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Biphasic influence of PGE_2 on the resorption activity of osteoclast-like cells derived from human peripheral blood monocytes and mouse RAW264.7 cells

Anne-Helen Lutter [a,b,*], Ute Hempel [a], Ursula Anderer [b], Peter Dieter [a]

[a] Institute of Physiological Chemistry, Medical Faculty Carl Gustav Carus, Dresden University of Technology, Fiedlerstraße 42, 01307 Dresden, Germany
[b] Department of Cell Biology and Tissue Engineering, Brandenburg University of Technology Cottbus-Senftenberg, Großenhainer Straße 57, 01968 Senftenberg, Germany

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ABSTRACT

Osteoclasts are large bone-resorbing cells of hematopoietic origin. Their main function is to dissolve the inorganic component hydroxyapatite and to degrade the organic bone matrix. Prostaglandin E_2 (PGE_2) indirectly affects osteoclasts by stimulating osteoblasts to release factors that influence osteoclast activity. The direct effect of PGE_2 on osteoclasts is still controversial. To study the influence of PGE_2 on osteoclast activity, human peripheral blood monocytes (hPBMC) and mouse RAW264.7 cells were cultured on osteoblast-derived extracellular matrix. hPBMC and RAW264.7 cells were differentiated by the addition of macrophage colony-stimulation factor and receptor activator of NFκB ligand and treated with PGE_2 before and after differentiation induction. The pit area, an indicator of resorption activity, and the activity of tartrate-resistant acid phosphatase were dose-dependently inhibited when PGE_2 was present *ab initio*, whereas the resorption activity remained unchanged when the cells were exposed to PGE_2 from day 4 of culture. These results lead to the conclusion that PGE_2 treatment inhibits only the differentiation of precursor osteoclasts whereas differentiated osteoclasts are not affected.

1. Introduction

Remodeling of bone tissue is the concerted action of the two key players - osteoblasts and osteoclasts (OC) – collaborating to couple resorption and bone formation. Osteoblasts evolve from mesenchymal stem cells and differentiate into bone-forming cells that deposit hydroxyapatite crystals and collagen into the osteoid. Upon terminal differentiation, these cells are embedded into the bone matrix as osteocytes, which are smaller in cell size and lack particular organelles, such as mitochondria, the Golgi apparatus, and endoplasmic reticulum [1]. OC evolve from hematopoietic stem cells and differentiate into multinucleated cells with a variable number of nuclei, ranging from 1 to 100. Differentiation is dependent on specific factors, such as receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (MCSF) [2]. The function of OC requires a tight connection to the bone matrix (formation of a resorption unit/sealing zone) and to osteoblast-like cells (activation of RANK via an interaction with osteoblast-derived RANKL). Fully differentiated active OC are able to degrade inorganic and organic components of the extracellular bone matrix by secreting hydrochloric acid and several proteases (cathepsin K, matrixmetalloproteinases, and tartrate resistant acid phosphatase (TRAP)) via the ruffled border membrane into the resorption lacunae surrounded by the sealing ring [3].

Prostanoids are important mediators that have manifold functions in biological processes such as fever, pain, inflammation, tumorigenesis, gastrointestinal protection, vascular circulation, and bone metabolism [4]. For bone remodeling, PGE_2 is a potent, multifunctional and complex regulator [5]. PGE_2 effects are mediated by four different G-protein-coupled receptor (GPCR) subtypes: EP1, EP2, EP3, and EP4. EP1 receptor signaling increases the intracellular Ca^{2+} levels and activates protein kinase C (PKC) via the G_q protein [6,7]. The EP2 and EP4 subtypes are the most frequent EP subtypes in bone cells [8] and activate G_s proteins,

Abbreviations: αMEM;, Alpha Minimum Essential Medium; cAMP, cyclic adenosine monophosphate; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; hPBMC, human peripheral blood mononuclear cell; MCSF, macrophage colony-stimulating factor; OC, osteoclast; ODEM, osteoblast-derived extracellular matrix; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PI3K, posphatidylinositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; NaOH, sodium hydroxide; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase; Tris–HCl, Tris (hydroxymethyl)aminomethane hydrochloride

* Correspondence to: BTU Cottbus-Senftenberg, Großenhainer Str. 57, 01968 Senftenberg, Germany.
E-mail address: lutter@b-tu.de (A.-H. Lutter).

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which stimulate cyclic adenosine monophosphate (cAMP) formation followed by the activation of cAMP-dependent protein kinase A (PKA). EP4-mediated signaling involves further the extracellular signal-regulated kinases (ERK) 1 and 2 and posphatidylinositol-3-kinase (PI3K) [9]. EP3 acts via G_i proteins, inhibiting cAMP generation [10]. The effects of PGE_2 on bone remodeling are not yet fully understood. Whether PGE_2 causes the activation or inhibition of bone resorption seems to be strongly dependent on the experimental setting. Using *in vivo* or *in vitro* models, working with coculture systems or osteoclasts alone and evaluating precursor cells or mature osteoclasts can result in different findings. During the osteoclastogenic differentiation process, hematopoietic precursor cells go through TRAP-positive, resorption-inactive to TRAP-positive, and resorption-active mature OC phenotypes [11]. However, the analysis of functionally active OC should not only be restricted to TRAP activity, but should also include testing the physiological function of OC, which is their resorption activity. In the presented study, we wanted to clarify the role of PGE_2 in the bone remodeling process with respect to the controversially discussed influence of PGE_2 on OC precursors and mature OC. Therefore, human and mouse OC precursors cultured in the presence of MCSF and/or RANKL on osteoblast-derived extracellular matrix (ODEM) were treated with PGE_2 simultaneously with plating or at day 4 after plating. On day 10 after seeding, the cells were analyzed for morphology, TRAP activity, pit area formation, and the expression of *ep2* and *ep4*.

2. Materials and methods

2.1. Cell Culture and induction of osteoclastic differentiation

The RAW264.7 cell line (ATCC® TIB-71™, ATCC via LGC Standards, Wesel, Germany) was used for osteoclast differentiation. The cells were cultured in DMEM (Biochrom, Berlin, Germany) containing 10% heat-inactivated FBS and 20 U penicillin//20 μg/ml streptomycin (Biochrom). For differentiation, 30,000 RAW264.7 cells/well were plated in 24-well polystyrene culture plates coated with ODEM. ODEM preparation was described by Lutter et al. [12]. Bare 24-well plates were used as a control. The cells were cultured in 0.5 ml α-MEM (Biochrom) containing 20% heat-inactivated FBS, 2 mM glutamine, 20 U penicillin//20 μg/ml streptomycin and 40 ng/ml of mouse RANKL (kindly provided by B. Hoflack, BioZ, Dresden, Germany). The medium was changed every 2–3 days. PGE_2 (Sigma-Aldrich, Deisenhofen, Germany) treatment was started at day 0 or at day 4 in a concentration of 1 μM, 5 μM or 10 μM PGE_2. PGE_2 was added with every medium change.

Primary human PBMC were isolated from donor blood as previously described [13]. Briefly, the hPBMC were isolated from peripheral blood by Ficoll-gradient centrifugation. The resulting cell suspension was sorted for dendritic cells using autoMACS (purity $>95\%$) (Miltenyi Biotech, Bergisch Gladbach, Germany). For preparation of hPBMC, the residual unsorted suspension was used. The cells were further purified by adhesion, adding α-MEM conditioned with 1 ng/ml MCSF produced by cell line 5637 (ATCC® HTB-9™). For the experiment, 2.5×10^5 hPBMC/well were plated in 24-well polystyrene culture plates coated with ODEM in α-MEM (Biochrom) containing 20% heat-inactivated FBS, 2 mM glutamine, 20 U penicillin//20 μg/ml streptomycin, 10 ng/ml human RANKL (R&D Systems, Wiesbaden, Germany) and 10 ng/ml human MCSF. The medium was changed every 2–3 days. PGE_2 treatment was started at day 0 or at day 4 at a concentration of 1 μM, 5 μM or 10 μM. PGE_2 was added with every medium change.

2.2. Fluorescence staining

hPBMC and RAW264.7 cells were stained for F-actin and nuclei. Before staining, the cells were fixed in 4% paraformaldehyde (w/v) (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS) for 10 min at 25 °C and washed in PBS. The cells were permeabilized in PBS (Biochrom) containing 0.1% Triton X-100 (Ferak, Berlin, Germany) for 20 min and non-specific binding was blocked by incubation with blocking solution (PBS containing 1% bovine serum albumin and 0.05% Tween-20) for 10 min This was followed by incubation with Phalloidin-AlexaFluor 488 (Sigma-Aldrich) diluted in blocking solution for 1 h at 25 °C. Next, DAPI (4′,6-diamidino-2-phenylindol) (Roche, Mannheim, Germany) was used for 15 min at 25 °C for staining the nuclei. The stained cells were then embedded in Mowiol 4–88 (Sigma-Aldrich, Deisenhofen, Germany). The staining was visualized using an AxioPhot fluorescence microscope; digital images were acquired with an AxioCam HRm camera working with AxioVision software version 4.6 (Zeiss, Oberkochen, Germany). The fluorescence signals were detected with the following optical settings: Alexa488 excitation 450–490 nm, emission 515–565 nm; DAPI excitation 365 nm, emission 420 nm.

2.3. Activity of tartrate-resistant acid phosphatase (TRAP)

TRAP activity was assessed by the lysis of cells with TRIS/HCl (10 mM, pH 7.5) buffer containing 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), 1% Triton X100, 0.25 μM aprotinin (Sigma-Aldrich) and 0.1 mM PMSF (Boehringer, Mannheim, Germany). The release of p-nitrophenolate from p-nitrophenyl phosphate (Roche) via TRAP activity was determined by measuring the absorbance at 405 nm with a microplate spectrophotometer (Benchmark Plus, Biorad, Munich, Germany). The reaction was stopped after 60 min of incubation at 37 °C by the addition of 100 mM NaOH. The amount of released p-nitrophenolate is directly proportional to the enzyme activity. In parallel, the protein concentration was quantified using the Bradford method [14], with bovine serum albumin as a standard. TRAP activity was normalized to the protein concentration to obtain the specific enzyme activity.

2.4. Localization of TRAP

TRAP staining was performed using the leukocyte acid phosphatase kit (Sigma-Aldrich). Briefly, the cells were washed with PBS, fixed with fixative solution for 1 min, washed with H_2O and stained with TRAP staining solution containing tartrate for 1 h. The stained cells were then embedded in Mowiol 4–88. The purple staining was visualized with a light optical microscope (Zeiss, Jena, Germany).

2.5. Resorption activity

For quantitation of the resorption pit area, calcium phosphate was visualized using histochemical von Kossa staining [15,16] and analyzed densitometrically. Briefly, decellularized ODEM was thoroughly washed with H_2O and incubated with 5% silver nitrate solution (Merck, Darmstadt, Germany) for 1 h. After the next washing step with H_2O, the plates were developed with 1% pyrogallol (Sigma-Aldrich) in H_2O for 5 min and air-dried. The complete area of the stained plates was scanned with transmitted light at 600 dpi (Powerlook 1000, Umax). Image analysis was performed with Image Quant 5.1. The optical density of cell-free ODEM (after von Kossa staining visible as a complete black area) was used as a reference (0% resorption, 100% black area after von Kossa staining). For quantitation of the pit area (resorption lacunae are visible as