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Effects of Prostaglandin E₂ and Lipopolysaccharide on Osteoclastogenesis in RAW 264.7 Cells

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Abstract

Introduction—Prostaglandins (PGs) can act on both hematopoietic and osteoblastic lineages to enhance osteoclast formation.

Methods—We examined PGE₂ stimulated osteoclastogenesis in RAW 264.7 cells and the role of endogenous PGE₂ in lipopolysaccharide (LPS) stimulated osteoclastogenesis.

Results—RANKL (1–100 ng/ml) increased formation of osteoclasts, defined as tartrate resistant acid phosphatase multinucleated cells, with peak effects at 30 ng/ml. Addition of PGE₂ (0.01–1.0 μM) to RANKL (30 ng/ml) dose dependently increased osteoclast number 30 to 150%. Use of NS-398 (0.1 μM) or indomethacin (Indo, 1.0 μM) to block endogenous PG synthesis had little effect on the response to RANKL alone but significantly decreased the response to PGE₂. Addition of LPS (100 ng/ml) to RANKL increased osteoclast number 50%, and this response was significantly decreased by NS-398 and Indo. RANKL and PGE₂ produced small, additive increases in COX-2 mRNA levels, while LPS produced a larger increase. PG release into the medium was not increased by RANKL and PGE₂ but markedly increased by LPS.

Conclusion—We conclude that RANKL stimulated osteoclastogenesis can be enhanced by PGE₂ and LPS though direct effects on the hematopoietic cell lineage and that these effects may be mediated in part by induction of COX-2 and enhanced intracellular PG production.

Keywords

Cyclooxygenase; RANKL

Introduction

Prostaglandin E₂ (PGE₂) and bacterial lipopolysaccharide (LPS) are known to promote osteoclast formation in marrow cultures and co-cultures of hematopoietic spleen cells and osteoblastic stromal cells [1,2,3,4]. The effect of LPS in these cultures may be dependent on stimulation of PGE₂ production in osteoblastic cells [4]. In addition PGE₂ can have a direct effect on the production of osteoclasts from hematopoietic precursors in spleen cell cultures

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treated with receptor activator of NF κ B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) [5]. Moreover both PGE₂ and LPS have been shown to stimulate the inducible cyclooxygenase (COX-2) in a transformed cell line with macrophage characteristics, RAW 264.7 [6,7,8,9,10]. Cultured RAW 264.7 cells treated with RANKL can produce tartrate resistant acid phosphatase positive (+) multinucleated cells (TRAP+MNC) that have the characteristics of osteoclasts. RAW 264.7 cells express the PGE₂ receptors EP2 and EP4, which are implicated in stimulation of osteoclastogenesis [11].

In the present study we confirmed that PGE₂ could increase osteoclast formation in RANKL treated cultures and that LPS had similar effects. Osteoclast formation in response to these agonists was reduced by inhibitors of prostaglandin synthesis. RANKL and PGE₂ produced small increases in COX-2 mRNA levels which were additive, while LPS produced a larger increase. The mRNA levels for the constitutive enzyme, COX-1, were not increased. Prostaglandin release into the medium before and after treatment with arachidonic acid to increase PGE₂ production was not significantly increased by RANKL and PGE₂ but markedly increased by LPS. These results indicate that osteoclastogenesis can be enhanced by PGE₂ and LPS though direct effects on the hematopoietic cell lineage. These effects may be mediated in part by induction of COX-2 and enhanced intracellular prostaglandin production.

Material and Methods

Materials

Culture dishes and plates were from Corning (Corning, NY). PGE₂ and NS-398 were obtained from Cayman Chemical (Ann Arbor, MI). LPS (from *E. coli* serotype 055:B5) was from Sigma (St Louis, MO). Recombinant murine RANKL was obtained from R & D Systems (Minneapolis, MN). α -Minimum essential medium (α MEM) and fetal calf serum were from Invitrogen (Carlsbad, CA). Leukocyte acid phosphatase A kit for TRAP staining was from Sigma (St. Louis, MO). PGE₂ EIA kits were from Assay Designs (Ann Arbor, MI) and Cayman Chemicals (Ann Arbor, MI). Real-time PCR primers were from Applied Biosystems (Foster City, CA). Other chemicals and reagents were from analytical grade and obtained from Sigma.

Cell Culture

The RAW 264.7 monocyte/macrophage cell line was cultured at 37°C in 5% CO₂ atmosphere in Dulbecco modified Eagles medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (HIFCS). For osteoclast cultures, RAW cells were seeded in a 48 well plate at a density of 5×10^3 cells per well and cultured for 4 days in α MEM with 10% HIFCS in the presence of 30 ng/ml of RANKL unless otherwise stated. To examine the effects of PGE₂ and LPS on osteoclastogenesis, cells were plated in 48 well dish and culture in α MEM and treated with PGE₂ or LPS in the presence of RANKL. Half of the media was changed on the 3rd day. After 4 days (unless otherwise stated) cultures were fixed for 30 min with 2.5% glutaraldehyde and stained for TRAP. TRAP+MNC containing 3 or more nuclei were counted as osteoclasts. Total number of osteoclasts was counted in 4 wells.

Quantitative real time PCR (qPCR) analysis

For RNA extraction RAW 264.7 cells were plated in 6-well dishes at 6×10^4 cells per ml in α MEM with 10% HIFCS. Cells were grown for 2 days before they were treated for 1, 4 and 24 h with RANKL, PGE₂, LPS or a combination. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) following the manufacturer's instructions. Two to five μ g of total RNA was converted to cDNA by the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following company recommended protocol. qPCR was performed for different genes in separate wells (singleplex assay) of 96- well plate in a reaction

volume of 20 μ l. GAPDH served as the endogenous control for all experiments. Three replicates of each sample were amplified using Assays-on-Demand Gene Expression (Applied Biosystems, Foster City, CA) to give one measurement for a sample. Predesigned unlabeled gene-specific PCR primers and TaqMan dye-labeled probes were purchased from Applied Biosystems. Primers were tested for equal efficiency over a range of target gene concentration before use. All primers were designed to cross exon-exon boundaries. The PCR reaction mixture (including 2X TaqMan Universal PCR Master Mix, 20X Assays-on-Demand Gene Expression Assay Mix, 100 ng of cDNA) was run in Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. The relative quantification of target gene expression in a test sample to a control calibrator sample ($\Delta\Delta C_t$ Method) was used for data analysis.

PGE₂ measurements

RAW 264.7 cells were cultured for 2 days in α MEM with 10% HIFCS in a 24 well dish at 6×10^4 cells per ml. Treatments were pulsed in for 2 h; cells were washed well twice with serum-free media and then media were collected after 4 h. Arachidonic acid (10^{-5} M) was pulsed in for 15 min. and media was collected again. The PGE₂ levels were measured in the media using the commercially available PGE₂ enzyme immunoassay kits from Assay Designs (Ann Arbor, MI) and Cayman Chemicals (Ann Arbor, MI) according to the company's recommended protocols.

Statistical analysis

The results are expressed as the mean $\pm$ SE. Statistical significance of differences among means was determined by analysis of variance (ANOVA) with post-hoc comparison of more than 2 means by the Bonferroni method using SigmaStat for osteoclast numbers or by t-test for mRNA induction (Jandel Scientific, San Rafael, CA).

Results

RANKL produced a dose related increase in osteoclast formation in RAW 264.7 cells (figure 1). The effects of 30 and 100 ng/ml were not significantly different. PGE₂ at 10^{-6} M produced an increase in osteoclast number at all doses of RANKL. In the experiment illustrated in figure 2 this was significant at 3 and 30 ng/ml of RANKL. The effects of PGE₂ were dose related with a significant increase at 0.01 μ M in some experiments and at 0.1 μ M in all experiments (figure 3 and 4). The effects of 1 μ M of PGE₂ ranged from 30–150% in different experiments. The time course for osteoclast formation (figure 4A) showed a peak of osteoclast numbers at 4 d. In contrast to the previous studies using spleen cells [5], there was no early inhibition or prolongation of osteoclast survival and there was no difference in osteoclast size in cultures treated with PGE₂ compared to those treated with RANKL alone (Fig. 4A). One day of treatment with PGE₂ was sufficient to produce an increase in osteoclast number at 4 d and the effects were not significantly different from those of 4 d of continuous treatment (figure 4B). PGE₂ alone without RANKL did not increase osteoclast number (data not shown).

The effects of LPS were similar in magnitude to those of PGE₂ in RANKL treated RAW cells. LPS at 100 ng/ml produced a significant increase in osteoclast number of 60–80% (figure 5 and 6). The effects of both LPS and PGE₂ were reduced by the addition of NS398 (0.1 μ M) at a concentration which blocks COX-2 mediated prostaglandin production but may also affect production from COX-1 [12] (figure 6 and 7). Indomethacin (1 μ M), which blocks both COX-2 and COX-1, also inhibited PGE₂ stimulated osteoclastogenesis (figure 7). In this experiment the effect of PGE₂, although reduced, was still significant in the presence of inhibitors of prostaglandin synthesis and there was a small non-significant reduction in osteoclast number in cultures treated with RANKL alone. Since there is evidence that the prostaglandin F pathway

might be involved in induction of COX-2 [12], we tested $\text{PGF}_{2\alpha}$ and the selective agonist fluprostenol (figure 8). $\text{PGF}_{2\alpha}$ had a small stimulatory effect, which was reduced by NS-398. However, fluprostenol, at a concentration that activates the FP receptor, had no effect.

COX-2 mRNA levels were significantly increased by RANKL and PGE_2 , while the effect of LPS was substantially greater (Table 1). The combination of RANKL and PGE_2 appeared to be additive while RANKL did not further enhance the response to LPS. COX-1 levels were unaffected. A time course study showed that the effects of RANKL and PGE_2 were transient, while LPS produced sustained increases in COX-2 levels at 24-hours (data not shown). Prostaglandin production was examined in cultures that were treated with RANKL, PGE_2 , LPS or combinations for 2 hours in serum free medium, washed, cultured for 4 hours and then treated with arachidonic acid (10 μM) for 15 minutes. PGE_2 released into the medium before adding AA was markedly increased by LPS and slightly but not significantly increased by RANKL plus PGE_2 . After adding AA there was a further increase in PGE_2 in all cultures, but there was no significant effect of RANKL, PGE_2 or the combination compared to control, while the values were further increased in LPS treated cultures (Table 2). In another experiment in which the cultures were not serum deprived the results were similar (data not shown).

Discussion

The present study confirms and extends previous results on the effects of RANKL, PGE_2 and LPS on osteoclastogenesis in RAW 264.7 cells. Enhancement of RANKL induced osteoclastogenesis by PGE_2 and LPS was quite similar, although the induction of COX-2 and the production of PGE_2 was far greater in LPS treated cultures. Moreover NS-398, a selective COX-2 inhibitor, and indomethacin, an inhibitor of both COX-2 and COX-1, both reduced the responses to PGE_2 and LPS to a similar extent. Prostaglandin synthesis inhibitors had only a small and variable effect on the response to RANKL alone. These results suggest that endogenous PGE_2 production may play a role in osteoclastogenesis in this cell line. Previous studies have shown that prostaglandins can enhance osteoclast production in non-transformed hematopoietic precursors [2], but have not defined a role for endogenous prostaglandins. On the other hand, prostaglandin production by osteoblasts is clearly associated with an increase in the production of RANKL and increased osteoclast formation by co-cultured hematopoietic cells [13]. There was a substantial difference in medium PGE_2 between control, RANKL or PGE_2 cultures and LPS treated cultures yet PGE_2 and LPS produced similar prostaglandin dependent increases in osteoclastogenesis. This suggests that the intracrine effects of prostaglandins may be important in this stimulation. Prostaglandin receptors are present on the nuclear as well as the cell membrane and the synthetic enzymes are located near the nuclear membrane as well [14,15,16,17].

There are few studies of the specific pathways by which prostaglandin directly enhances osteoclastic differentiation. Protein kinase A and phosphorylation of a transforming growth factor β activated kinase 1 (TAK-1) have been implicated [2]. Based on this it is likely that the effects in RAW 264.7 cells are mediated by activation of the EP2 or EP4 receptors that increase cAMP [2,18]. Both receptors have been identified in these cells, although in osteoclast precursors from the spleen the EP2 receptor seems to play the key role in the effect of prostaglandin to increase osteoclastogenesis [5]. Further studies on the specific receptor and signal transduction pathways need to be carried out in non-transformed osteoclast precursors.

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Figure 1. Effect of RANKL on osteoclast formation in RAW 264.7 cell cultures

RAW cells were treated with increasing doses of RANKL (1–100 ng/ml) for 4 days. Cells were fixed and stained for TRAP. TRAP⁺ cells with more than 3 nuclei were counted as osteoclasts. RANKL produced a dose related increase in the osteoclast number. Values are means $\pm$ SEM. ^a significant effect of RANKL treatment, $P < 0.05$, ^b significantly different from next lower dose by t-test.

Figure 2. Effect of PGE₂ (1 μ M) on osteoclastogenesis in RAW 267.4 cells treated with different concentrations of RANKL

Conditions were the same as for figure 1. Cells were treated with 3–100 ng/ml of RANKL in the presence or absence of PGE₂. Values are means $\pm$ SEM. ^a Significant effects of PGE₂, $P < 0.05$.

Figure 3. Effect of increasing doses of PGE₂ (0.001– 1.0 μM) in RAW 264.7 cells treated with RANKL (30 ng/ml) for

Conditions were the same as for figure 1 Cells were treated with 30 ng/ml of RANKL alone or in the presence of increasing concentrations of PGE₂. Values are means +/- SEM. ^aSignificant effects of PGE₂, P<0.05.

Figure 4.

(A) Time course for the effect of different concentrations of PGE₂ on osteoclastogenesis in RAW 264.7 cells treated with 30 ng/ml RANKL: Cells were treated with 0.01 μM, 0.1 μM and 1.0 μM of PGE₂ for 3, 4 and 5 days. ^aSignificant effect of treatment, P<0.01; ^b P<0.05.

(B) Effect of PGE₂ (1 μM) given for 1 d or continuously on RAW 264.7 cells treated with RANKL (30 ng/ml) for 4 d: Cells were treated with PGE₂ and RANKL for 1 day and then with RANKL alone for 3 days or PGE₂ was given continuously for 4 days in the presence of RANKL. ^aSignificantly effect of PGE₂, P<0.01.

Figure 5. Effect of LPS on osteoclastogenesis in RAW 264.7 cells treated with 30 ng/ml RANKL
Cells were treated with 1, 10 and 100 ng/ml of LPS and PGE₂ (1 μM) in the presence of RANKL. ^a Significant effect of LPS or PGE₂, $P < 0.01$, ^b $P < 0.05$.

Figure 6. Effect of NS398 (0.1 μM) on stimulation of osteoclastogenesis by PGE₂ (1 μM) and LPS (100 ng/ml) in RAW 264.7 cells treated with RANKL (30 ng/ml)
 Cells were treated with PGE₂ and LPS in the presence of RANKL ± NS398 for 4 days. ^a
 Significantly different from RANKL alone, P<0.01.

Figure 7. Effects of PGE₂ (1 μM) on osteoclastogenesis in RAW 264.7 cells treated with RANKL (30 ng/ml), with or without NS-398 (0.1 μM) or indomethacin (1 μM)
Conditions were the same as for figure 1. ^a Significantly different from RANKL alone, P<0.01. ^b Significant effect of NS398 or Indomethacin, P<0.05.

Figure 8. Effects of PGF_{2α} (1 μM) and fluprostenol (flup, 0.01 μM) compared with PGE₂ (1 μM) on RAW 264.7 cells treated with RANKL (30 ng/ml) for 4 days, with and without NS-398 (0.1 μM). Conditions were the same as for figure 1. ^aSignificantly different from RANKL alone, P<0.01; ^b Significant effect of NS 398, P<0.05.

TABLE 1

Changes in COX-2 mRNA levels after treatment with RANKL, PGE₂, LPS, RANKL + PGE₂ and RANKL + LPS and analyzed by qPCR. Cells were subjected to the treatments for 1h. Each RQ value for COX-2 was calculated as fold induction relative to the control. Single data values were pooled from 2 independent experiments.

Treatment (Time 1 h)	COX-2	COX-1
Control (N=5)	1.00 ± 0.19	1.00 ± 0.11
RANKL (N=6)	5.59 ± 1.47 ^a	0.77 ± 0.08
PGE ₂ (N=5)	2.98 ± 0.45 ^a	0.92 ± 0.21
LPS (N=3)	46.23 ± 2.04 ^a	0.79 ± 0.27
RANKL + PGE ₂ (N=6)	7.52 ± 2.02 ^a	0.88 ± 0.12
RANKL + LPS (N=3)	38.78 ± 3.34 ^a	0.88 ± 0.31

The data are the means ± SEM values from N Cultures from 2 independent experiments.

^aSignificantly different from control, P<0.05.

TABLE 2

Changes in medium PGE₂ levels after treatment with RANKL, PGE₂, LPS, RANKL + PGE₂ and RANKL + LPS. Cells were treated for 2h, washed with serum-free media and cultured in serum free media. Media were collected after 4 h. Arachadonic acid (AA) (10⁻⁵ M) was pulsed in for 15 min and media were again collected.

Treatment	PGE ₂ conc. before adding AA (nM)	PGE ₂ conc. after adding AA (nM)
Control	10.2 ± 7.2	91.7 ± 15.9
RANKL	9.1 ± 4.7	105.4 ± 16.1
PGE ₂	11.6 ± 4.9	101.0 ± 12.2
LPS	996.7 ± 74.3 ^a	1667.9 ± 89.2 ^a
RANKL + PGE ₂	23.3 ± 12.7	99.6 ± 12.1
RANKL + LPS	1206.5 ± 58.8 ^a	2636.5 ± 810.0 ^a

The data are the means ± SEM from 4 independent cultures.

^aSignificantly different from control, P<0.05.