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# Bone resorptive activity of osteoclast-like cells generated in vitro by PEG-induced macrophage fusion

Andrea Murillo[1], Carlos A Guerrero[1*], Orlando Acosta[1] and Carmen A Cardozo[2]

[1] Physiological Sciences Department, Universidad Nacional de Colombia, Bogota AA 14490, Colombia
[2] Institute of Biotechnology, Universidad Nacional de Colombia, Bogotá AA 14490, Colombia.

**ABSTRACT**

Normal bone remodeling is maintained by a balance between osteoclast and osteoblast activity, whereas defects in osteoclast activity affecting such balance result in metabolic bone disease. Macrophage-macrophage fusion leading to multinucleated osteoclasts being formed is still not well understood. Here we present PEG-induced fusion of macrophages from both U937/A and J774 cell lines and the induced differentiation and activation of osteoclast-like cells according to the expression of osteoclast markers such as tartrate resistant acid phosphatase (TRAP) and bone resorptive activity. PEG-induced macrophage fusion, during the non-confluent stage, significantly increased the osteoclastogenic activity of macrophages from cell lines compared to that of spontaneous cell fusion in the absence of PEG (polyethylene glycol). The results shown in this work provide evidence that cell fusion *per se* induces osteoclast-like activity. PEG-fused macrophage differential response to pretreatment with osteoclastogenic factors was also examined in terms of its ability to form TRAP positive multinucleated cells (TPMNC) and its resorptive activity on bovine cortical bone slices. Our work has also led to a relatively simple method regarding those previously reported involving cell co-cultures. Multinucleated osteoclast-like cells obtained by PEG-induced fusion of macrophages from cell lines could represent a suitable system for conducting biochemical studies related to basic macrophage fusion mechanisms, bone-resorption activity and the experimental search for bone disease therapeutic alternatives.

**Key terms:** bone resorption, macrophage fusion, osteoclast activity, osteoclast differentiation, PEG, RANKL.

## INTRODUCTION

Bone constantly undergoes turn-over resulting from bone-resorption by osteoclasts and the synthesis of bone matrix by osteoblasts (Boyle *et al*., 2003, Theill *et al*., 2000). Adult skeleton bone is continuously renewed by a number of stimuli making up the bone remodeling process (Boyce and Xing 2008, Yao *et al*., 2008). Osteoclast function is controlled by inflammatory cytokines, growth factors and hormones produced by supporting cells such as stromal cells and osteoblasts (Udagawa *et al*., 1990, Wada *et al*., 2006). Alteration of these factors can lead to an imbalance between osteoclast and osteoblast activities, which can cause bone abnormalities such as osteoporosis (Wada *et al*., 2006, Wagner and Matsuo, 2003). Characterizing factors implicated in controlling osteoclast function is central to understanding skeletal abnormalities; these would include the receptor for activator of NK-kB (RANK) (Anderson *et al*., 1997), its ligand RANKL (Anderson *et al*., 1997) and the decoy receptor for RANKL, osteoprotegerin (OPG) (Yasuda *et al*., 1998). RANKL and RANK play essential roles in regulating bone turnover through osteoclast activity (Anandarajah, 2009, Boyce and Xing 2008); such bone-resorbing activity degrades bone matrix, which is primary composed of collagen I extracellular matrix (ECM) embedded in a mineralized milieu (Jurdic *et al*., 2006). RANKL binding to its receptor triggers osteoclast development from hematopoietic progenitor cells and also activates mature osteoclasts. RANKL binding to RANK is negatively regulated by OPG, which causes inhibition of bone turnover by osteoclast activity (Wada *et al*., 2006).

Osteoclasts are large, specialized multinucleated cells formed by the proliferation, differentiation and fusion of precursor cells belonging to the monocyte/macrophage lineage (Boyle *et al*., 2003). Multinucleation is an essential step during osteoclast differentiation as mononucleated macrophages are unable to resorb bone efficiently. Osteoclast multinucleation is induced by the cell-cell fusion of mononucleated osteoclasts, thereby playing a critical role in controlling bone mass and osteoblastic activity

Correspondence to: *Carlos A. Guerrero, Telephone number: 57-1-3165000 EXT 15052, FAX: 57-1-3165000 EXT 15053
Email: caguerrerof@unal.edu.co

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(Iwasaki *et al*., 2008). Osteoclast resorption depends on the formation of the F-actin-rich structure called the sealing zone; however, the osteoclast actin cytoskeleton is organized *in vitro* into a different structure referred to as podosomes (Jurdic *et al*., 2006). RANKL, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF-α) and IL-1α stimulate osteoclast survival (Fuller K *et al*., 1993, Lacey *et al*., 2000, Lee *et al*., 2001, Lee *et al*., 2002), whereas transforming growth factor-β (TGF-β) and estrogens promote osteoclast apoptosis (Kameda *et al*., 1997, Murakami *et al*., 1998). In turn, RANKL expression can be stimulated by prostaglandin E2, dexamethasone, parathyroid hormone (PTH), IL-1 and TNF-α, or calcitriol [vitamin D, 1α,25-dihydroxyvitamin D3, 1,25-$(OH)_2D_3$], whereas TGF-β and strogens attenuate RANKL expression (Suda *et al*., 2003, Theill, 2002, Walsh and Choi 2003). In particular, calcitriol plays a key role in bone resorption by stimulating osteoclast formation and activity (Mee, 1996); activated macrophages have been found to synthesize vitamin D3 (Evans *et al*., 2006, Mawer *et al*., 1990). Vitamin D3 receptors have also been described on osteoblasts, osteoblast progenitors and macrophages (Freedman 1999, Morgan *et al*., 1996, Suda *et al*., 1999, Tetlow and Woolley, 1999). Osteocalcin, osteopontin, CYP24 and CYP1a-hydroxylase, D9K and D28K calbindin, integrin b3, fibronectin, c-fos, PTHrP and p21 genes are up-regulated by vitamin D3 (Jones *et al*., 1998); by contrast, PTH and IL-2 genes are down-regulated (Jones *et al*., 1998). PTH regulates resorption, inducing the production of factors in osteoblasts/stromal cells that are soluble or bound to the cell surface. These factors act on mature osteoclasts to induce their activity or on osteoclast progenitors to increase their proliferation (Lee and Lorenzo, 1999).

RANKL and M-CSF are required for inducing the expression of genes typifying osteoclast lineage, such as genes encoding tartrate resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CT) and integrin β3, thereby leading to the development of mature osteoclasts (Brockstedt *et al*., 1993, Nakchbandi *et al*., 2000). Bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) have been identified as having powerful anabolic effects on bone formation (Itoh *et al*., 2001). BMP2 is found among the BMPs and it increases osteoclast formation from macrophages derived from bone marrow (Bais *et al*., 2009, Itoh *et al*., 2001). Several osteoclast differentiation and activity inhibitors have been developed as candidates for treating bone abnormalities (Kwak *et al*., 2004, Murakami *et al*., 1997, Woo *et al*., 2000). Antiresorptive agents have been used for treating osteoporosis, alendronate being one of the most widely prescribed drugs for treating this bone disorder (Kamel, 2007). However, coupling bone resorption and formation has become a major cause of difficulties arising in therapeutic approaches (Fuller *et al*., 1993).

The experimental establishment of primary cultures or cell-lines from osteoclasts has been found to be difficult as these cells do not reproduce. Two approaches have been reported for obtaining osteoclast-like cells. The first (Vigenery, 2000) involves culturing macrophages in high confluence at pH 3, which induces spontaneous macrophage fusion. The other involves co-culturing osteoblasts and hematopoietic cells (Itoh *et al*., 2001), spleen cells and stromal cells (Liu *et al*., 2002), peripheral blood mononuclear cells and periodontal ligament cells (Kansaki *et al*., 2001), osteoclast precursor cells and the stromal cell-line (Kamolmatyakul *et al*., 2001), mouse osteoblasts and mouse bone marrow cells (stimulated with D3, PGE2) (Jimi *et al*., 1999), epithelial stellated reticule cells and dental follicle cells (Nakchbandi *et al*., 2000) and the human osteosarcoma line (SaOS-2) and hematopoietic cells (stimulated with PTH, dexamethasone) (Matsuzaki *et al*., 1999). The effects that cell confluence or stimulus with cytokines and/or hormones might have on osteoclast-like cell fusion and formation cannot be separated when using these approaches.

Cell fusion is an event in which the membranes of the cells being fused approach each other, come into contact and, after the lipids from their bilayers have become mixed, a fusion pore is then formed which expands, allowing contact between cytoplasms (Chernomordik *et al*., 1997, Chernomordik *et al*., 1998). However, the molecular mechanism of multinucleated osteoclast formation from macrophage fusion is not well understood, even though several glycoproteins have been involved in mediating both membrane attachment and fusion (Helming and Gordon, 2007). The energy barrier existing during the time when cells come together must be overcome for fusion to be carried out; this would require the expression of proteins having fusion activity (Vignery, 2000). Macrophage fusion can be induced by cytokines and growth factors among other stimuli, N-glycosylation being an important factor contributing towards efficient macrophage fusion (Helming and Gordon, 2007).

Cell fusion can be induced by the hydrophilic polymer, polyethylene glycol (PEG), which seems to favor close contact between membranes resulting from the dehydration produced on membrane surfaces (Lentz and Lee, 1999). Furthermore, binding of PEG-cholesterol derivatives to J774 cell plasma membrane has been reported to affect phagocytic intake by inducing a decrease in cell

surface hydrophobicity (Vertut-Doi et al., 1996). Our work explored the hypothesis that chemically fusing macrophage cell lines by PEG treatment, during the non-confluent stage, induces osteoclast differentiation as evidenced by the expression of osteoclast markers such as TRAP and bone resorption activity. PEG-fused macrophage response to pretreatment with osteoclastogenic factors was also examined in terms of their TRAP and resorption activities. A relatively simple method based on PEG-induced macrophage fusion was also used that could represent a suitable system for carrying out basic and applied studies related to bone metabolism. PEG has not been used to date for analyzing resorptive activity following macrophage fusion, this being the first report to our knowledge.

## MATERIALS AND METHODS

### *Reagents*

PTH, dexamethasone, RANKL and BMP-2 were purchased from SIGMA and calcitriol from ICN. PTH, RANKL and BMP-2 were each diluted in DMEM from SIGMA. Dexamethasone and vitamin D3 were diluted in SIGMA DMSO. DEMEM RPMI medium were supplemented with 10% (v/v) fetal bovine serum (FBS) from Bio Whitaker. Different molecular weights (1,450, 3,000, 6,000 or 8,000) of polyethylene glycol (PEG) were purchased from SIGMA. PEG was prepared at 50% (P/V) in PBS, prepared with SIGMA reagents. All remaining reagents were from SIGMA, unless otherwise stated. Bovine cortical bone slices (4 x 4 x 0.1 mm) were prepared as previously described (Collin-Osdoby *et al*., 2003, Salo 2002).

### *Cultures*

Adherent cells from human macrophage line U937/A (Puentes *et al*., 2000) and mouse macrophage line J774 (both being a gift from Fundacion Instituto de Inmunología de Colombia - FIDIC) were used in PEG-induced cell fusion and resorption activity assays. Both cell-lines were maintained in RPMI medium supplemented with 10% (v/v) (FBS) at 37°C in a 5% $CO_2$ atmosphere. Mouse fibroblast cell-line CD40L (a gift from the Instituto Nacional de Cancerología de Colombia) and mouse skin fibroblast primary cultures, obtained according to McCarthy *et al*. (McCarthy *et al*., 1988), were kept in DEMEM and used as control.

### *Immunohistochemistry*

Cells treated with PEG and cultured for 5 days were fixed in 4% paraformaldehyde (Carlo Erba Reagenti) in PBS for 40 minutes at 4°C and then stained with Mayer hematoxylin for five minutes to compare multinucleated and mononucleated cell morphology and to determine the number of nuclei. After two washes with PBS, cells were stained with eosin for 30 sec. Antibodies (2 mg/ml) against cathepsin K, carbonic anhydrase, pump ATPase, calcitonin and integrin $\alpha_V\beta3$ receptor (all from Santa Cruz Biotechnology) were used for identifying typical osteoclast markers. PEG-fused cells (100,000 cells) were cultivated for 5 days on coverslips and fixed in 4% paraformaldehyde for 1 hour before treatment with 0.1% Triton X-100 in PBS for 30 min. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Then cells were blocked with 1% albumin for 30 min and incubated with the primary antibody for 1 h at 37°C or overnight at 4°C. After three PBS washes, cells were incubated with the peroxidase-conjugated secondary antibody (1:500) (Santa Cruz Biotechnology) at room temperature for 1h. The reaction was developed with 3-amino-9-ethyl-carbazole (AEC) (Harlow 1988).

### *Cell fusion*

Around 5-6 x $10^6$ cells from 80%-confluent U937/A (Puentes *et al*., 2000) and J774 (TIB-67™) macrophage lines were collected to determine PEG-induced fusion stability. Cells were detached with 5 mM PBS-EDTA for 25 minutes at 37°C and spun at 600 *g* for 7 min. The supernatant was skimmed off and the excess was dried on sterile gauze so that no medium was left to prevent PEG dilution. Forty mL PEG (50% w/v), having a 1,450, 3,000, 6,000 or 8,000 molecular weight, was added to the cell pellet for 3, 5, 10, 12 or 15 min. PEG 3000 for 4 min was used for the CD40L mouse fibroblast line and fibroblasts obtained from primary culture. After incubation with PEG, 50 mL DMEM was added every 30 sec for 10 min and 100 mL every 30 sec for an additional 5 min. The fusion technique, medium volumes and times were proportionally determined according to those stated for hybridoma fusion (Burdon 1986). Once PEG had been diluted, cells were spun for 10 min at 600 *g*, the supernatant was discarded and cells were washed once with medium and suspended again in 150 ml RPMI medium containing 10% FBS to be seeded on sterile coverslips that had previously been placed in 6-well culture plates. Cells were left overnight to allow attachment to coverslips before 2 ml RPMI medium with FBS was added. Fusion stability was determined at different times during a 5-day period. After different times had elapsed, the cells were fixed in 4% paraformaldehyde to be stained with hematoxylin. Cells were fixed in cold

acetone/ethanol (50/50 v/v) to evaluate TRAP enzyme activity. Adherent and non-adherent PEG-fused multinucleated cells were counted using a Euromex inverted microscope at × 40 magnification. Osteoclast formation was followed by counting the number of TRAP positive multinucleated cells (TPMNC) adhering to coverslips in 6-well plates. The number of MNC or TPMNC cells was also compared with the total number of cells (TPMNC plus MNC) and expressed as a percentage. Mononucleated cells were counted by selecting fields at × 10 magnification and then increasing the magnification to × 40. Multinucleated cell morphology and the number of their nuclei were determined by optical microscopy as described above. Microscopic determination was carried out in all cases by selecting representative and comparable fields on coverslips.

*Preparing and co-culturing mouse bone-marrow cells*

Mouse bone-marrow derived cells were obtained according to (Reddy *et al*., 2001). Briefly, bone marrow was obtained from aseptically removed mouse (ICR strain) tibiae by flushing with 1 ml DMEM using a tuberculin syringe fitted with a 27-gauge needle. The bone marrow-derived cells were washed twice, suspended in DMEM-10% FBS and depleted of stromal cells adherent to plastic by incubating the bone marrow cell suspension in sterile 10 cm tissue culture dishes for 2 h. Non-adherent bone marrow cells ($10^5$/ml) were plated on 35-mm tissue culture dishes in DMEM supplemented with 20% FBS and 100 pg/ml GM-CSF. The cells remaining on the bottom of the dishes were considered to be bone marrow-derived macrophages (BMM). The cultures were incubated at 37°C in a 5% $CO_2$-air atmosphere for 7 days. *Colony forming unit-granulocyte macrophage* (*CFU*-GM) (>40 cells) numbers were scored using a microscope. The BMM (2.0 x $10^6$ cells/ml) were then plated in 48-well plates in DMEM-10% FBS supplemented with 0.01 $\mu$M 1,25-$(OH)_2$ $D_3$ for 7 days. These cells (2.0 x $10^4$ cells/well) were placed on bovine cortical bone slices in 24-well plates and co-cultured with mouse erythrocyte-free spleen cells (5.0 x $10^4$ cells/well), prepared according to (Yamagishi *et al*., 2001). Cells were co-cultured for 7 days at 37°C in a humid 5% $CO_2$ - air atmosphere and the osteoclast-like cells so formed were then assayed for TRAP and bone resorption activity.

*TRAP and bone resorption pit assays*

TRAP activity histochemistry was carried out as previously described (Vanderkenken *et al*., 2005). Briefly, cells adhering to coverslips or bone slices were fixed with a 0°C pre-chilled ethanol/acetone (50:50 v/v) mixture for 40 minutes at 4°C. Cells were then placed in a solution containing hexazotized pararosaniline, sodium nitrite, sodium tartrate (13 mM) and naphtol AS-B1 phosphate in veronal acetate buffer at pH 5.2. Cells were left for 3 hours at 37°C, washed in PBS and TRAP-positive fused cells (those containing more than 3 nuclei) were identified by their typical reddish color. Cells were then counted under a light microscope at × 40 magnification. Osteoclast-like cells or PEG-fused U937/A cells were seeded on 0.5 mm thick UV-sterilized bovine cortical femur bone slices that had been placed on 24-well plates for assaying their bone-resorbing activity. Non PEG-fused cell TRAP and resorption activities were evaluated as control. The number of PEG-fused TPMNC and mononucleated cells present on the bone slices were counted. Cells were then completely removed from the bone slices with lysis buffer (RIPA: 150 mM NaCl, 1% Nonidet-P40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8) for ten minutes together with mechanical detachment using a soft-bristled brush. Bone slices were stained with Coomassie blue for 20-30 minutes, left to dry and then stained with hematoxylin for 5 minutes, followed by two washes with PBS. The resorption pits were counted using an optical microscope at × 40 magnification (VanGuard) and the total surface area of the resorption pits was calculated by selecting 10 representative fields.

*Hormone and cytokine treatment*

The effect of osteoclastogenic hormones and cytokines on TRAP activity exhibited by PEG-fused macrophage cells was determined in U937/A cells seeded on coverslips. The cell inoculum density was adjusted to obtain an 80% confluent culture after 5 days of seeding. Cells were treated with PTH (0.01 mM), 1a,25$(OH)_2D_3$ (0.0025 mM) or PTH plus 1a,25$(OH)_2D_3$ during this time (at 0.01 and 0.0025 mM, respectively). Cells were then fixed and stained for TRAP activity. Cells cultured in the same conditions, but without hormone or cytokine stimulation, were used as control. Alternatively, cells cultured and stimulated in the conditions described above were detached with PBS-EDTA 5 mM at the end of the 5-day treatment and fused using PEG 6000 (50%) for 12 min. Cells were then seeded on coverslips and left in incubation for 48 hours. Cells were seeded on coverslips as controls without being PEG-fused but stimulated with hormones or cytokines, as indicated above. Cells were fixed at the end of the incubation time and stained for TRAP activity. The number of adhered TPMNC and mononucleated

cells and that of cells found in supernatant (dead multinucleated and mononucleated cells) were counted in all cases.

PEG-fused cell TRAP and bone resorptive activity were analyzed on bone slices in the presence of PTH (0.0025, 0.005 and 0.01 mM), vitamin D3 (0.0025, 0.005 and 0.01 mM), dexamethasone (0.0025, 0.005 and 0.01 mM), RANKL (10, 20, 40 and 80 ng/ml) or BMP2 (10, 20, 50 and 100 ng/ml). Cells in logarithmic growth phase were treated for 4 days in Falcon plastic culture flasks with the above concentrations; PEG-6000-induced fusion was then carried out as described above. Cells were seeded on cortical bone slices and cultured for 5 days. Control cells were seeded without being stimulated and PEG-fused; a control was also used, consisting of PEG-fused cells without previous hormone or cytokine treatment. Pre-stimulated cells were left without being PEG-fused for each concentration of hormone or cytokine tested, in an attempt to distinguish the effects of hormone or cytokine treatment from those of PEG. The number of TPMNC/mm$^2$ adhering to bone slices was determined by light microscopy as indicated above. Bone resorption pit formation was evaluated for each treatment, as described above.

*Statistical analysis*

Data was expressed as the mean ± standard error from at least three independent experiments for each experimental condition. The differences between groups were analyzed using Duncan's method. The SAS GLM repeat measurement procedure was used for determining whether the number of TPMNC that became fused while using PEG was related to the other variables (time and PEG molecular weight). A 95% significance level was used (*alpha* = 0.05).

## RESULTS

*PEG 6000 and 3000 were efficient agents for fusing U937/A and J774 cells, respectively*

To determine PEG cell fusion ability and its efficiency, 80% confluent U937/A cells were fused using PEG (MW:1,450, 3,000, 6,000 and 8,000) for 3, 5, 10, 12 and 15 min. Cells were then seeded on coverslips, fixed after 48-hour incubation and stained to determine TRAP activity. Cells seeded on coverslips without being PEG-treated were used as a control for these experiments. PEG treatment for J774 cells was carried out for 2, 4, 6, 8 and 10 min. The number of adherent TPMNC and MNC and cells found in supernatant (i.e. dead fused and non-fused cells) was counted to evaluate the effect of PEG.

PEG treatment of U937/A cells increased TPMNC percentages at all times tested compared to that of control cells. However, major average deviation was found in the percentage of TPMNC for all PEG treatments (data not shown), except for the PEG-6000 treatment, which induced the greatest formation (7%-9%) of PEG-fused TPMNC after 12-15 min treatment, whereas TPMNC in non-PEG-treated control cells was lower than 1% for all times tested (Fig. 1A). The percentage of detached multinucleated cells ranged from 1% to 2.5% for all PEG molecular weights tested (data not shown). The PEG inducing the greatest J774 cell fusion efficiency (about 5%) was that having a 3,000 molecular weight when it was used for 4-6 min; however, high TPMNC percentage deviation was evident (Fig. 1B). Average TPMNC percentage in control cells lacking PEG treatment was less than 2% and 1%-4% fluctuations were observed for detached multinucleated cells at all PEG molecular weights assayed (data not shown).

*Osteoclast-like cells were formed by PEG treatment*

PEG-fused U937/A cells were fixed using paraformaldehyde and incubated with specific antibodies against cathepsin K, carbonic anhydrase, ATPase pump and calcitonin and integrin $\alpha_V\beta3$ receptor to test for osteoclast-specific antigens. All these antigens reacted positively, suggesting that these osteoclast-specific proteins are present in cells fused using PEG (data not shown). Figure 2A shows PEG-fused U937/A macrophages that were cultured on coverslips for 48 h and then stained with haematoxylin-eosin after staining for TRAP activity (indicated by arrows). Optical microscopy revealed multinucleated cells having a well-defined membrane containing the nuclei within them. Figure 2B shows PEG-fused U937/A cells that were treated with a hypotonic solution and stained with Giemsa (Verma and Babu, 1989); nuclei were visible within a single cell membrane in these cells. TPMNC from PEG-fused macrophages were observed on bovine cortical bone slices (Fig. 2D) but not on those in which non PEG-fused U937/A macrophages were cultured as control (Fig. 2C). The resorption activity of PEG-fused macrophages cultured on bovine cortical bone slices was observed in the electron microscope as areas of resorption pits (Fig. 2F,G), which were absent in bone slices on which no U937/A cells were seeded (Fig. 2E).

*TRAP activity was stably expressed in PEG-fused U937/A cells*

A kinetic assay was conducted to determine the time when PEG-fused cells from 80%-confluent

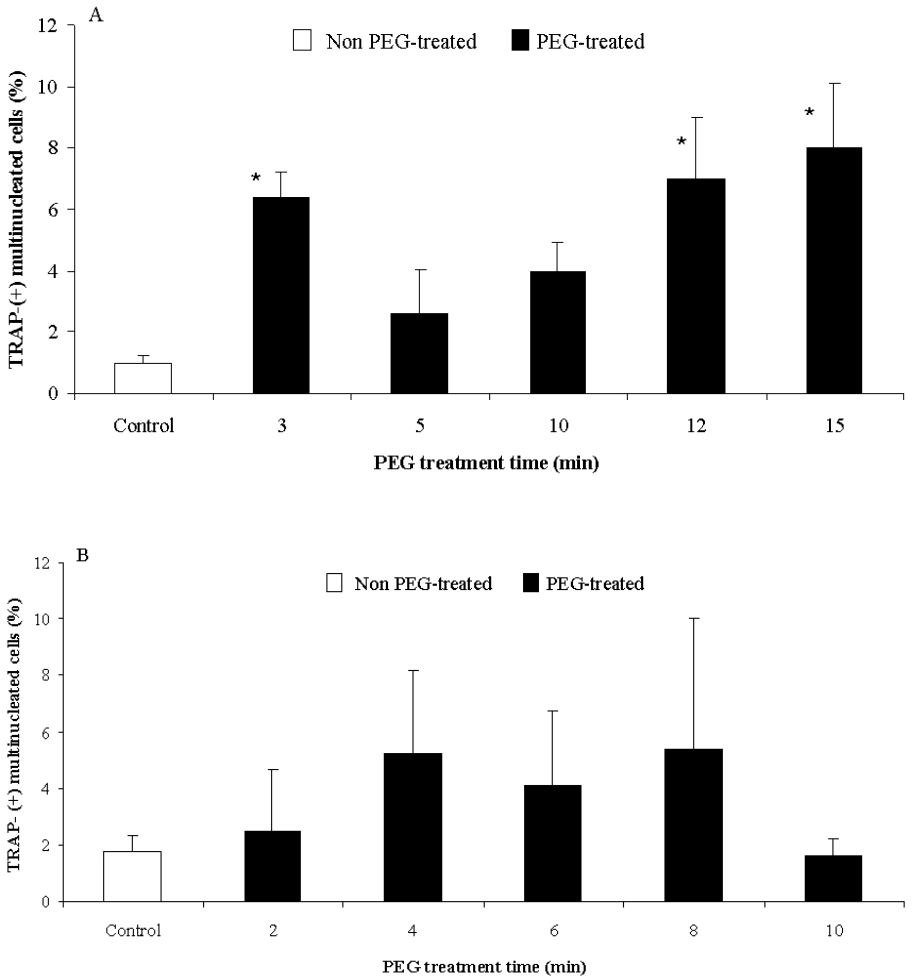


**Fig. 1** Fusion efficiency of U937/A and J774 cell lines treated with PEG. U937/A (**a**) and J774 (**b**) cells at 80% confluence were detached and treated with PEG 6000 for 3, 5, 10, 12 and 15 min, and with PEG 3000 for 2, 4, 6, 8 and 10 min, respectively. The proportion (%) of attached TPMNC was determined at 48 hours post-fusion on coverslips and expressed as percentage of the total population of cells (TPMNC plus MNC). The averaged proportion (%) of TPMC for cells without PEG-treatment over all times assayed was used as a control. Results are presented as mean ± SEM (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

macrophage line U937/A showed TRAP activity. Once fused U937/A cells had adhered, they were harvested and then fixed at times ranging from 2 h to 5 days. TRAP activity was already present in adhered multinucleated cells 2 h post-fusion; the number of these TPMNC remained nearly constant during the first 24 h of culture. However, the number of TPMNC seemed to show a relative increase between 48 and 72 h, returning to their previous values by day 4 and 5 (Fig. 3A). The TPMNC number for non-PEG treated control cells essentially remained constant throughout the times being studied. Given that the number of TPMNC for PEG-treated cells remained essentially unchanged 2-24 h post-PEG fusion, but showed a slight increase 48-72 h, this number was determined at 48 h post-fusion in subsequent experiments when complete and stable cell adhesion also occurred. PEG was not toxic for cells after fusion had occurred since the remaining non-fused mononucleated cells (MNC) continued to increase in number (mainly after 8 h culture) (Fig. 3B). The number of MNC for non-PEG treated control cells showed a similar growth profile to that of PEG-treated cells (data not shown). Taking mean MNC increase 48 h post-fusion for both PEG- and non-PEG-treated cells into account then the ratio of the number of TPMNC/number of TPMNC plus the number of MNC was corrected by a factor of 10 before being expressed as a percentage.

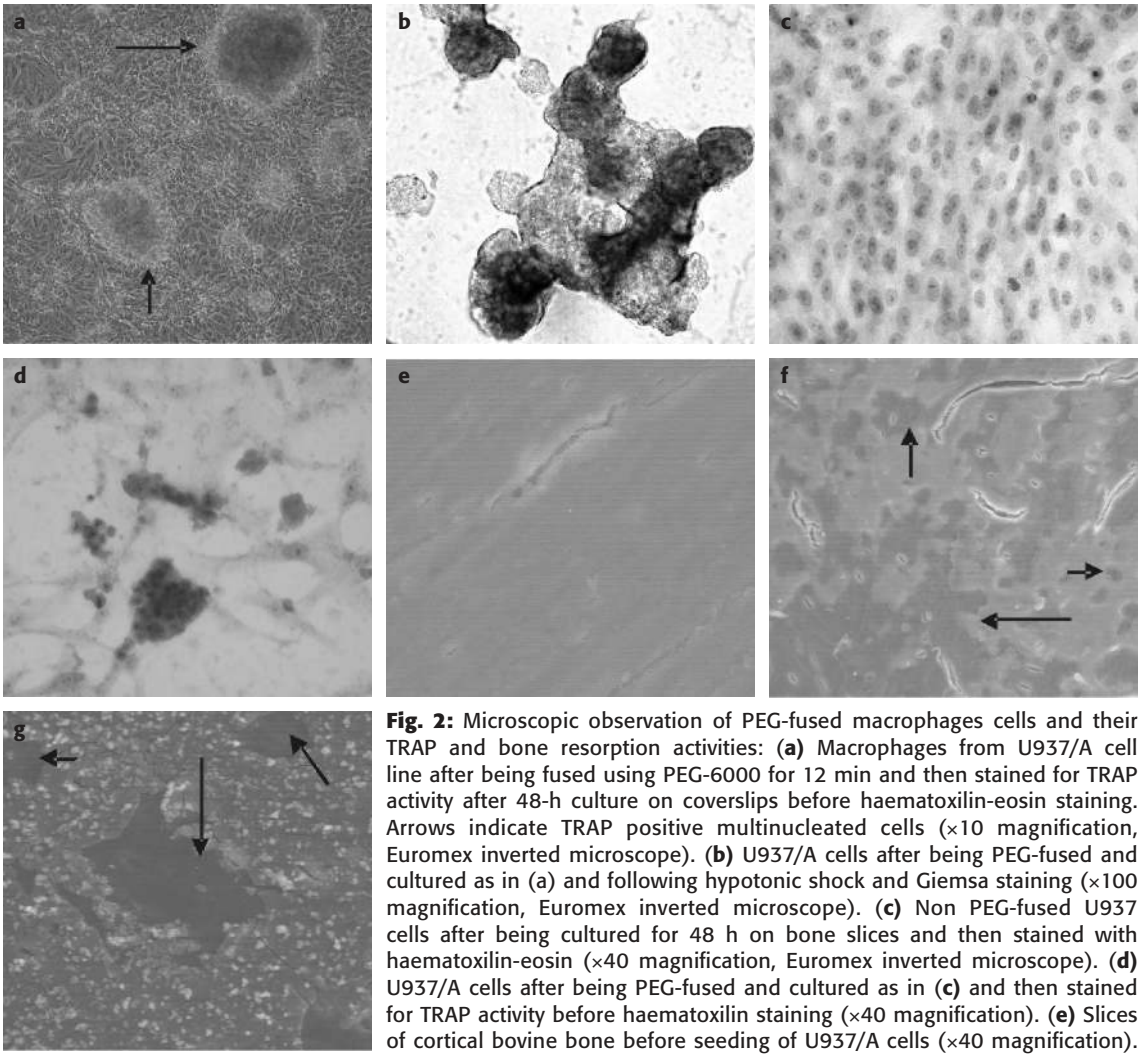


**Fig. 2:** Microscopic observation of PEG-fused macrophages cells and their TRAP and bone resorption activities: (**a)** Macrophages from U937/A cell line after being fused using PEG-6000 for 12 min and then stained for TRAP activity after 48-h culture on coverslips before haematoxilin-eosin staining. Arrows indicate TRAP positive multinucleated cells (×10 magnification, Euromex inverted microscope). (**b)** U937/A cells after being PEG-fused and cultured as in (a) and following hypotonic shock and Giemsa staining (×100 magnification, Euromex inverted microscope). (**c)** Non PEG-fused U937 cells after being cultured for 48 h on bone slices and then stained with haematoxilin-eosin (×40 magnification, Euromex inverted microscope). (**d)** U937/A cells after being PEG-fused and cultured as in (**c)** and then stained for TRAP activity before haematoxilin staining (×40 magnification). (**e)** Slices of cortical bovine bone before seeding of U937/A cells (×40 magnification). (f) (microscope x40 magnification) and (g) (Quanta 200 Phillips FEI Electron Microcope 300x magnification) Slices of cortical bovine bone on which U937/A were cultured and then detached before microscope observation. Resorption pit electron indicated by arrows.

*Cells having lineages other than the monocyte/macrophage line fused using PEG but were TRAP negative*

It was also determined whether PEG-induced fusion of cells from non-monocyte/macrophage lineage was able to induce TRAP activity. This activity was compared to that of PEG-fused cells from 80% confluent U937/A and J774 cell lines; CD40L line mouse fibroblasts and mouse skin fibroblast primary cultures were used for this comparison. These cells formed more than 3% multinucleated cells after PEG treatment (data not shown). However, essentially none of these multinucleated cells (0.015%-0.03%) were TRAP positive (Fig. 4). By contrast, PEG-fused TPMNC percentages for U937/A and J774 cell lines were found to be 7% and 4.1%, respectively. These results suggest that TRAP activity present in multinucleated cells following PEG-induced fusion was specific for cells having monocyte/macrophage lineage.

*The number of TPMN increased in macrophages cultured at high confluence*

U937/A cells were fused by treatment with PEG-6000 for 12 min at different cell confluences (20%, 40%, 60%, 80% and 100%) to determine the effect of culture cell confluence on TPMNC percentages. Once this time had elapsed, cells were seeded on coverslips; they were fixed after 48-hour incubation

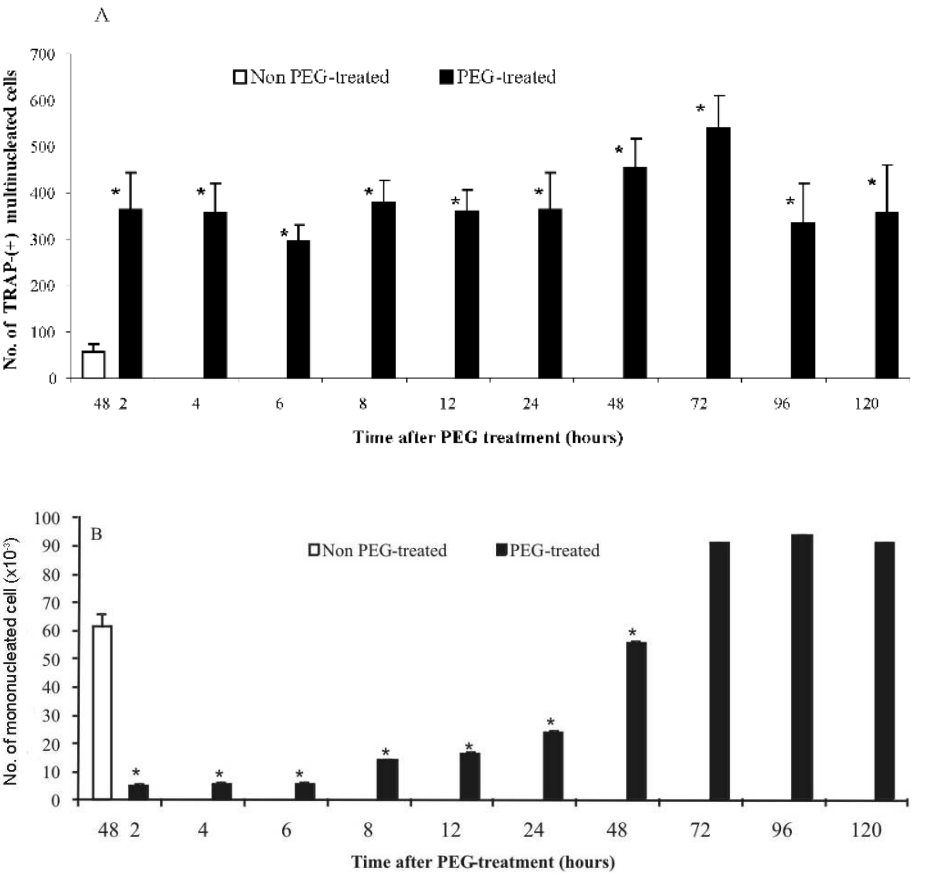


**Fig. 3** Accumulation of TRAP positive multinucleated cells and mononucleated cells. PEG 6000-fused U937/A cells were seeded on coverslips and then fixed and stained for TRAP activity after being cultured for 2 hours to 5 days. The number of TRAP positive multinucleated cells (**a**) and mononucleated cells (**b**) was determined under a light microscope by selecting representative and comparable fields on coverslips. The average number of TPMNC for non-PEG treated cells over all times assayed was used as a control (**a**). Values for TRAP positive multinucleated cells are shown as mean ± SEM (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

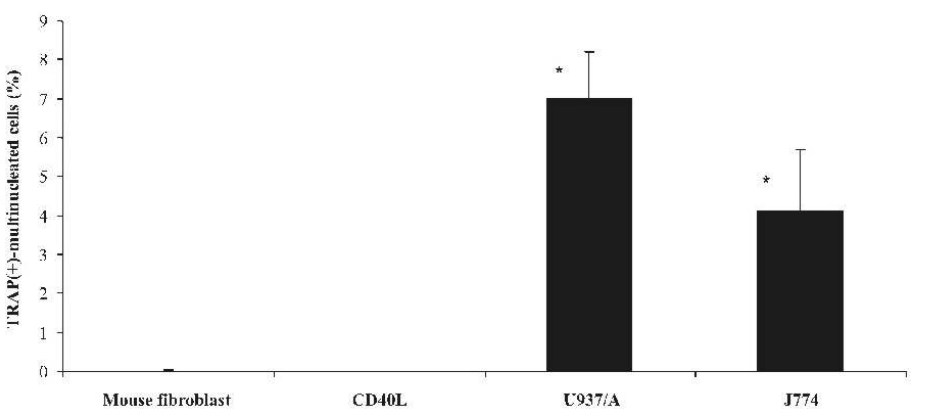


**Fig. 4** Forming TPMNC in different cell types. Mouse fibroblasts, CD40L fibroblasts, and U937/A and J774 cells at 80% confluence was fused by PEG-6000 treatment for 12 min, seeded on coverslips and then their TRAP activity in multinucleated cells at 48-h post-PEG induced fusion. TPMNC are expressed as percentage of total cell population. Histograms show mean ± S.E.M (n=3) (*P-value = 0.0225 < alpha = 0.05). The asterisk indicates significant differences from controls.

and stained for determining TRAP activity. Cells at 100% confluence, but lacking PEG treatment, were used as control; this was done for quantifying spontaneous fusion due to high cell confluence, leading to a very low TPMNC percentage (0.03%) being observed. No significant differences for PEG-treated cells were observed (Duncan test) due to 20%-80% confluence when comparing results in terms of TPMNC percentage (Fig. 5), whereas the TPMNC percentage increased almost 5 times, giving 100% confluence regarding that for 20-80% confluence. Based on these results, subsequent experiments were done at 80% confluence to separate the effect which 100% confluence might produce on cell fusion. The net effect of PEG on TPMNC formation could thus be determined.

*PTH treatment increased TPMNC percentage, whereas calcitriol had no effect*

It has been reported that PTH and vitamin D3 induce monocyte/macrophage differentiation into osteoclasts when they are added to osteoblast and bone marrow cell co-cultures (Reddy *et al*., 2001, Yamagishi *et al*., 2001). However, there are no studies regarding PTH or vitamin D3 activity in the U937/A cell line in terms of their effect on TRAP activity. On the other hand, it has been reported that PTH can enter already differentiated macrophages and be excreted from them (Diment *et al*., 1989). In the present study we wanted to address the question of whether PTH and vitamin D3 were affecting TRAP activity and fusion efficiency when applied to the U937/A tumor cell line before PEG-induced fusion.

The concentrations described in Materials and Methods were used for determining the effect of PTH and vitamin D3 treatment on fusion and TRAP activity. After a 5-day treatment on coverslips, cells were fixed and stained to determine TRAP activity and TPMNC percentage. Non PEG-fused U937/A cells (but PTH and/or calcitriol treated) showed no significant differences in TPMNC percentage when compared to non-fused cells lacking hormone treatment ($p=0.2355 > 0.05$) (Fig. 6). By contrast, U937/A cells stimulated for 5 days with PTH or vitamin D3 or with PTH plus vitamin D3 before PEG-induced fusion, showed increased TPMNC percentage regarding non-fused control cells lacking hormone treatment (Fig. 6). Furthermore, PTH pre-treatment of cells before PEG-induced fusion resulted in a 7-fold increase in TPMNC percentage compared to PEG-fused cells lacking PTH pre-treatment, whereas vitamin D3 or PTH plus vitamin D3 treatment produced only a slightly non-significant 1.2- and 1.8-fold increase in TPMNC percentage, respectively, compared to PEG-fused control cells without hormone treatment.

*PEG-fused macrophages had more resorptive activity than PEG-fused fibroblasts*

U937/A cells were fused using PEG to determine whether PEG-fused cells showed osteoclast-like activity; they were seeded on slices from bovine cortical bone and kept in culture for 1 to 5 days. Once this time had elapsed, they were stained for TRAP activity; cells were detached and resorption pit surface area was quantified (see Materials and Methods). The number of TPMNC/mm$^2$ (20 cells/mm$^2$) for PEG-fused U937/A cells determined on bone slices was significantly different from that

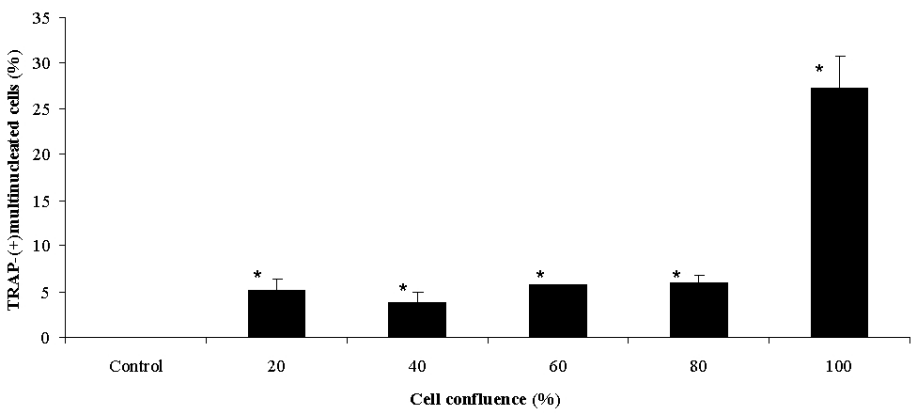


**Fig. 5** Fusion efficiency of U937/A and J774 cells treated with PEG at different degrees of cell confluence. U937/A cells were PEG-6000-fused for 12 min at different cell confluences (20% to 100%) and seeded on coverslips. Cells at 100% confluence and without PEG-treatment were used as a control. The proportion of TPMNC was expressed as percentage of the total cell population. Histograms show mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

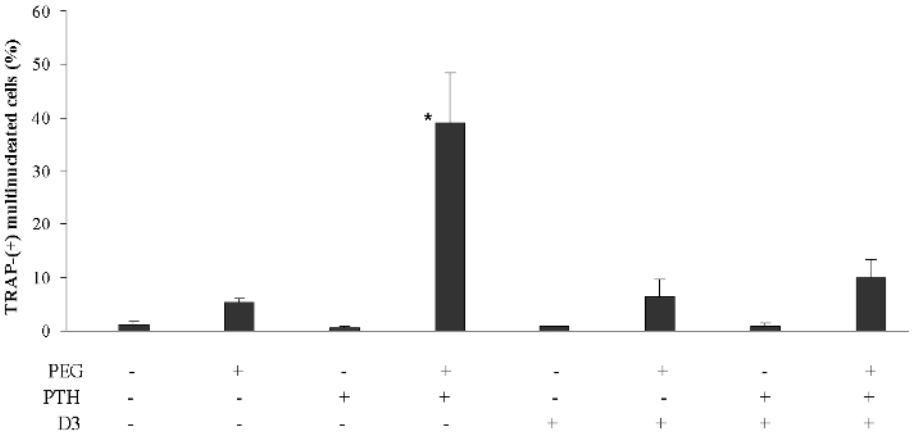


**Fig. 6** Effect of PTH and vitamin D3 treatment on forming TPMNC. U937/A cells were treated with PTH (0.01 mM), calcitriol (0.0025 mM) or PTH plus calcitriol (at 0.01 and 0.0025 mM, respectively) for 5 days. After this, cells were fixed and stained for TRAP activity. Cells cultured in the same conditions, but without hormone stimulation, were used as a control. Alternatively, cells cultured and hormone-treated as described above were PEG-fused after 5-day culture. PEG-fused cells without hormone pre-treatment were used as a control. The proportion of TPMNC on coverslips at 48 h post-fusion was expressed as percentage of the total cell population. Results are presented as the mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

observed for non-PEG-fused control cells (1 cells/mm$^2$ averaged over all times assayed), even though no significant differences were found from days 1 to 5 for PEG-fused cells (Fig. 7A) (p-value=0.8742 > *alpha* =0.05). Similarly, the number of TPMNC/mm$^2$ for PEG-fused J774 cells increased from day 5 to 15 compared to that of control cells (1 cells/mm$^2$), showing values of 2, 9 and 17 TPMNC/mm$^2$ on day 5, 10 and 15, respectively (Fig. 7C) (p-value=0.0015 < *alpha* =0.05).

Total resorption pit area produced by PEG-fused U937/A cells increased about 2.2 times (0.83 mm$^2$) on day 5 compared to the total area recorded on days 1 to 4 (0.37 mm$^2$) (Fig. 7B) (p-value=0.0761 > *alpha* =0.05), whereas non-PEG-fused control cells produced a 0.052 mm$^2$ total resorption area when averaged over all the times assayed. No statistically significant differences were found for PEG-fused J774 cells between the total resorption pit area quantified on day 5 (0.82 mm2) and day 10 (0.97 mm$^2$), whereas a significantly reduced resorption pit area was observed (0.17 mm$^2$) on day 15 (Fig. 7D). PEG treatment significantly increased fused cell resorption activity for all assayed times regarding non-PEG-fused control cells (0.052 mm$^2$).

Mouse skin fibroblasts were PEG-fused for testing whether resorptive activity was specific to the U937/A and J774 macrophage cell-lines. Once cells had been PEG-fused, they were seeded on slices from bovine cortical bone and kept in culture for 5 days, fixed, stained for TRAP activity, detached and the resorption pit area was quantified. Non PEG-fused cells were used as control in all cases. It was observed that the U937/A cells showed about 15 times more PEG-fused TPMNC/mm$^2$ than the J774 cell line and 30 times more compared to mouse fibroblasts (Fig. 8A); there was nearly double the number of TPMNC/mm$^2$ for PEG-fused J774 cells compared to fibroblasts. On the other hand, considering resorptive activity for any of the three type of cells tested, PEG-fused cells presented greater resorptive activity compared to their respective controls (non-PEG fused cells) when comparison was made in terms of total resorption pit area (Fig. 8B). Both PEG-fused cell lines had similar resorptive activity when comparing U937/A line resorptive activity to that of the J774 line, but both cell lines had about 4.7 times greater activity compared to that of PEG-fused fibroblasts.

Osteoclast-like cell differentiation was carried out by co-culturing spleen cells and bone marrow-derived macrophages as described in Materials and Methods to compare the resorptive activity of the PEG-fused cells obtained in our model with those mentioned in the literature. It was observed that the number of TPMNC/mm$^2$ for osteoclast-like cells was nearly similar to that of PEG-fused U937/A cells, whereas resorption pits produced by osteoclast-like cells were remarkably deeper than those of PEG-fused U937/A cells; however, total resorption pit areas were indistinguishable for both types of cells (data not shown).

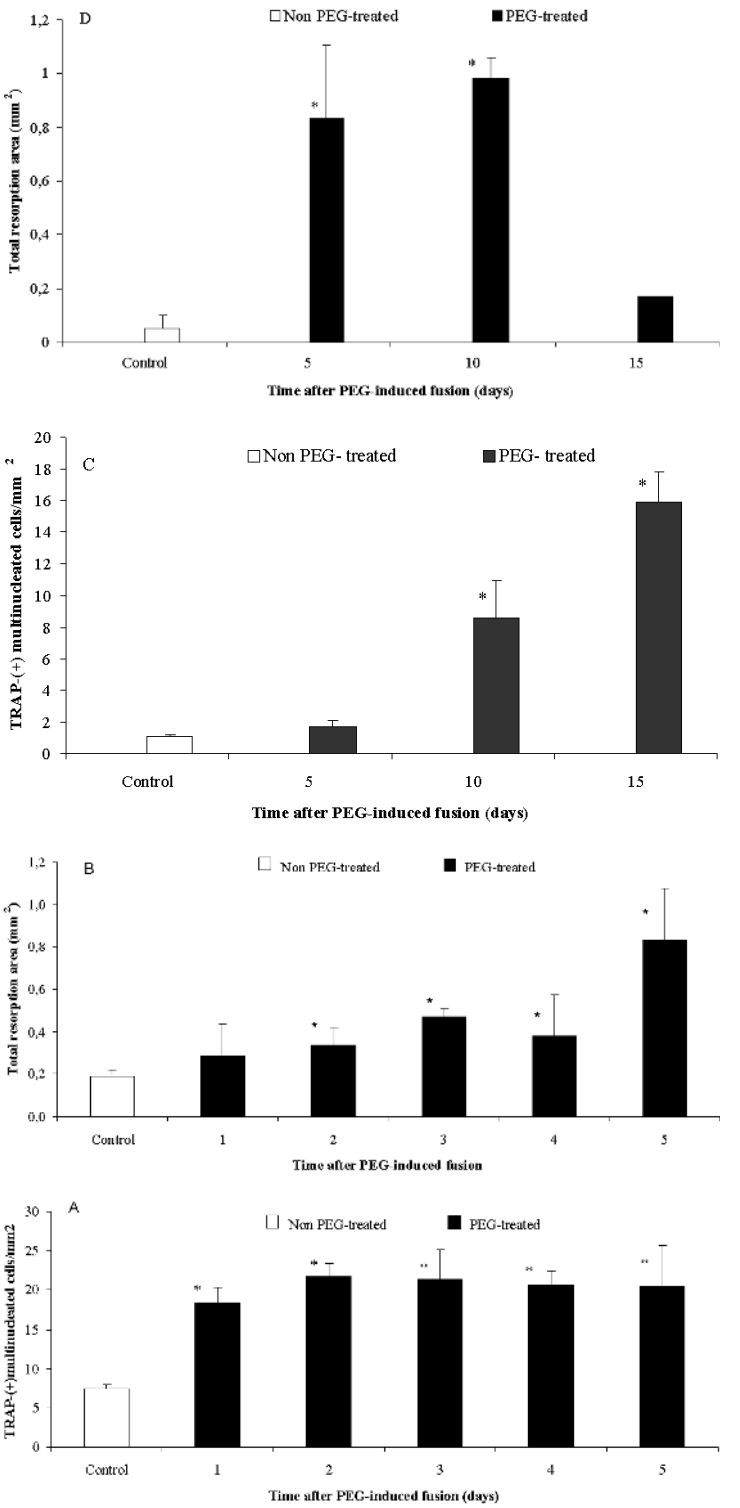


**Fig. 7** Bone resorptive activity of PEG-fused U937/A and J774 cells. PEG-fused U937/A (**a**, **b**) and J774 (**c**, **d**) cells were seeded on slices from bovine cortical bone and the number of TPMNC/mm$^2$ determined through 5 (**a**) and 15 (**c**) days. Cells were removed from bone slices with lysis buffer and the total stained surface area (mm$^2$) of the resorption pits was determined for U937/A (**b**) and J774 (**d**) cells using an optical microscope at ×40 magnification. The average number of TPMNC/mm$^2$ and the average resorption pit area (mm$^2$) over all times tested were used as a control. Results are presented as mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

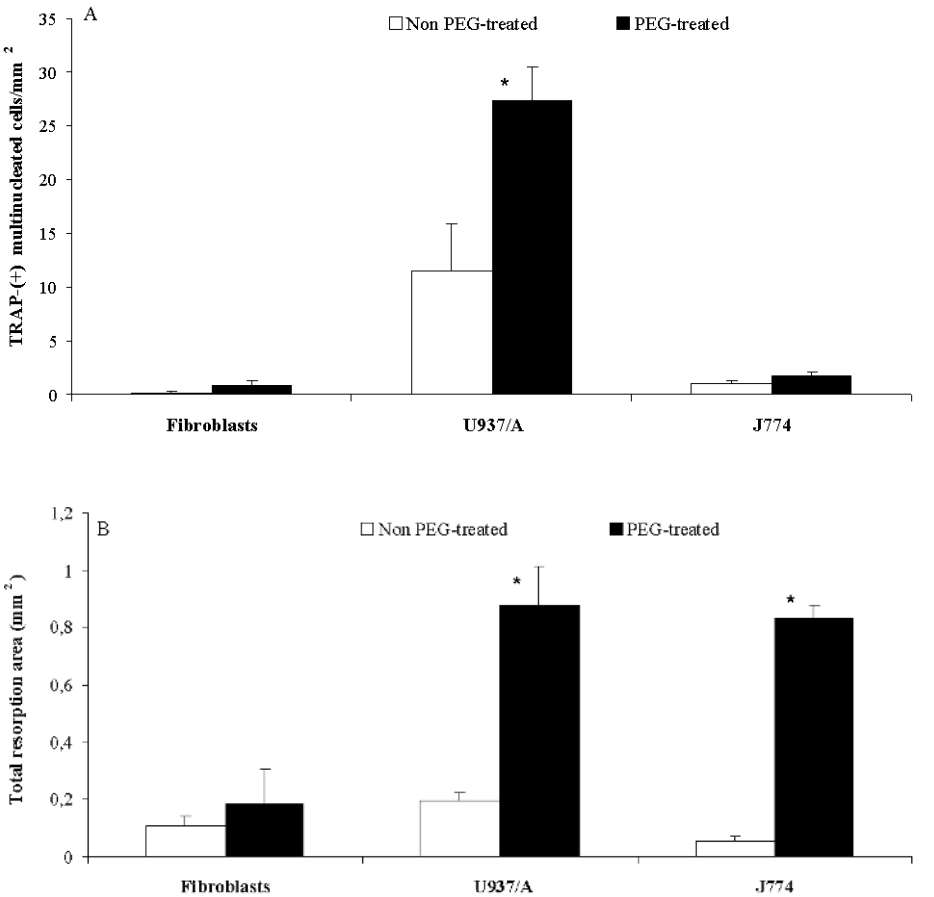


**Fig. 8** Bone resorptive activity of PEG-fused macrophages and PEG-fused fibroblasts. U937/A, J774 and mouse fibroblast cells were seeded on bovine cortical bone slices after being fused by PEG-6000 treatment. After 5-day culture, cells were stained for TRAP activity (number of TPMNC/mm2) (**a**), detached and the total resorption pit area ($mm^2$) (**b**) measured. Non PEG-fused cells were used as control for the respective cell type. Values are shown as mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

*PTH treatment increased PEG-fused cell resorptive activity whereas calcitriol and dexamethasone reduced such activity*

To our knowledge, no studies have been made about the dexamethasone effect on the U937/A tumor cell line; however, this it was required for human osteoclast-like multinucleated cell formation in whole blood mononucleated cell cultures (Matsuzaki *et al*., 1999). U937/A macrophages were thus stimulated with PTH, vitamin D3 or dexamethasone for 4 days to determine whether these hormones had any effect on TPMNC formation and their resorptive ability (see Materials and Methods). Following the 4-day treatment, cells were fused by PEG treatment, seeded on bovine cortical bone slices, cultured for 5 days, fixed and stained for TRAP activity. PEG-fused TPMNC/$mm^2$ were counted and detached according to the procedure described in Materials and Methods. Once cells had been removed, the bone slices were stained and resorption pit areas were quantified by optical microscopy.

When the number of TPMNC/$mm^2$ for PEG-fused control cells without being hormone-treated was compared to that of the PEG-fused and hormone-treated cells, it was found that, except for 0.005 mM $1,25(OH)_2D_3$-treatment, any hormone concentration used did not lead to a higher number of TPMNC (Fig. 9A). Comparison between non PEG-fused control cells without hormone treatment and hormone-treated cells without PEG-induced fusion showed a dramatic decrease in the TPMNC number for all hormone treatments. Moreover, pre-treatment with PTH, calcitriol or dexamethasone before PEG-induced fusion reduced the number of TPMNC/$mm^2$ compared to that of PEG-fused cells that had not been stimulated (Fig. 9A) (p-value = 0.0001 < *alpha* = 0.05 between treatments). However, a greater number of PEG-fused TPMNC/$mm^2$ was observed

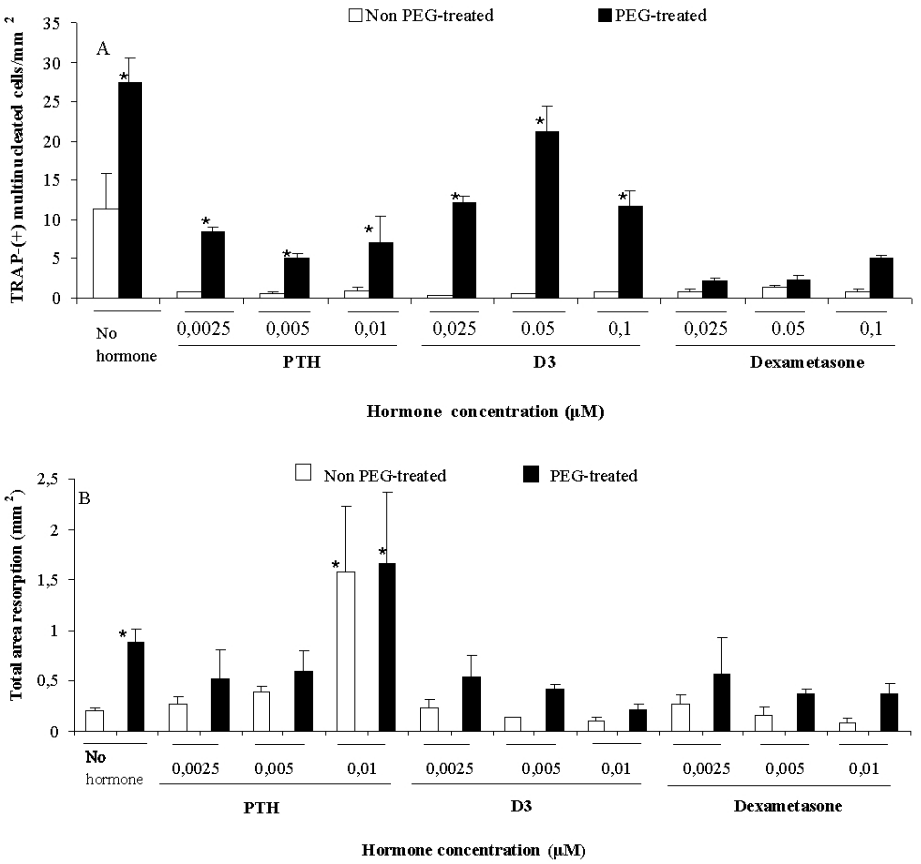


**Fig. 9** Effect of hormones on the bone resorptive activity of TPMNC. U937/A cells were stimulated for 4 days with PTH (0.0025, 0.005 and 0.01 mM), vitamin D3 (0.0025, 0.005 and 0.01 mM) and dexamethasone (0.0025, 0.005 and 0.01 mM), fused by PEG-6000 treatment and seeded on bovine cortical bone slices. After 5-day culture on bone slices, TPMNC/mm$^2$ (**a**) were counted, detached and bone slices stained for total resorption pit area (mm$^2$) (**b**). Cells without being stimulated and PEG-fused, and PEG-fused cells without previous hormone treatment were used as a control. Values are presented as mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant difference from controls.

with a 0.005 mM vitamin D3 dose when compared to the effect of doses used for the remaining hormone pre-treatments (p-value = 0.0001 < *alpha* = 0.05 between treatments). On the other hand, the cells which were not hormone-stimulated but PEG-fused showed 4 times more TPMNC/mm$^2$ compared to the average for PEG-fused cells which had been pre-stimulated with PTH. Similarly, pre-stimulating cells with dexamethasone decreased the number of TPMNC/mm$^2$ 9.3 times regarding cells which had not been stimulated but had been PEG-fused.

It was found in all cases that fusing cells by PEG treatment after hormone pre-stimulation led to a slightly greater resorption pit area than that of the respective cells which had been pre-stimulated but not PEG-fused, except for cells treated with PTH (0.01 mM) which exhibited similar resorption activity whether PEG-fused or not. It was also observed that the averaged resorption activity of these cells pre-treated with PTH (0.01 mM) was almost 2 times greater than that shown by control cells which had been PEG-fused without pre-stimulation (Fig. 9B). By contrast, when cells pre-treated with different doses of vitamin D3 or dexamethasone and then PEG-fused were compared to those which had been PEG-fused without any pre-treatment it was observed that the resorptive activity was roughly reduced with increasing hormone concentrations. A direct correlation between the number of TPMNC/mm$^2$ and the resorption pit area was not always found.

*RANKL and BMP2 did not boost PEG-fused cells' resorptive activity*

A large number of reports have supported the role of RANKL and BMP2 in inducing osteoclast differentiation from monocytes/macrophages when

these cytokines are added to osteoblast and bone marrow-derived cell co-cultures. Nevertheless, RANKL-treated macrophages enhanced their survival and showed increased allogeneic T-cell activation and phagocytic activity [60]. Osteoclast differentiation can also be induced from BMM preparations by treating them with GM-CSF and RANKL (Kwak *et al*., 2004). Members of the BMP family have been shown to have anti-proliferative effects on their target cells (Amedee *et al*., 1994, Blessing *et al*., 1996). It is not known if RANKL or BMP2 modify TRAP activity or resorption activity when applied to the U937/A tumor cell line. U937/A macrophages were thus treated with both cytokines before PEG-induced fusion to determine whether RANKL and BMP2 cytokines might have any effect on such activity.

U937/A macrophages were stimulated for 4 days with RANKL and BMP2 (as described in Materials and methods) and then fused by PEG treatment. Pre-stimulated but non-PEG-fused cells were used as control for each cytokine concentration assayed. After 4-day treatment, cells were PEG-fused, seeded on slices of bovine cortical bone, left in incubation for 5 days, fixed and stained for TRAP activity. PEG-fused TPMNC/mm$^2$ were counted and detached as described above. After removing the cells, the bone slices were stained and resorption pit areas quantified using an optical microscope. Cells without cytokine pre-treatment and PEG-induced fusion, as well as PEG-fused cells without cytokine pre-treatment, were used as control for both the number of TPMNC/mm$^2$ and resorption pit area determination.

Figure 10A shows that there was a reduced number of TPMNC/mm$^2$ for cells pre-stimulated with RANKL, whether PEG-fused or not, when comparing them to PEG-fused cells which had not been stimulated with RANKL. Nor were there any differences in the number of PEG-fused TPMNC/mm$^2$ when comparing applied doses of RANKL. On the other hand, there was a reduction in resorption pit area for cells pre-stimulated with RANKL (whether PEG fused or not) when comparing them to PEG-fused cells which had not been stimulated with RANKL (Fig. 10B). Regardless of whether PEG-induced fusion was performed, the resorption activities of cytokine-treated cells were the same or lower than that of control cells without being PEG-fused and cytokine treated, except for 10 ng/ml RANKL-treated PEG-fused cells (Fig. 10B).

BMP2 pre-stimulated cells that had then been PEG-fused also showed a reduction in the number of TPMNC/mm$^2$ when comparing them to PEG-fused cells that had not been pre-stimulated with BMP2 (Fig. 10C). However, the number of TPMNC/mm$^2$ for BMP2 pre-treated and PEG-fused cells was the same as that for control cells lacking BMP2 treatment and PEG-induced fusion (Fig. 10C). There were no significant differences between the number of TPMNC/mm$^2$ for non PEG-fused BMP2-treated cells and their PEG-fused counterparts, except for cells treated with 20 ng/ml BMP2 which had been PEG-fused (Fig. 10C). Regarding resorption activity, BMP2 pre-treated cells showed reduced PEG-fused and non-fused cell resorptive ability regarding PEG-fused cells lacking BMP2 pre-treatment (Fig. 10D). Nevertheless, BMP2-treated and PEG-fused cells' resorption activity was no higher than that exhibited by the control cells without BMP2 treatment and PEG-induced fusion (Fig. 10D).

## DISCUSSION

This study has described osteoclast-like cell formation by PEG-induced fusion of macrophages from the U937/A and J774 cell lines. This model is presented as an alternative to approaches involving macrophage-fusion at low pH or cell co-cultures. The immunochemistry approach used in our model revealed that PEG-fused U937/A macrophages expressed cathepsin K, carbonic anhydrase, vacuolar proton pump $H^+$-ATPase, calcitonin receptor and integrin b3, thereby supporting the idea that they are multinucleated cells having resorptive activity. Our results also suggest that macrophage fusion using PEG induced an increase in TRAP staining which was already present from 2 hours post-fusion and became more or less stable for the 5 days that the experiment lasted. Given that we determined TRAP activity immunocytochemically instead of quantifying the corresponding transcript, it was not possible to determine whether increased TRAP staining resulted from increased gene expression, enzyme quantity or activity. PEG-induced fusion increased TRAP-positive multinucleate cells forming in U937/A and J774 cells; however, the mechanism by which PEG fusion induced TRAP still remains unknown, except that PEG seems to induce changes in the J774 cell surface hydrophobicity (Vertut-Doi et al., 1996). Although PEG-induced fusion has been described in hybridoma production, the TRAP activity expression in our model seemed to be specific to the monocyte/macrophage line, given that skin fibroblast primary culture and CD40L fibroblasts became fused; however, all fused cells were essentially TRAP negative.

One of the models used for obtaining osteoclast-like cells was based on culturing macrophages (primary or from a cell line) at maximum confluence and low pH (Han *et al*., 2000). We reinterpreted these results, assuming that cells at 100% confluence could favor fusion, probably because proliferation is detained, or because cells leave the cell cycle and

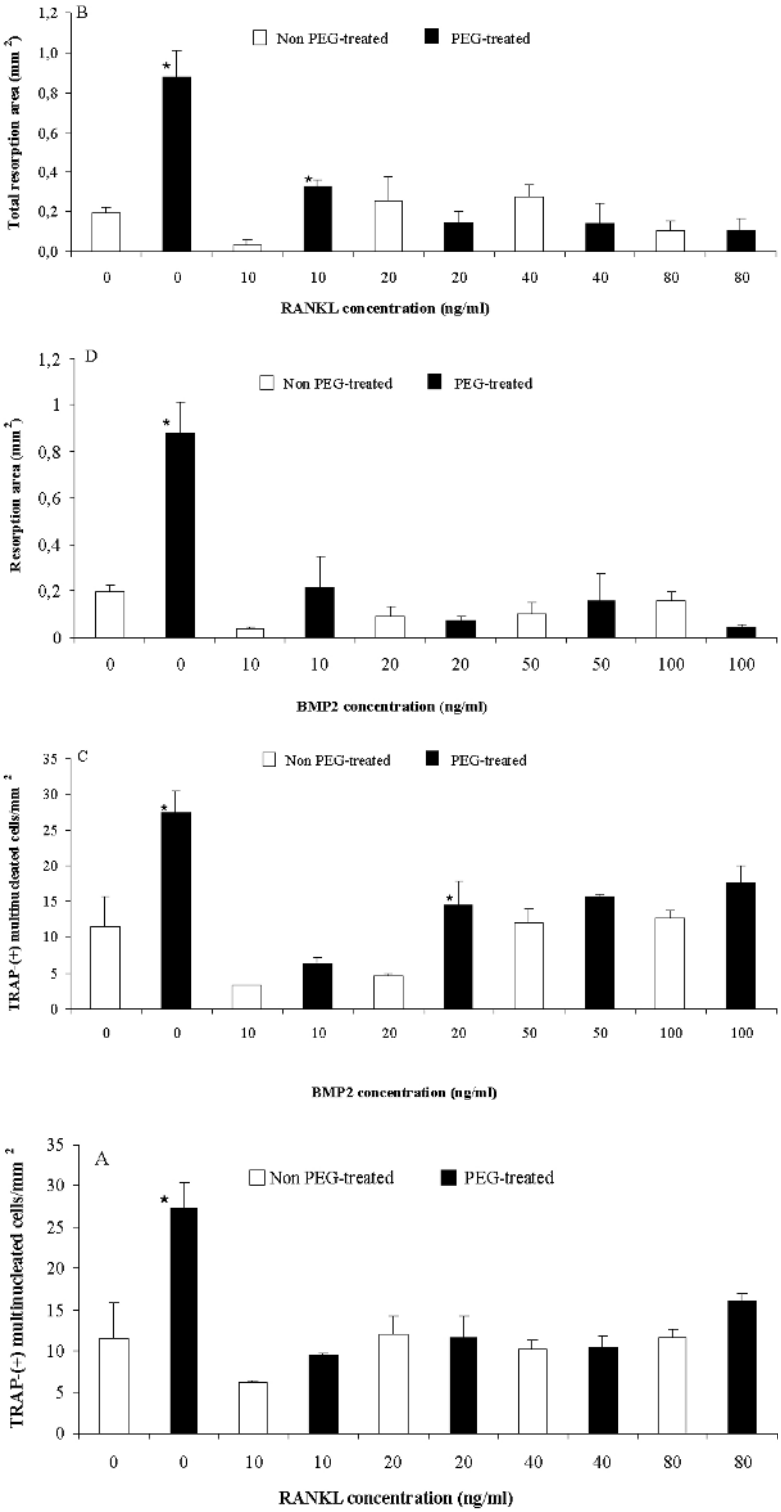


**Fig. 10** Effect of RANKL and BMP2 on bone resorptive activity of TPMNC. U937/A cells were stimulated during 4 days with RANKL (10, 20, 40 and 80 ng/ml) (**a**, **b**) or BMP2 (10, 20, 50 and 100 ng/ml) (**c**, **d**) and then PEG-fused and cultured on bovine cortical bone slices for 5 days. Cells were stained for TRAP activity (TPMNC/mm$^2$) (**a**, **c**) detached and total resorption pit areas (mm$^2$) (**b**, **d**) quantified. Cells without being stimulated and PEG-fused, and PEG-fused cells without previous hormone treatment were used as a control. Results are presented as mean ± S.E.M (n=3) (*P<0.05 cf control). The asterisk indicates significant differences from controls.

enter Go, an event that could lead to changes in the expression of cell membrane proteins implicated in cell-cell recognition during macrophage fusion. However, our results showed that spontaneous cell fusion without using PEG, even at 100% confluence, led to a negligible percentage of TPMNC on coverslips compared to cell fusion at 20%-80% confluence when using PEG. The percentage of TPMNC increased almost 5 times for PEG-fused cells at 100% confluence compared to averaged values for other confluence percentages (Fig. 5). This result at least suggests that cell membrane protein changes induced by 100% confluence could probably promote increased PEG-induced cellular fusion and the expression of osteoclast-specific markers, such as TRAP activity. It has been described that the macrophage fusion receptor (MFR) is expressed during the first moments of bone marrow macrophage spontaneous cell fusion, this being a macrophage fusion-mediating receptor (Saginario *et al*., 1998); its ligand, CD47, is also expressed (Han *et al*., 2000). However, it is still poorly understood which molecular mechanisms allow macrophages to fuse with each other (Vignery 2005).

Given that cells at 80% confluence, treated with PTH, fused by PEG treatment and seeded on coverslips, increased the percentage of TRAP positive multinucleated cells compared to PEG-fused cells lacking PTH treatment, it is suggested that this hormone probably stimulates the expression of or activates molecules implicated in fusing membranes. This result was surprising, as it is known that osteoblasts/stromal cells, but not osteoclast precursors, are normally PTH target cells (Suda *et al*., 1999, Okada *et al*., 2000). PTH/PTHrP (PTH/PTHrP1R) type 1 receptor expression has not been reported in U937/A macrophages and thus we did not investigate it in our work. However, it has been reported that PTH can enter a macrophage cell and is excreted after having produced a differentiation in the cell (Diment *et al*., 1989) and, given that PTH induced an effect on the U937/A line, it can be supposed that this cell line expresses it, given that the result was clear regarding the controls (i.e. vitamin D3 used in the experiment did not lead to the same effect). However, we observed a different effect regarding PTH when PTH pre-treated U937/A cells were PEG-fused and seeded on bone slices instead on coverslips. Bone substrate seemed to reduce the PTH effect on the number of TPMNC by contrast with the effect observed on coverslips.

Vitamin D3 has been used in several studies (Freedman, 1999, Kukita *et al*., 1993, Manabe *et al*., 2001, Otsuka *et al*., 2000, Udagawa *et al*., 1990, Yamagishiet *et al*., 2001) for obtaining osteoclast-like cells from bone marrow cells or in hematopoietic cell and stromal cell/osteoblast co-cultures with a significant increase in the number of osteoclast-like cells. However, this hormone is known in relation to promoting macrophage differentiation (Tanaka *et al*., 1991) and when it is applied to U937 cells it can induce them to differentiate to monocytes (Passmore *et al*., 2001). The results of this work showed that vitamin D3 does not influence spontaneous (lacking PEG treatment) cell fusion or TRAP enzyme expression on coverslips. No differences were found regarding PEG-fused cells lacking pre-treatment when cells were pre-stimulated with calcitriol and then PEG-fused. When vitamin D3 was used together with PTH, it was found that vitamin D3 seemed to reduce the PTH's inducer effect on coverslips. Our results thus differed from those reported in hematopoietic cells and stromal cell/osteoblast co-cultures; however, our model's conditions (PEG-induced fusion of a tumor line) were different and were not comparable to the co-culture model. It is also known that vitamin D3 has anti-proliferative effects on several types of tumor cells, which has been demonstrated both *in vivo* and *in vitro* (Konety *et al*., 2001), and is due to an increased apoptosis rate (Hisatake *et al*., 2001, Moffatt *et al*., 2001). Bearing in mind that the U937/A cell line is tumoral, it is possible that vitamin D3 reduces proliferation, which is in accordance with what was observed in the culture.

Bone matrix resorption is another marker of a cell having osteoclastic activity. It was found that PEG-fused U937/A cells produced resorption pits that increased their areas from day 1 through day 5 compared to non-PEG-fused control cells. It was very interesting that resorption pit areas observed in bone slices for PEG-fused U937/A cells were similar to those found for osteoclast-like cells produced by co-cultures, except that the resorption pits produced were deeper than those observed for PEG-fused U937/A cells. Similarly, J774 line cells seeded on bone slices showed resorptive ability. This cell line's resorptive activity presented no significant differences when compared to that of the U937/A cell line (Fig. 7). However, when the number of TPMNC was compared, it was observed that the U937/A line formed a significantly greater number than the J774 line (Fig. 7). This suggested that TRAP activity was not directly related to resorptive activity and that this activity could be a particular characteristic of each line.

PTH, calcitriol and dexamethasone pre-treatment reduced TPMNC forming on bone slices, whether cells had been PEG-fused or not. However, the average decrease in the number of TPMNC for PTH and vitamin D3-treated cells was much less when they had been PEG-fused compared to their respective non-PEG-fused controls and even to

dexamethasone-treated and PEG-fused cells. This could indicate that hormone pre-treatment reduced the percentage of spontaneous cell fusion and that PEG-treatment cannot completely restore the number of TPMNC observed for PEG-fused cells without hormone pre-treatment. These results also suggest the U937/A cells' differential response to the hormones used here in terms of TRAP activity expression.

Regarding the bone resorption activity of the three hormones evaluated, PTH had the greatest effect on resorptive ability, this being twice as much as that for control cells, whereas vitamin D3 and dexamethasone-treatment had no significant effect on increasing resorption pit areas. It should be stressed that no systematic correspondence was found between the number of TPMNC cells and bone resorption activity. For instance, vitamin D3-treated cells did not produce a resorption pit area proportional to their number of TPMNC when compared to the resorption pit areas produced by cells lacking hormone treatment; this suggested that TRAP activity is not always a prediction of resorptive activity. It could not be ruled out that in the present model we were producing cell sub-populations when U937/A macrophages became fused using PEG and that not all TPMNC carried out resorption activity. It could also have been possible that different cell sub-populations were affected differently when stimulated with PTH, vitamin D3 and dexamethasone. It has been reported that different osteoclast sub-populations can be found *in vivo* in bone according to their size and resorptive ability (Lees *et al*., 2001).

RANKL, at all concentrations tested here, reduced U937/A cell TPMNC formation and resorption activity independently of being PEG-fused or not. This result was surprising as it is known that RANKL has been described as being the main cytokine involved in osteoclast differentiation and activation in hematopoietic cell and stromal cell/osteoblast co-cultures. However, its use as an osteoclastogenic factor in a single cell-line system in the absence of another cytokine has not been reported. RANKL augmented macrophage activity, especially as antigen-presenting cells, suggesting its new role in immune regulation (Park *et al*., 2005). It is probable that, on having applied RANKL to U937/A cells, it induced them to differentiate to monocytes as happens with vitamin D3.

It is possible that this cytokine has a synergic effect with other molecules *in vivo* or in co-culture and that its individual effect (as evaluated in this experiment) could be different.

Similar behavior to that shown by RANKL treatment was found in BMP2-treated cells. In this case, all the concentrations assayed decreased the number of TPMNC and PEG-fused cells' resorptive activity after being cytokine pre-treated compared to non-cytokine treated and PEG-fused control cells. It has been reported that macrophages from the J774 cell line (Yamagiwa *et al*., 2001) express BMP2, but that their function is not known. It is known that BMP2 in osteoblasts activates the expression of genes involved in bone formation (Itoh *et al*., 2001). It has been reported that members of the BMP family have been shown to have anti-proliferative effects on their target cells (Amedee *et al*., 1994, Blessing *et al*., 1996). It is probable that this hormone induces monocyte/macrophage differentiation (as discussed for vitamin D3 retarding the expression of membrane molecules implicated in fusion). It is also possible that the expression of genes related to cell fusion, TRAP activity and bone resorption was inhibited to some extent when adding BMP2. It surprised us that neither RANKL nor BMP2 showed an osteoclastogenic effect in this work. In addition, the resorptive activity shown by U937/A cells, which had not been cytokine and PEG-treated, suggested that spontaneous fusion *per se* induces macrophage resorptive activity and that such activity usually becomes significantly inhibited when cells are pre-treated with cytokines.

Our work has also led to a relatively simple method regarding those previously reported involving cell co-cultures (Itoh *et al*., 2001, Jimi *et al*., 1999, Kamolmatyakul *et al*., 2001, Kansaki *et al*., 2001, Liu *et al*., 2002, Nakchbandi *et al*., 2000). Our method could be suitable for obtaining multinucleated cells that can be used for biochemical studies related to basic mechanisms concerning macrophage fusion, bone resorption activity and the experimental search for bone disease therapeutic alternatives. Some approaches within this context are currently being conducted by our group.

## ACKNOWLEDGMENTS

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