loss seen in multiple myeloma, rheumatoid arthritis and post-menopausal osteoporosis.

A third facet explored the osteoclastic actions of IL-6 at the receptor level. We demonstrated that the monoclonal anti-IL-6R antibody, MT-18, inhibited bone resorption and triggered cytosolic Ca^{2+} signals. This action is not seen when the IL-6R is activated by IL-6, which indicates for the first time, that the same IL-6R can trigger signals differentially not only to activate, but also to inhibit, osteoclastic bone resorption.

We next corroborated the IL-6R specificity of MT-18 by investigating the competing actions of soluble human IL-6R (shIL-6R) and IL-6. Soluble hIL-6R is a recombinant truncated receptor that binds both MT-18 and IL-6 (Taga *et al.* 1989). We expected that soluble hIL-6R would displace MT-18 from osteoclastic IL-6Rs. Conversely, by interacting with the IL-6R, IL-6 should prevent MT-18 access to the receptor. We observed that IL-6 and shIL-6R both reversed the inhibitory effect of MT-18 on bone resorption and prevented MT-18-induced Ca^{2+} signaling. Together, the results strongly suggest that the novel anti-resorptive effects of MT-18 were exerted through an IL-6R.

Finally, MT-18-induced Ca^{2+} signals showed latency and their transient phase persisted in Ca^{2+} -free, EGTA-containing solutions consistent with a release of intracellularly stored Ca^{2+} . The plateau elevations that followed were abolished in Ca^{2+} -free extracellular solutions or were aborted by the Ca^{2+} channel blocker, Ni^{2+} . They thus resembled responses triggered by hormones, cytokines and growth factors that activate phospholipase C to trigger IP_3 formation and Ca^{2+} release (Berridge 1993, Hughes *et al.* 1991, Rogers & Hammerman 1993, Yang *et al.* 1993). To our knowledge, this is the first demonstration of IL-6R-mediated Ca^{2+} signaling in any cell.

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