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TNF-α stimulates alkaline phosphatase and mineralization through PPARγ inhibition in human osteoblasts

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ABSTRACT

The aims of the present study were to determine whether prostaglandins (PGs) and PPARγ are involved in the stimulation of tissue-non-specific alkaline phosphatase (TNAP) activity and mineralization by TNF-α in human osteoblasts. We used osteoblasts differentiated from MSCs from three different donors and MG-63 osteoblast-like cells. Inhibition of prostaglandin synthesis with the cyclooxygenase (COX) inhibitor indomethacin or the specific COX-2 blocker NS-398 abolished mineralization in the absence and presence of 1 ng/ml of TNF-α, suggesting that PGs were involved. The TNAP inhibitor levamisole abolished TNF-α effects on mineralization, suggesting that PGs were involved in TNAP expression and mineralization. TNF-α stimulated expression of COX-2 and PG E synthase before that of TNAP, but expression of PG D synthase later suggesting that PGE_2 and $PGF_2\alpha$ but not 15d-PGJ_2 were involved in TNF-α effects. However, both PGE_2 and $PGF_2\alpha$ dose-dependently inhibited mineralization indicating that endogenous PG are required for mineralization but that TNF-α does not increase mineralization by increasing PG synthesis. Interestingly, TNF-α inhibited PPARγ expression and binding activity to PPRE consensus sequences independently of 15d-PGJ_2. Inhibition of PPARγ activity with GW-9662 mimicked TNF-α effects in MG-63 cells, indicating that TNF-α stimulates mineralization by inhibiting PPARγ in osteoblasts. In MSC-derived osteoblast cultures, inhibition of PPARγ dropped TNAP expression and mineralization. Treatment of MG-63 cells with conditioned media from MSC-derived osteoblasts or MSC-derived adipocytes treated or not with GW-9662 revealed that TNF-α inhibition of PPARγ in undifferentiated MSCs and/or adipocytes was responsible for the decreased expression of TNAP in osteoblasts. In conclusion, TNF-α increases TNAP expression and stimulates mineralization by inhibiting PPARγ in osteoblasts, but PPARγ in adipocytes or undifferentiated MSCs controls the secretion of a factor leading to TNAP stimulation in osteoblasts.

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Introduction

Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are major cytokines responsible for bone loss in many inflammatory diseases. In rheumatoid arthritis for instance, bone loss is blocked by therapies blocking TNF-α or IL-1β (for review see [1]). TNF-α and IL-1β promote bone loss by activating osteoclastogenesis, which occurs both directly [2,3] and also through the expression by stromal cells of receptor activator of nuclear factor κB ligand and macrophage colony stimulating factor [4,5]. Both cytokines also contribute to decrease bone mineral density by inhibiting osteoblast differentiation and bone formation, probably mainly by inhibiting RUNX2 expression and function [6–8].

However, in contrast to their effects on bone loss, TNF-α and IL-1β are strongly suspected to induce ectopic calcification. For example, inflammatory cytokines are believed to play a role in the development of vascular calcification occurring during atherosclerosis and diabetes type 2, or during aortic valve disease [9–11]. Importantly, a recent article reported that in *ldlr*−/− diabetic mice, inflammation and aortic calcification are reduced upon treatment with the TNF-α inhibitor infliximab, whereas weight gain, hyperglycemia, hypercholesterolemia, or hyperleptinemia remain unaffected [12]. Underlying mechanisms in the calcifying effects of TNF-α and IL-1β likely rely on the stimulation of tissue-non-specific alkaline phosphatase (TNAP) expression in vascular smooth muscle cells (VSMCs) [13–16] and myofibroblasts [12,17].

Abbreviations: COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GP, glycerophosphate; IL, interleukin; MSC, mesenchymal stem cell; PG, prostaglandin; NF-κB, nuclear factor-κB; NSAID, non-steroidal anti-inflammatory drug; PGDS, PG D synthase; PGES, PG E synthase; PPARγ, peroxisome proliferator-activated receptorγ; PPRE, PPARγ response element; RT-PCR, reverse transcriptase polymerase chain reaction; RA, retinoic acid; RXR, retinoid X receptor; TNAP, tissue-non-specific alkaline phosphatase; TNF, tumor necrosis factor; VD, vitamin D; VSMC, vascular smooth muscle cell.

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We recently showed that TNF-α and IL-1β stimulate TNAP activity and mineralization independently of RUNX2, whereas they decrease collagen expression by inhibiting RUNX2 in human osteoblasts [6]. The stimulation of TNAP by TNF-α in human osteoblasts has been recently confirmed by other independent studies [18,19]. Since mineralization merely requires the co-expression of a collagen network as a template for crystal deposition and TNAP to inactivate pyrophosphate ions, which are potent calcification inhibitors [20], we proposed the very simple hypothesis that inflammatory cytokines may initiate ectopic calcifications by stimulating TNAP independently on RUNX2 in virtually any tissue containing a fibrillar collagen, such as the vascular wall. This hypothesis appears supported by the fact that overexpression of TNAP under the control of the type I collagen promoter induces vascular calcification [20].

Vascular calcification is recognized as an independent predictor of cardiovascular disease and mortality in the general population (reviewed in [21]). Identifying the molecular mechanisms responsible for TNAP expression in response to cytokines appears therefore important. In the present study, we particularly focused on the involvement of prostaglandins (PGs) and peroxisome proliferator-activated receptorγ (PPARγ) in the effects of cytokines on TNAP expression and mineralization. TNF-α and IL-1β are indeed known to stimulate via NF-κB expression of cyclooxygenase (COX)-2, PGE synthase (PGES) and PGF synthase (PGFS), resulting in the synthesis of PGE_2 and $PGF_{2\alpha}$, which display pro-inflammatory effects. In a second step, both cytokines stimulate expression of PGD synthase, allowing the production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), the most potent naturally occurring PPARγ ligand [22]. 15d-PGJ_2 eventually initiates the resolution of inflammation, mainly by activating PPARγ, which competes with NF-κB [23,24].

The importance of PGs in bone formation has been highlighted in many reports (reviewed in [25]). Non-steroidal anti-inflammatory drugs (NSAIDs) have indeed been reported to inhibit fracture healing in animal studies [26], and in humans, NSAIDs significantly reduce the rate of spinal fusion [27]. In cultured mouse cells, indomethacin decreases TNAP activity and mineralization without significantly affecting RUNX2, collagen type I and osteocalcin levels [28]. PGE_2 in particular may play an important role in mineralization [29], but other PGs such as $PGF_{2\alpha}$ also likely contribute to osteoblast functions [30]. Finally, 15d-PGJ_2, by binding and activating PPARγ, may also modulate TNAP expression and mineralization [23,31–33].

In the present study, we show that prostaglandins are necessary for basal mineralization in human osteoblasts, but that TNF-α stimulates TNAP expression and mineralization by inhibiting PPARγ expression and activity independently from 15d-PGJ_2.

Materials and methods

Chemicals

Cell culture plastic ware was purchased from D. Dutscher (Brumath, France). Dulbecco's minimum essential medium (DMEM), α-MEM, fetal calf serum (FCS), L-glutamine, penicillin, streptomycin (P/S), trypsin/EDTA, and Extract-All reagents were from Eurobio (Les Ulis, France). Indomethacin, NS-398, $_{1,25}(OH)_2$Vitamin D3 (VD3), vitamin C, β-glycerophosphate (β-GP), levamisole, prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were obtained from Sigma-Aldrich Corporation (St Quentin Fallavier, France). DNase I, Taq DNA polymerase and SYBR green mix were from Roche Diagnostic (Meylan, France). Random primers were obtained from TibMolBiol (Berlin, Germany). Superscript II reverse transcriptase and dNTPs were purchased from Invitrogen (Cergy Pontoise, France). TNF-α was purchased from R&D Systems (Lille, France). GW-9662 was from Tebu-bio (Le Perray en Yvelines, France).

Cell cultures

To investigate osteoblast differentiation, cells from three donors were used. Cells consisted in purified MSCs from two healthy donors [a 34-year-old female and a 36-year-old male (Lonza, Walkersville, USA; certified positive for CD29, CD44, CD105 and CD166, and negative for CD14, CD34 and CD45)] and also in MSCs obtained from trabecular bone explants prepared from the iliac crest bone harvested during pelvic osteotomy in one patient with Legg–Perthes–Calve disease, as previously described [6]. According to our regional ethics committee, surgeons asked informed consent from the children's parents. Legg–Perthes–Calve disease is an idiopathic avascular necrosis of the femoral head, which does not affect the iliac crest. Bone marrow was extracted as previously published in details [34]. Briefly, a small part of the body iliac crest was removed at the end of the surgery in order to close without creating tension of the iliac crest. This fragment of bone was sent to the laboratory, where it was minced into small pieces, and extensively washed with PBS to obtain the bone marrow cells. Cells were isolated by centrifugation, and stromal cells were separated from non-adherent cells by several washes in culture. MSCs were seeded at a density of 5000 cells per cm^2 and routinely cultured in DMEM containing 10% FCS, 1% P/S, and 1% L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in air. Cells were subcultured at approximately 80–90% confluence with trypsin/EDTA.

To induce osteoblast differentiation, MSCs were seeded at 5000 cells per cm^2 in DMEM and at confluence, medium was replaced by an osteogenic medium, consisting of DMEM with 10% FCS, containing 10^{-8} M $_{1,25}(OH)_2$VD3, 50 μM vitamin C and 10 mM β-GP [6,35]. Media were changed every 2 or 3 days. To induce adipocyte differentiation, MSCs at confluence were placed in an adipogenic medium consisting of DMEM with 10% FCS supplemented with 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 50 μM indomethacin.

MG-63 "osteoblast-like" cells were routinely grown in DMEM containing 10% FCS, 1% P/S, and 1% L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in air. To optimize expression of osteoblast markers, MG-63 cells at confluence were also cultured in an osteogenic medium, consisting of DMEM with 10% FCS, containing 10^{-8} M $_{1,25}(OH)_2$VD3, 50 μM vitamin C and 10 mM β-GP.

To investigate the effects of TNF-α on PPARγ binding activity at 24 h or RNA levels at 48 h, MSCs or MG-63 cells were treated 7 days after addition of osteogenic medium, with 1 ng/ml of TNF-α. To assess the involvement of PPARγ, the specific inhibitor GW-9662 (100 μM) was added 2 h before TNF-α. To study the effects on mineralization, TNF-α, GW-9662, indomethacin, levamisole, NS-398, PGE_2, or $PGF_2\alpha$ were added in the osteogenic medium from confluence, and was replaced with each medium change, every 3–4 days, for 14 days.

To investigate the effects of conditioned medium from MSC-derived osteoblasts or adipocytes on *TNAP* expression in MG-63 cells, osteoblast or adipocyte differentiation was induced as described above. On day 7 after confluence, cells were treated or not with GW-9662 and media were collected 48 h later. Then, conditioned media were mixed with fresh osteogenic media (50% v/v) and added on MG-63 cells cultured for 7 days after confluence. MG-63 cells were treated with conditioned media for 48 h.

RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted using Extract-All reagent according to the manufacturer's instructions. Briefly, lysis of the cells in Extract-All was followed by centrifugation at 12,000 *g* for 15 min, at 4 °C in the presence of chloroform. The upper aqueous phase was collected, and the RNA was precipitated by addition of isopropanol and centrifugation at 12,000 *g* for 10 min, at 4 °C. RNA pellets were washed with 75% ethanol, dried and reconstituted in sterile water. Total RNA was

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