Role of EP_3 and EP_4 Prostaglandin Receptors in Reorganization of the Cytoskeleton in Mature Human Osteoclasts

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ABSTRACT. ***Objective.*** Osteoclasts are central to the pathophysiology of several bone diseases. Prostaglandin E_2 (PGE_2) is well known to influence osteoclasts indirectly, but its direct action on osteoclasts is still controversial and the relevant receptors are unknown. We investigated the distribution and function of EP receptors in human mature osteoclasts.

Methods. Osteoclasts were extracted from femurs and tibias of human fetuses obtained from legal abortions. *In situ* hybridization and immunohistochemistry were used to detect the presence of EP_1, EP_2, EP_3, and EP_4 receptors on these cells. Actin staining and fluorescent microscopy were used to detect the effects of receptor activation on the cytoskeleton.

Results. Only EP_3 and EP_4 receptors were detected at the RNA and protein level in osteoclasts. These receptors were functional: PGE_2 decreased the number of osteoclasts presenting an actin ring; 11-deoxy-PGE_1, an EP_2 and EP_4 agonist, also decreased the number of tartrate-resistant acid phosphatase-positive cells with an actin ring; sulprostone, an EP_3-specific agonist, had no effect on this variable but increased the number of cells with lamellipodia.

Conclusion. Mature human osteoclasts present 2 subtypes of EP receptors, namely EP_3 and EP_4, that mediate different actions of PGE_2 on these cells: activation of the EP_4 receptors inhibits actin ring formation and activation of the EP_3 receptors increases the number of lamellipodia. Activation or inhibition of these receptors by specific agents could be used to study and influence osteoclast function. (J Rheumatol 2004;31:1598–606)

Key Indexing Terms:
PROSTAGLANDINS BONE OSTEOCLASTS

Osteoclasts are large, multinucleated cells of hematopoietic origin that share a common differentiation pathway with monocytes and macrophages[1]. Their principal if not only function is to resorb bone and other calcified tissues. The resorption cycle is a multistep process during which the actin cytoskeleton of osteoclasts undergoes drastic reorganization. In nonresorbing osteoclasts, podosome-like structures are located throughout the whole bone-facing surface and in the extremities of lamellipodia[2]. Prior to resorption, there is an intense accumulation of podosomes to the paramarginal region of the bone-facing surface, forming a dense belt-like structure, the actin ring[3]. This structure is essential to the formation of the resorption lacunae and to bone resorption[4].

Osteoclasts are central to the pathophysiology of several human bone diseases, such as osteoporosis[5], osteopetrosis[6], and Paget's disease[7]. They also play an important role in the focal subchondral and marginal bone erosions seen in rheumatoid arthritis (RA)[8]. RA synovial tissues produce a variety of proinflammatory cytokines, growth factors, and lipid mediators that may influence osteoclast formation and activity[9]. Furthermore, it is reported that cyclooxygenase-2 is upregulated in such pathologies and that its activation leads to an increase in the release of prostaglandins (PG) in the synovial environment[10]. PG are potent, multifunctional, and complex regulators of bone metabolism; depending on the model or the species studied, these lipid mediators may modulate bone formation[11-13] and/or resorption[14,15]. Endogenous PG appear to play important roles in the normal development and maintenance of bone tissue, as well as in a number of bone related diseases[16]. PGE_2, the major prostaglandin produced in bone[17], potently stimulates bone formation *in vivo*[13], increases the skeletal response to mechanical loading[18], and decreases bone loss in experimental models[19]. In different situations, however, PGE_2 may increase bone resorption in humans[14,20] and in animal models[21-26]. PGE_2 also promotes osteoclast differentiation *in vitro*[27], but may also inhibit osteoclast motility[28]. These apparently paradoxical effects of PG probably reflect the

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different distribution of prostaglandin receptors in different models.

PGE_2 exerts its cellular effects through activation of 4 different G protein-coupled receptor subtypes: EP_1, EP_2, EP_3, and EP_4. In previous studies, we described the presence of functional EP_4, IP, FP, and TP receptors in human osteoblasts in culture[29]. More recently, using immunohistochemistry in human bone slices, we confirmed the presence of the EP_4 receptor in human osteoblasts in normal and pathological bone and showed that osteoclasts from fetal, pagetic, and osteoporotic bone present EP_3 and EP_4 receptors[30]. The functions of these receptors, however, remain unknown. Our objectives in this study were to develop a model of mature human osteoclasts in culture, to determine if the distribution of EP receptors in these cells corresponds to the findings *in situ*, and to analyze the effects of their activation on the osteoclast cytoskeleton.

MATERIALS AND METHODS

Cell isolation. Mature osteoclasts were extracted from femurs and tibias of human fetuses (16 to 20 weeks of gestation) obtained from legal abortions after the mother signed an informed consent, as required by Quebec's civil law. We used an adaptation of the method described by Chambers, *et al*[31] for the preparation of neonatal rabbit osteoclasts. Briefly, femurs and tibias were removed, cleaned of adherent soft tissues, and cut longitudinally. Each bone was curetted with a scalpel blade into 5 ml of α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), in a 100 mm Petri dish. The media were vortexed for 30 s, sedimented for 1 min, and aliquots of the cell suspension were transferred to 96-well plates containing devitalized bovine cortical bone discs or to 6-well plates containing glass coverslips. The plates were incubated 45 min at 37°C with 5% CO_2 and rinsed 3 times with phosphate buffered saline (PBS) to remove nonadherent cells. Cells were kept in α-MEM 10% FBS at 37°C with 5% CO_2 and used after 20 h in culture for all assays, except when passed on bone slices for the resorption assays, which were done after 48 h.

Characterization of osteoclastic phenotype. Four osteoclastic markers were studied: (1) presence of tartrate-resistant acid phosphatase (TRAP); (2) presence of 3 nuclei or more; (3) response to calcitonin; and (4) formation of actin rings. Cells seeded on microscope slides were washed twice with PBS and fixed for 10 min with 3.7% formaldehyde in PBS, then permeabilized with 0.1% Triton X-100 in PBS. The presence of TRAP was determined with the use of a leukocyte-acid-phosphatase kit (Sigma, St. Louis, MO, USA) with 50 mM L-tartrate according to the manufacturer's protocol. The cells were then rinsed twice with PBS and incubated 40 min at room temperature with 0.3 µM rhodamine conjugated phalloidin (Molecular Probes, Eugene, OR, USA) and with 0.26 ng/ml bisbenzimide H 33342 (Sigma). After incubation, the cells were rinsed twice in PBS and then mounted with Geltol (Immunon, Detroit, MI, USA). To detect the presence of functional calcitonin receptors, the cells were seeded on round 25 mm coverslips overnight in the same culture medium, then washed twice with Tyrode buffer containing 0.1% bovine serum albumin (BSA) and loaded with 2 µM of the calcium probe Fluo-3 AM (Molecular Probes) diluted in Tyrode with 0.1% BSA for 45 min. They were rinsed and incubated in Tyrode without BSA to allow hydrolysis of the probe for 15 min. Coverslips were mounted in an experimental bath containing 1 ml of Tyrode, and the fluorescence level was observed with a scanning confocal microscope (Noran Instruments, Middleton, WI, USA). Specimens were excited at 488 nm and the emitted fluorescence was measured at 525–550 nm. Data were acquired before and after the addition of 1 µM human calcitonin (Calbiochem, La Jolla, CA, USA). To detect bone resorption activity, the cells were seeded on devitalized bovine cortical bone slices, kept in culture with the same culture medium with the pH adjusted to 6.9 at 37°C, 5% CO_2, for 48 h in the presence or absence of 1 µM calcitonin, washed twice with PBS, and fixed for 2 min in 1:1 ethanol-acetone mix. The slices were stained for TRAP as described, and the number of TRAP-positive cells recorded. The bone slices were air dried, cleaned with NH_4OH, and air dried before they were coated with gold by sputter coating and examined by scanning electron microscopy. Total resorption area was calculated using the Mocha imaging software and the resorption area per osteoclast calculated according to the number of TRAP-positive cells detected on the slice.

In situ hybridization. Specific sequences for prostaglandin EP_1, EP_2, EP_3, and EP_4 receptors and their complementary homologs were labeled with digoxigenin according to the supplier's protocol (Roche Diagnostics, Laval, QC, Canada). The sequences used for probe synthesis were 5'-AGATGCACGACACCACCATGATACCGACAAGCT-3' for the EP_1 receptor, 5'-TCCGACAACAGAGGACTGAACGCATTAGTCTCA-GAACAGG-3' for the EP_2 receptor, 5'-ACATGATCCCCATAAGCT-GAATGGCCGTCTCGGTCGTGAT-3' for the EP_3 receptor, and 5'-TTCATGTACGTGGCGATGGTCACCGGGCTCACCAACAAAGT-3' for the EP_4 receptor. Cells were washed twice with PBS, fixed 2 min in 1:1 acetone:methanol, and rinsed with PBS. The hybridization mixture (probe in 40% formamide, 10% dextran sulfate, 1× Denhardt, 4× SSC, 10 mM DTT, and 1 mg/ml tRNA) was applied to the slide and covered with a glass coverslip. Hybridization was done at 40°C for 18 h in a moist chamber. After hybridization, the slides were washed at 22°C for 10 min in 2× SSC solution, 10 min in 1× SSC, and 10 min in 0.5× SSC. Another wash of 60 min in 0.5× SSC was done at 40°C. The digoxigenin-labeled probe was detected using anti-digoxigenin alkaline phosphatase conjugated antibody (Roche Diagnostics, Québec, Canada). Slides were washed 3 times with buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with the antibody diluted in 0.3% Triton X-100 and 1% normal goat serum at 22°C for 3 h. The slides were then washed 2 times in buffer 1, incubated 10 min in buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$), and developed in 0.33 mg/ml nitroblue tetrazolium, 0.175 mg/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 0.24 mg/ml levamisole for 8 h in the dark. The reaction was stopped with TE (10 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA) for 10 min and slides were mounted. Negative controls included treatment with RNase (100 µg/ml in 2× SSC at 37°C for 60 min before hybridization) and noncomplementary probes.

Immunostaining. Cells were fixed 2 min in 1:1 acetone:methanol and endogenous peroxidase blocked by incubation in 0.5% H_2O_2/methanol for 30 min. The slides were washed in PBS and microwaved for 4 min in 0.1 M citric acid solution, pH 7.4, to release masked epitopes. Nonspecific binding was blocked by incubating the sections with 10% goat serum and 5% BSA/PBS for 20 min. After a brief wash in PBS, slides were incubated overnight at 4°C with the specific antiserum (1/1000) diluted in 10% goat serum, 0.1% BSA/PBS in a humidified chamber. The antisera were raised in rabbits against oligopeptides specific for the receptor subtypes studied and found to be specific under the experimental conditions used[30]. The slides were then washed 5 min in PBS 1% Triton X-100 and 5 min in PBS, blocked with 10% goat serum for 20 min and incubated with 1:200 dilution of biotinylated anti-rabbit IgG for 30 min at room temperature. After washing 5 min with PBS 1% Triton X-100 and 5 min with PBS, development was done with the ABC kit and DAB substrate according to the supplier's protocol (Vector Laboratories, Hamilton, ON, Canada). The slides were then counterstained with hematoxylin.

Actin staining. Twenty hours after osteoclast isolation, α-MEM was replaced with new medium containing 2 mM indomethacin to inhibit endogenous PG synthesis. Cells were incubated 1 h and stimulated for 5 min with different agonists (PGE_2, 11-deoxy-PGE_1, butaprost, and sulprostone) (Cayman Chemical, Ann Arbor, MI, USA), then fixed in 3.7% formaldehyde for 10 min, washed twice with PBS, permeabilized in PBS 0.1% Triton X-100 for 5 min, and washed with PBS. Cells were stained for TRAP, washed with PBS, and incubated at room temperature with 5 µM

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rhodamine conjugated phalloidin (Molecular Probes), diluted in PBS for 20 min, rinsed twice with PBS, and mounted. Sections were examined with a Zeiss Axioskop-2 microscope and a spot camera. The Spot-32 program was used to obtain the images. The number of TRAP-positive cells with an actin ring or lamellipodia was expressed as percentage of control.

Statistical analysis. All data are presented as means ± SEM of n different observations. P values < 0.05 were considered statistically significant. Differences between points in the same curve were compared using Mann-Whitney statistical tests.

RESULTS

Characterization of the osteoclastic phenotype. Between 10,000 and 25,000 cells were obtained from the tibia and femurs of each fetus; TRAP-positive, multinucleated cells represented 4.2 ± 0.6% (n = 6) of the cells in culture (Figure 1A, i). After 20 h in culture on coverslips, 45.8 ± 3% of the TRAP-positive cells presented an actin ring (Figure 1A, ii). To further characterize the phenotype of the multinucleated cells, we studied the response of these cells to calcitonin, a hormone known to elevate both intracellular cyclic adenosine monophosphate (cAMP) and calcium concentrations in osteoclasts[32]. Because of the technical limitations to perform intracellular cAMP level measurements in a heterogeneous cell population, we chose to determine the changes induced by 1 µM human calcitonin, a concentration known to elicit the maximum intracellular calcium response from the calcitonin receptor[32,33] in intracellular calcium levels in single cells. As shown in Figure 1B, human calcitonin induced an increase in the intracellular calcium level in multinucleated cells. The level of fluorescence is expressed in pseudocolor on a scale from 0 to 255, the latter being the maximum fluorescence. The increase in intracellular calcium lasted at least 2 min after stimulation, the maximum time the cells were observed. After the last acquisitions of intracellular calcium reading, we performed a nuclear staining to ensure that the cells studied were multinucleated (data not shown). All cells responding to calcitonin were found to be multinucleated. Finally, using scanning electron microscopy, we observed that our heterogeneous cell cultures were able to form resorption pits when cultured on devitalized bovine cortical bone slices (Figure 1C). In control, untreated cultures the total bone resorption area per bone slice was 102 ± 6.5 nm^2, with a calculated area per osteoclast of 0.55 ± 0.05 nm^2. When the cells were treated with 1 µM calcitonin, total resorption area decreased to 20 ± 4.3 nm^2 and the area per osteoclast to 0.25 ± 0.07 nm^2 ($p < 0.05$, n = 3).

Expression of PGE_2 receptors in human osteoclasts. *In situ* hybridization showed that human mature osteoclasts in culture expressed mRNA for the EP_3 (Figure 2A) and the EP_4 (Figure 2D) PG receptors. No staining was obtained when the cells were incubated with complementary probes (Figures 2B, 2E) or after treatment with RNase (Figures 2C, 2F). The presence of the EP_3 and EP_4 receptor proteins was also confirmed using immunohistochemistry (Figures 3A, 3C, respectively). Controls using the antisera preadsorbed

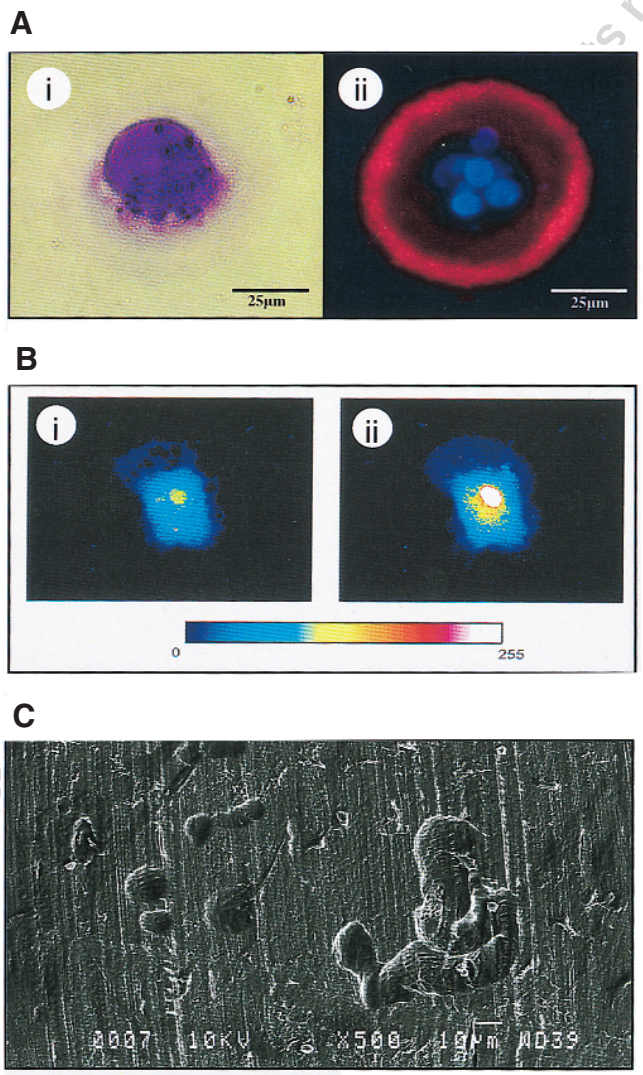

Figure 1. Phenotypic characterization of osteoclasts. (A, i) Multinucleated cells showing purple staining confirming expression of TRAP. (A, ii) Actin ring (red) and nuclei (stained blue by bisbenzimide H 33342) in the same cell shown in panel i. (B) Intracellular calcium level determination using FLUO-3 probe showing a multinucleated cell before (i) and 1 min after (ii) addition of 1 µM human calcitonin. Fluorescence levels indicate intracellular calcium concentration and are expressed in pseudocolor on an intensity scale from 0 to 255; these results are representative of 3 independent assays. (C) Scanning electron microscopy showing resorption pits by cells incubated 48 h on devitalized bone slices.

with the respective immunization peptides are shown in Figures 3B and 3D and confirm the specificity of the antisera. Virtually all multinucleated cells in the preparations stained positive for EP_3 and EP_4 receptors in both immunohistochemistry and *in situ* hybridization. EP_1 and EP_2 mRNA and proteins were not detected in these cells (data not shown).

Action of activated receptors on the actin cytoskeleton of osteoclasts. In our preparations 45.8 ± 3% of the TRAP-positive multinuclear cells without treatment presented actin rings after 20 h in culture (Figures 4B, 4D). In the same

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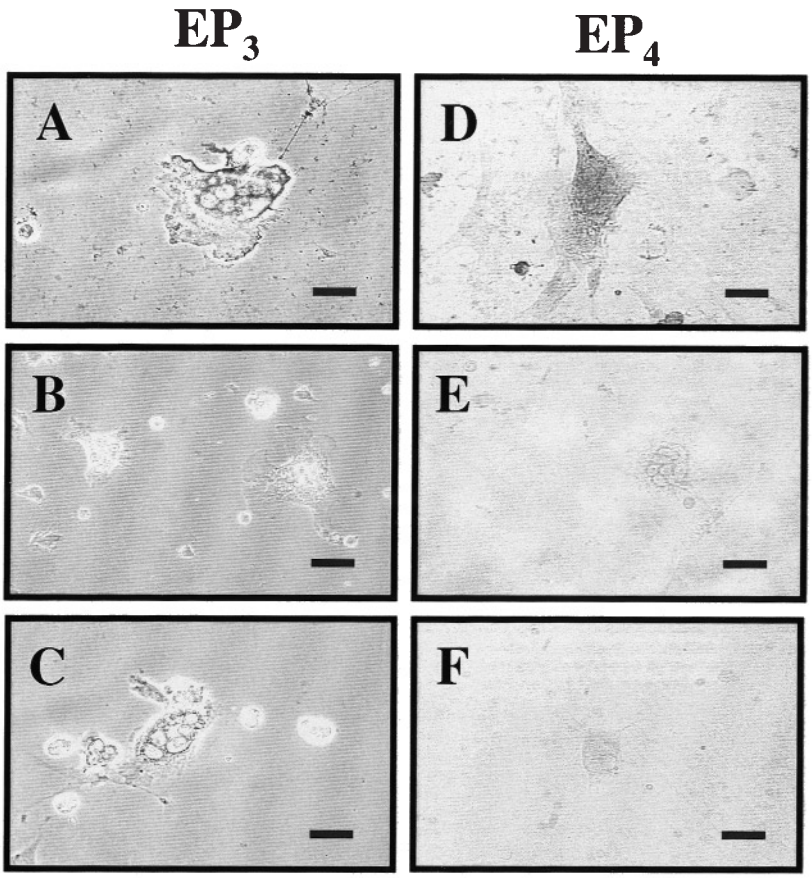

Figure 2. In situ hybridization performed on mature human osteoclasts in culture showing the expression of EP_3 and EP_4 mRNA. (A) Cells were hybridized with an oligonucleotide probe complementary to the EP_3 mRNA (5'-ACATGATCCCCATAAGCTGAATGGCCGTCTCG-GTCGTGAT-3'). (B) Hybridization performed with a noncomplementary probe against EP_3 mRNA (5'-TAGTGTGGCTCTGCCGGTAAGTCGAATACCCCTAGTACA-3). (C) Negative control hybridized with a complementary EP_3 mRNA probe after RNAse treatment. (D) Human osteoclasts were hybridized with an oligonucleotide probe complementary to the EP_4 mRNA (5'-TTCATGTACGTGGCGATGGTCACCGGGCTCACCAACAAAGT-3'). (E) Hybridization performed with a noncomplementary probe against EP_4 mRNA (5'-GCCT-GTCGTGACGAGTCTGTCAGTTTCCTGTAGAAGACGG-3). (F) Negative control hybridized with a complementary EP_4 mRNA probe after RNAse treatment. Results are representative of cell preparations from 4 different fetuses. Bars = 25 µm.

conditions, 16.25 ± 6.96% of the cells presented lamellipodia (Figures 4A, 4C). We considered the presence of actin rings and lamellipodia mutually exclusive, i.e., cells presenting transitional cytoskeletal structures were not counted in any of these categories. However, we seldom found cells with both well defined actin rings and lamellipodia.

As shown in Figure 5A, PGE_2 induced a significant concentration-dependent decrease of the percentage of TRAP-positive cells with an actin ring. In the same experimental conditions, there was no significant effect of PGE_2 in the percentage of TRAP-positive cells with lamellipodia.

To determine the EP receptors implicated in this effect, we stimulated osteoclasts with specific agonists for PG receptors. Incubation with 11-deoxy-PGE_1 (Figure 5B), an EP_2 and EP_4 agonist, showed a concentration-dependent decrease in the percentage of TRAP-positive cells with an actin ring. 11-deoxy-PGE_1 had no effect on the percentage of TRAP-positive cells with lamellipodia. To distinguish between the EP_2 and EP_4 receptors, we stimulated osteoclasts with butaprost, a selective agonist for the EP_2 receptor, which had no effect on the actin cytoskeleton (Figure 5C). As shown in Figure 5D, sulprostone, an EP_3-specific agonist, had no effect on the percentage of TRAP-positive cells with an actin ring, but increased the percentage of TRAP-positive cells with lamellipodia.

DISCUSSION

Understanding the exact role of prostaglandins on the control of bone metabolism is difficult because of conflicting effects reported in the literature. Indeed, depending on the model studied, on the prostaglandins used,

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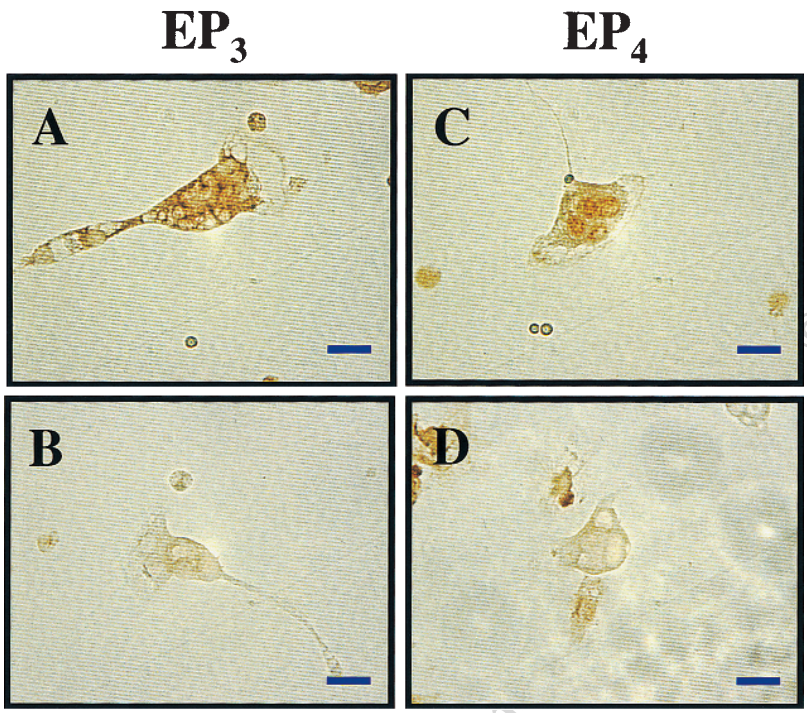

Figure 3. Immunohistochemistry showing the localization of the EP_3 and EP_4 receptors on human osteoclasts in culture. (A) Staining with a rabbit anti-human EP_3 receptor antiserum and (B) with the same antiserum preadsorbed with human EP_3 peptide. (C) Staining with a rabbit anti-human EP_4 receptor antiserum and (D) with the same antiserum preadsorbed with human EP_4 peptide. Results are representative of cell preparations from 3 different fetuses. Bars = 25 µm.

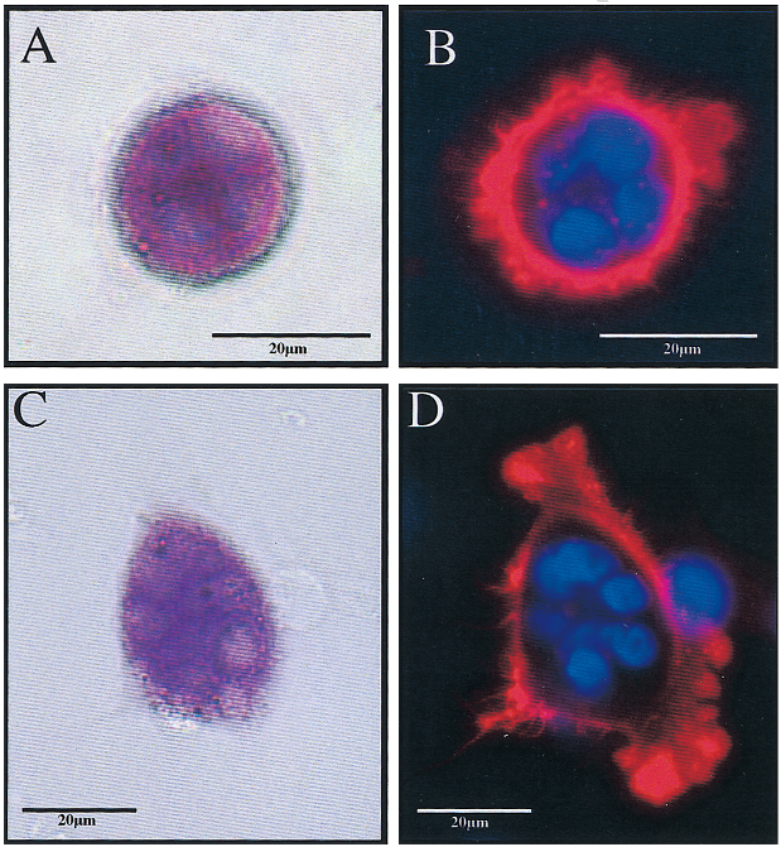

Figure 4. Osteoclasts with an actin ring or lamellipodia. Multinucleated cells were fixed and actin filaments were stained with rhodamine-conjugated phalloidin. (A) Purple staining showing expression of TRAP. (B) F-actin staining with rhodamine-conjugated phalloidin showing an actin ring in the same cell shown in (A). (C) Purple staining showing expression of TRAP. (D) F-actin staining with rhodamine-conjugated phalloidin showing lamellipodia of the same cell shown in (C).

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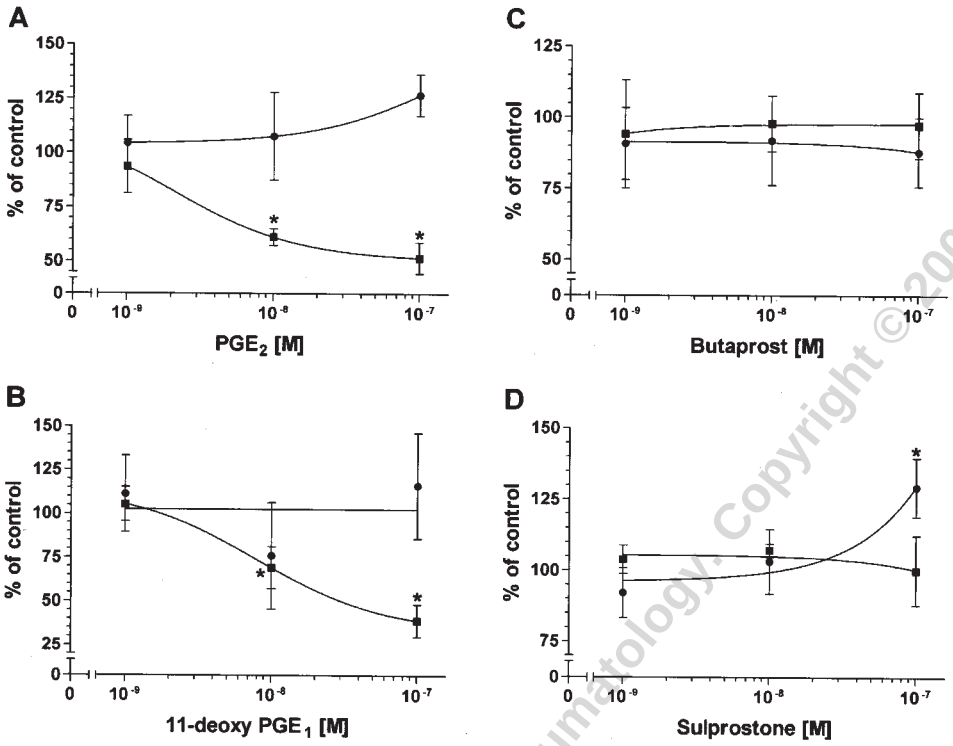

Figure 5. Effect of PG receptor agonists on actin organization in mature human osteoclasts in culture. Cells were fixed and actin filaments were stained with rhodamine-conjugated phalloidin after stimulation for 5 min with different concentrations of (A) PGE_2, (B) 11-deoxy-PGE_1 (EP_2/EP_4 agonist), (C) butaprost (EP_2 agonist), and (D) sulprostone (EP_3 agonist). Results represent the percentage [100 × (absolute number of multinucleated TRAP-positive cells showing an actin ring or lamellipodia in the treated preparation/absolute number of multinucleated TRAP-positive cells showing an actin ring or lamellipodia in the untreated control preparation)] of cells showing an actin ring (■) or lamellipodia (●) compared to their own controls, defined as 100%. In the control groups, the average for total number of osteoclasts per coverslip was 58.03 ± 4.61, that for cells presenting an actin ring was 18.6 ± 0.98, and that for cells with lamellipodia was 15.8 ± 3.8. Points represent the means and error bars the SEM. N = 4 for PGE_2, 8 for butaprost, 6 for 11-deoxy PGE_1, and 5 for sulprostone. *$p < 0.05$.

and on the experimental design, these effects may be anabolic or catabolic. It is possible that differences in the distribution of the types and subtypes of prostaglandin receptors, depending on the species studied and on the stage of differentiation of the cells, may contribute to these difficulties. Moreover, systemic and local factors can act either directly on osteoclasts to control their activity, or indirectly via interactions with osteoblasts or stromal cells that, in turn, may produce soluble or cell-surface factors that affect osteoclasts. Our objectives were thus to establish a model of mature human osteoclasts in culture, and to determine the distribution of the subtypes of PGE_2 receptors in these cells and the effects of direct activation of the EP receptors on the cytoskeleton.

Mature osteoclasts are rarely obtained from adult bones, even when the tissues are from severely osteoporotic patients. Although bones from Paget's disease present high numbers of osteoclasts in histological sections, we avoided this source because of low availability and the possibility that the disease could influence the distribution of the EP receptors. Fetal bones were thus chosen as the source of osteoclasts because of the availability of the tissue and the relatively high number of mature osteoclasts isolated, probably a consequence of the high remodeling rate of the fetal tissue. Although osteoclasts represented a small percentage (4.2%, corresponding to 400–1000 osteoclasts per fetus) of the cells in the heterogeneous cultures obtained with this method, these cells were found to be multinuclear and TRAP-positive, to present functional calcitonin receptors, to form actin rings, and to resorb bone, confirming their osteoclastic phenotype. It is interesting, however, that bone resorption could only be observed when a high number of osteoclasts (> 200 multinucleated cells) were cultured on each cortical bone slice. Although this characteristic severely limits the use of this model for the study of the effect of different interventions on bone resorption, the model is useful for the study of mature human osteoclasts at the individual cell level. One should also consider that fetal cells may not represent their adult counterparts. However, as far as we know, this is the only model of authentic mature

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human osteoclasts that were not generated by *in vitro* differentiation, and it seems fair to suppose that they are the closest model to adult mature osteoclasts.

In a study using immunohistochemistry to identify the subtypes of PGE_2 receptors present in bone cells in tissue samples from adult normal, osteoporotic, and pagetic individuals as well as from human fetuses, we showed that osteoclasts present both EP_3 and EP_4 receptors[30]. We used the present model to study the functions mediated by these receptors, and the first step was to determine if their distribution in fetal cells corresponded to that found in histological sections of adult bone. *In situ* hybridization demonstrated the presence of EP_3 and EP_4 mRNA in isolated fetal human osteoclasts, and this distribution was confirmed at the protein level with immunohistochemistry. These results clearly show that the model presents the same EP receptors as *in situ* osteoclasts, suggesting that, at least for the study of PG receptors, isolated fetal human osteoclasts can be used as a representative model of adult cells.

It is interesting from the immunocytochemical results with isolated cells that the EP_3 and EP_4 receptors are not only present at the plasma membrane, but that there is also a strong nuclear signal. Although we cannot exclude the possibility that the nuclear localization may be due to an artifact, recent studies describe the presence of functional nuclear EP_3 and EP_4 receptors in neonatal porcine brain and adult rat liver[34], and functional EP_1 receptors in porcine brain and myometrium[35]. Also, the enzymes involved in the biosynthesis of PG, cyclooxygenase and PLA_2, have been found to be localized at the nuclear membrane of different cell types[36,37]. The existence of a PG transporter that facilitates the entrance of PG to the cytoplasm is more evidence supporting the existence and possible physiological relevance of nuclear PG receptors[38]. In human osteoclasts, the nuclear localization of these receptors should be confirmed by further studies.

Osteoclasts express unique cell adhesion structures called podosomes, which contain actin filaments. Podosomes are organized differently depending on the activity of the osteoclast: in bone-resorbing cells they form the actin ring necessary for bone resorption, and in motile osteoclasts they are organized in lamellipodia, the structure responsible for cell movement[3,39]. Prostaglandins appear to affect the actin cytoskeleton of many cell types[40,41]. In rat osteoclasts, PGE_2 was shown to decrease the number of cells presenting an actin ring[28], but nothing is known of its effects on the cytoskeleton of human osteoclasts or of the receptor subtypes implicated. To determine this, we stimulated the cells with different EP receptor agonists in the presence of indomethacin to avoid endogenous PGE_2 production, and determined the proportion of osteoclasts showing actin rings or lamellipodia. PGE_2, which activates all EP receptors, decreased the number of cells presenting actin rings. Reproduction of this result with 11-deoxy-PGE_1, an agonist acting on both EP_2 and EP_4 receptors, and its absence in cells treated with butaprost, an EP_2 agonist, strongly suggest that this effect is mediated by the EP_4 receptor. Since the presence of an actin ring indicates that osteoclasts are in a state of bone resorption[39], it is possible that the EP_4 receptor may inhibit bone resorption by reducing the number of cells with actin rings. Although the technical limitations of our model did not allow confirmation of this hypothesis in human osteoclasts, it has been shown in rabbit cells that PGE_2 inhibits bone resorption through the EP_4 receptor[42].

Stimulation of our cells with sulprostone, an EP_3 agonist, induced a concentration related increase in the percentage of cells with lamellipodia, suggesting that activation of the EP_3 receptor could have a positive effect in osteoclast motility. Interestingly, PGE_2, which activates both EP_4 and EP_3 receptors, induced only a slight and not statistically significant increase in the number of cells presenting lamellipodia. This could be explained considering the different second messengers coupled to these receptor subtypes. EP_4 receptors activate adenylate cyclase through G_s proteins, leading to an increase in intracellular cAMP[43]. In humans, all subtypes of EP_3 receptor cloned to date are coupled to G_i proteins and decrease intracellular cAMP[44]. Assuming this is the case in the cells we studied, it is possible to speculate that when both EP_3 and EP_4 receptors are activated by PGE_2, the adenylate cyclase-stimulating activity associated with the EP_4 receptor prevails. The net result would be an increase in intracellular cAMP concentration and the biological effects associated with it, in this case a decrease of the number of cells presenting actin rings. Since these effects are seen within 5 min, it is likely that PGE_2 is disrupting rings that are already in existence. However, it is interesting that increases in the intracellular concentration of cAMP can inhibit actin polymerization and formation of adhesion structures[45], raising the possibility that both mechanisms may lead to the observed results. The more pronounced effect of 11-deoxy-PGE_1 on the inhibition of actin ring formation compared to PGE_2 would support this explanation, as would the effect of sulprostone on the presence of lamellipodia. Thus, *in vivo* the effect of PGE_2 associated with the activation of the EP_3 receptor would prevail only in situations where the EP_4 receptor is not active or is downregulated. In this context, it is interesting that in our model the effect of 11-deoxy-PGE_1 on the actin ring was transient, with a maximal decrease (94%) after 5 min of stimulation. After 10 and 30 min, the decrease observed was 23.3% and 15.6%, respectively (data not shown). If the EP_4 receptor is rapidly downregulated after stimulation, it would be possible that the EP_3 effect would prevail after a short term EP_4-driven effect. It has been found in other models that PG transiently inhibit and subsequently increase osteoclastic bone resorption, which would be compatible with this hypothesis[46].

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Even if we cannot totally exclude participation of other cell types in our model, the fast reorganization observed in actin rings (less than 5 min) allows us to believe that these effects are indeed caused by activation of EP receptors on osteoclasts. It is known that PGE_2 can induce RANKL expression by osteoblasts[47], and we have shown that human osteoblasts express EP_4 receptor[29]. In the model used in this study, however, the low cell density did not allow extensive physical contact between cells, suggesting that osteoblasts, which need physical contact with osteoclasts for regulation of their activity[48], are not implicated in the observed effect.

Studies using EP_4 knockout mice showed that this receptor is implicated in lipopolysaccharide-induced bone resorption *in vivo*[49] and in PGE_2-induced bone resorption in mouse calvaria in culture[50]. In the last model, EP_4 and EP_2 but not other EP agonists also stimulate bone resorption[51]. It is possible that at least part of these proresorptive effects of EP_4 activation in mice are due to an increase in osteoclastogenesis or stimulation of osteoblasts[52]. However, our results suggest that direct activation of the EP_4 receptor on mature human osteoclasts could have an inhibitory effect on bone resorption.

We demonstrated for the first time that authentic mature fetal human osteoclasts *ex vivo* present 2 subtypes of EP receptors, EP_3 and EP_4, a distribution that corresponds to that found *in vivo*. Activation of EP_3 receptors leads to an increase in the number of osteoclasts presenting lamellipodia, while activation of EP_4 receptors reduces the number of cells presenting actin rings. Since formation of an actin ring is a necessary step to bone resorption, our results suggest that the EP_4 receptor could be implicated directly in the inhibition of bone resorption by mature human osteoclasts.

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