Life Sciences 84 (2009) 499–504

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# TNF-α and IL-1β inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells

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ARTICLE INFO

*Article history:*
Received 10 July 2008
Accepted 23 January 2009

*Keywords:*
TNF-α
IL-1β
MSC
Bone formation
Spondyloarthropathy

ABSTRACT

*Aims:* Joint inflammation leads to bone erosion in rheumatoid arthritis (RA), whereas it induces new bone formation in spondyloarthropathies (SpAs). Our aims were to clarify the effects of tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) on osteoblast differentiation and mineralization in human mesenchymal stem cells (MSCs).
*Main methods:* In MSCs, expression of osteoblast markers was assessed by real-time PCR and ELISA. Activity of tissue-nonspecific alkaline phosphatase (TNAP) and mineralization were determined by the method of Lowry and alizarin red staining respectively. Involvement of RUNX2 in cytokine effects was investigated in osteoblast-like cells transfected with a dominant negative construct.
*Key findings:* TNF-α (from 0.1 to 10 ng/ml) and IL-1β (from 0.1 to 1 ng/ml) stimulated TNAP activity and mineralization in MSCs. Addition of 50 ng/ml of IL-1 receptor antagonist in TNF-α-treated cultures did not reverse TNF-α effects, indicating that IL-1 was not involved in TNF-α-stimulated TNAP activity. Both TNF-α and IL-1β decreased *RUNX2* expression and osteocalcin secretion, suggesting that RUNX2 was not involved in mineralization. This hypothesis was confirmed in osteoblast-like cells expressing a dominant negative RUNX2, in which TNAP expression and activity were not reduced. Finally, since mineralization may merely rely on increased TNAP activity in a collagen-rich tissue, we investigated cytokine effects on collagen expression, and observed that cytokines decreased collagen expression in osteoblasts from MSC cultures.
*Significance:* The different effects of cytokines on TNAP activity and collagen expression may therefore help explain why inflammation decreases bone formation in RA whereas it induces ectopic ossification from collagen-rich entheses during SpAs.



## Introduction

Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are two major cytokines that lead to bone loss in many inflammatory diseases. In rheumatoid arthritis (RA) for instance, bone loss is reduced by therapies blocking TNF-α or IL-1β [for review see (Walsh et al. 2005)]. Interfering with TNF-α and IL-1β action may also be beneficial in preventing bone loss in diseases not usually considered as inflammatory, such as post-menopausal osteoporosis. Indeed, ovariectomized mice do not lose bone in absence of TNF-α (Ammann et al. 1997; Kimble et al. 1997) or IL-1 (Kimble et al. 1995; Kitazawa et al. 1994; Lorenzo et al. 1998). Moreover, bone resorption due to estrogen deficiency in women may be blocked by etanercept and anakinra, which are inhibitors of TNF-α or IL-1β respectively (Charatcharoenwitthaya et al. 2007).

The mechanisms used by TNF-α and IL-1β to promote bone loss include activation of osteoclastogenesis, which occurs both directly (Jimi et al. 1999; Kitaura et al. 2004) and also through the expression by stromal cells of receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony stimulating factor (MCSF) (Kitaura et al. 2005; Wei et al. 2005). It is also generally recognized that both cytokines contribute to decrease bone mineral density by inhibiting osteoblast differentiation and bone formation. TNF-α for instance has been reported to decrease RUNX2 expression (Gilbert et al. 2002) and promote its degradation (Kaneki et al. 2006).

In contrast to their effects on bone loss, inflammatory cytokines are strongly suspected to induce ectopic bone formation, for instance in arteries during atherosclerosis and type II diabetes, or during aortic valve disease (Demer 2002; Doherty et al. 2003; Helske et al. 2007). For instance, a recent article importantly 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 (Al-Aly et al. 2007). Underlying mechanisms may involve the reported positive effects of TNF-α and IL-1β on tissue-nonspecific

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doi:10.1016/j.lfs.2009.01.013

alkaline phosphatase (TNAP) expression by vascular smooth muscle cells (VSMCs) (Parhami et al. 2002; Shioi et al. 2002; Tintut et al. 2000, 2002) and myofibroblasts (Al-Aly et al. 2007; Kaden et al. 2005).

Inflammatory vascular calcification, as well as inflammatory bone loss, may have dramatic consequences [reviewed in (Doherty et al. 2003; Shao et al. 2006)]. A better understanding of the effects of inflammatory mediators on new bone formation in normal and ectopic sites is therefore warranted. In this context, aims of the study reported here were to determine TNF-α and IL-1β effects on osteoblast differentiation and mineralization in human mesenchymal stem cells (MSCs), which are osteoblast and VSMC precursors [reviewed in (Magne et al. 2005)]. In addition, our goal was also to investigate whether TNF-α effects on osteoblast differentiation and mineralization may depend on IL-1, since RANKL secretion induced by TNF-α in stromal cells requires the presence of IL-1 signaling (Wei et al. 2005).

## 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). Vitamin D3 (VD3), vitamin C, β-glycerophosphate (β-GP), p-nitrophenylphosphate, cetyl pyridinium chloride 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, IL-1β and IL-1 receptor antagonist (IL-1Ra) were purchased from R&D Systems (Lille, France).

### Cell culture

To investigate osteoblast differentiation, cells from four patients were used. Cells consisted in purified MSCs from a healthy 34-year-old female (Cambrex Bio Science, Walkersville, USA; certified positive for CD29, CD44, CD105 and CD166, and negative for CD14, CD34 and CD45) and also in bone marrow stromal cells obtained from trabecular bone explants prepared from the iliac crest bone harvested during pelvic osteotomy in three other patients (from 2.5 to 10 years-old) with Legg–Perthes–Calve disease. According to our regional ethic committee, surgeons asked informed consent to 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 (Anselme et al. 2000). 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. Purified MSCs or MSCs in bone marrow stromal cells were seeded at a density of 5000 cells/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. Osteoblastic differentiation was induced by culturing cells in an osteogenic medium, containing $10^{-8}$ M VD3, 50 μM vitamin C and 10 mM β-GP, as previously reported (Isaac et al. 2008). Media were changed every 2 or 3 days. To investigate the effects of cytokines, cells were treated from day 2 with TNF-α (0.1 to 10 ng/ml) and/or IL-1β (0.1 to 1 ng/ml) and cytokines were added in the medium at each change. To assess the effects of IL-1Ra, new medium with IL-1Ra was changed 2 h before addition of TNF-α and/or IL-1β.

To investigate whether cytokine effects were dependent on RUNX2, human SaOS-2 osteoblast-like cells were transfected with a construct encoding the RUNX2 DNA binding domain, fused to the CMV promoter, which was originally shown to act in a dominant negative manner (Ducy et al. 1999). Transfection and selection of clones were performed as already published in details (Bertaux et al. 2006, 2005).

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

Before RNA isolation, MSCs were seeded at 5000 cells/cm$^2$ and grown for 14 days in osteogenic medium in 25 cm$^2$ flasks. Selected SaOS-2 clones were seeded in 25 cm$^2$ flasks at 15,000 cells/cm$^2$ and cultured in α-MEM for 7 days. 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 quantified by spectrophotometer at 260 nm wave length and the integrity of RNA was controlled by the 28S/18S rRNA ratio after agarose gel electrophoresis. Contaminating DNA was removed from RNA samples in a 30 min digestion at 37 °C with DNase I. One microgram of each RNA sample was then used for reverse transcription performed under standard conditions with Superscript II reverse transcriptase and random hexamer primers in a 20 μl final volume. The reaction was carried out at 42 °C for 30 min and stopped with incubation at 99 °C for 5 min. The RT reactions were then diluted to 100 μl in water. 1 μl of stock cDNA template was used in subsequent PCR reactions.

### Quantitative PCR experiments

Quantitative PCR was performed using a LightCycler system (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Reactions were performed in 10 μl volume with 0.3 μM primers, 4 mM $MgCl_2$ and 1 μl of LightCycler-FastStart DNA Master SYBR Green I mix. Protocol consisted of a hot start step (8 min at 95 °C) followed by 40 cycles including a 10 s denaturation step (95 °C), a 10 s annealing step, and an elongation step at 72 °C varying from 15 s to 40 s. The primer sequences and PCR conditions for each cDNA are given in Table 1. Efficiencies of PCR were optimized according to Roche Diagnostic's recommendations on a standard sample expressing all studied genes. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and subsequent gel electrophoresis. Quantification data represented the mean of duplicate conditions. Relative quantification analyses were performed by RelQuant 1.01 Software (Roche Diagnostics, Meylan, France).

**Table 1**
Summary of primers used.

| cDNA | Forward and reverse primers | Ta (°C) | Product (bp) | Genbank |
|---|---|---|---|---|
| *18S* | F: 5′-ATTCCGATAACGAACGAGAC-3′<br>R: 5′-GCTTATGACCCGCACTTACT-3′ | 62 | 297 | X03205 |
| *RPLP0* | F: 5′-CGACCTGGAAGTCCAACTAC-3′<br>R: 5′-AGCAACATGTCCCTGATCTC-3′ | 60 | 289 | M17885 |
| *RUNX2* | F: 5′-GCTGTTATGAAAAACCAAGT-3′<br>R: 5′-GGGAGGATTTGTGAAGAC-3′ | 60 | 108 | NM_001024630 |
| *OC* | F: 5′-ATGAGAGCCCTCACACTCCTC-3′<br>R: 5′-GCCGTAGAAGCGCCGATAGGC-3′ | 57 | 293 | NM_199173 |
| *COL1A2* | F: 5′-GGACACAATGGATTGCAAGG-3′<br>F: 5′-TAACCACTGCTCCACTCTGG-3′ | 58 | 461 | NM_000089 |
| *GAPDH* | F: 5′-GTTCCAATATGATTCCACCC-3′<br>F: 5′-AGGGATGATGTTCTGGAGAG-3′ | 55 | 487 | M33197 |

Shown are the primer sequences, annealing temperatures (Ta), lengths of the corresponding PCR products, and Genbank accession numbers of the DNA sequences. F: forward; R: reverse; GAPDH: glyceraldehyde-3-phopshate dehydrogenase; OC: osteocalcin; RPLP0: acidic ribosomal phosphoprotein P0.