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Increased sensitivity of rheumatoid synoviocytes to Schnurri-3 expression in TNF-α and IL-17A induced osteoblastic differentiation

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ABSTRACT

Objective: To compare the effects of TNF-α and IL-17A on osteogenic differentiation of isolated fibroblast-like synoviocytes (FLS) from healthy donors, osteoarthritis (OA) and rheumatoid arthritis (RA) patients.
Methods: FLS were cultured in osteogenic medium, with and without TNF-α and/or IL-17A. Extracellular matrix mineralization was evaluated by alizarin red staining and alkaline phosphatase activity (ALP) measurement. mRNA expression was analyzed by qRT-PCR for Wnt5a, BMP2 and Runx2, genes associated with osteogenesis, for DKK1 and RANKL, genes associated with osteogenesis inhibition and Schnurri-3, a new critical gene in the cross talk with osteoclasts. IL-6 and IL-8 production was measured by ELISA.
Results: In osteogenic medium, matrix mineralization and increased ALP activity indicated that FLS can undergo osteogenic differentiation, which was increased with TNF-α and IL-17A. The expression of osteogenesis activators (BMP2 and Wnt5a) was increased with cytokines and that of the osteogenesis inhibitor DKK1 was decreased. There was no difference between all three cell types. In contrast, RA FLS were particularly sensitive to the synergistic increase of Shn3 with TNF-α and IL-17A. Levels of IL-6 and IL-8 were also higher for RA-FLS, compared to healthy and OA FLS.
Conclusion: IL-17A and/or TNF-α treatment favor an osteogenesis induction in isolated FLS, independent of their origin. RA-FLS were more sensitive to the synergistic increase of Schnurri-3 expression. Combined with the higher levels of inflammation, this may in turn activate osteoclastogenesis, leading to increased bone destruction seen in destructive arthritis.

Arthritis diseases are associated with various bone changes. Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are characterized by destruction of bone and cartilage, and defective repair, their combination leading to rapid destruction [1]. Osteoarthritis (OA) is characterized by simultaneous bone and cartilage destruction, and osteophyte formation, leading to a lower rate of destruction. Other conditions such as ankylosing spondylitis (AS) combine ectopic bone formation leading to syndesmophytes, combined with systemic bone loss [2].

Fibroblast-like synoviocytes (FLS) are the preponderant cells in the synovium, and play a key role in cytokine production that perpetuates inflammation and matrix changes. The origin of the expanding synoviocyte population is uncertain, but could be due to migration of mesenchymal stem cells (MSC) from the circulation or expansion of a stem cell pool in the synovium [3]. MSC from synovial membrane have shown their multi-lineage potential and differentiate into chondrocytes and osteoblasts [4,5].

The inflamed synovial environment in RA and PsA, and to some extend OA, is characterized by the presence of pro-inflammatory cytokines from monocytes such as IL-1β, IL-6, and TNF, and from T cells such as IL-17. Several studies showed that TNF-α and IL-17A inhibit osteoblastogenesis and activate osteoclastogenesis [6–11]. In contrast to these classical effects on bone loss, recent studies have indicated that TNF-α and IL-17A could increase osteogenesis in vitro [10,12,13]. Indeed culture of isolated synoviocytes in osteogenic medium leads to osteogenic differentiation with an increase in alkaline phosphatase and calcium deposits [5,14].

Long term exposure to inflammation leads to molecular changes in RA-FLS, which are long lasting, even maintained in long term cultures. To study the consequences of such exposure on osteogenic differentiation in FLS [15], our objective was to investigate the effects of IL-17 and TNF-α on FLS-osteogenic differentiation in RA-FLS compared to OA and healthy (H) FLS.

The results indicate that the three cell types do not appear to differ in terms of osteogenic differentiation. However RA-FLS were found to be highly sensitive to the effects of TNF-α and IL-17 on the overexpression

Abbreviations: FLS, fibroblast like synoviocytes; ALP, alkaline phosphatase; RA, rheumatoid arthritis; OA, AS, ankylosing spondylitis; OA, osteoarthritis; BMP-2, bone morphogenetic protein-2; Runx2, runt-related transcription factor-2; RANKL, receptor activator of nuclear factor kappa B ligand; Shn3, Schnurri-3; DKK-1, dickkopf 1; Wnt5a, wingless-type MMTV integration site family, member 5A..

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of Schnurri-3, a gene identified for its capacity to link osteoblasts and osteoclasts [16]. The resulting osteoclastogenesis associated with enhanced inflammation would explain the high degree of bone destruction which is characteristic of inflammatory arthritis as in RA and PsA.

1. Material and methods

1.1. Cell culture

FLS were obtained from the synovial tissue of hips or knees from 4 RA patients, 3 OA patients and 3 healthy donors who were undergoing knee or hip surgery. The RA patients fulfilled the American College of Rheumatology criteria of RA [17]. OA was diagnosed according to the typical clinical signs and symptoms according to the ACR criteria [18,19]. Healthy FLS obtained from patients with femur neck fractures. Each individual signed an informed consent and the protocol was approved by the committee for protection of persons participating in biomedical research under the number AC-2010-11-64.

FLS were isolated by enzyme digestion and cultured at 37 °C in DMEM medium (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum (Thermo scientific, Saint Aubin, France), 2 mM L-glutamine, 100 U/ml penicillin, streptomycin. FLS were cultured for 3 to 4 weeks to obtain sufficient number of cells for the experiments. Cells from each individual were kept separated for each experiment. For osteogenic differentiation, FLS were plated at a density of 5×10^3 cells/cm^2 and cultured in stem Xvivo Osteogenic/adipogenic base Medium (R&D systems, Lille, France), supplemented with 100 nM dexamethasone (Sigma, Saint Quentin-Fallavier, France), 10 mM β-glycerophosphate (Sigma) and 50 μM ascorbic acid (Sigma). FLS were differentiated for 21 days in the absence or presence of 1 ng/ml of TNF-α and/or 50 ng/ml of IL-17A (R&D systems, Lille, France). These concentrations were selected from previous studies as showing a synergistic interaction between the 2 cytokines. Half of the medium was changed every three days and FLS were used between passage 4 and 8. In total each experiment with cells from an individual lasted a total of 2 months.

1.2. Mineralization assay

Cells were washed twice with PBS, and fixed with 70% cooled ethanol for 1 h, then washed with water. Cells were stained for 20 min at ambient temperature with alizarin red (pH: 4.2, 40 min, Sigma, Saint Quentin-Fallavier, France) and examined under light microscope. The red color obtained referred to calcium deposit.

1.3. Alkaline phosphatase assay (ALP)

FLS seeded in 24-well plates were lysed with the assay buffer (Abcam, Paris, France). The protein contents in the lysates were determined using the Bradford protein assay (Sigma). The remaining lysate (10 μl) was mixed with 20 μl of MUP, used as a substrate (Abcam) in a 96-well plate, and incubated at room temperature for 30 min. Fluorescence intensity was measured at extension/emission 360/440 nm. The ALP activity was normalized to protein content and expressed as U/μg protein.

1.4. Enzyme-linked immunosorbent assays (ELISA)

IL-6 and IL-8 production were measured with commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems, Lille, France).

1.5. Quantitative RT-PCR analysis

RNA was purified using RNeasy kits (Qiagen, Les Ulis, France). The concentration of RNA was quantified by spectrophotometry (SmartSpec™3000, Biorad, Hercules, CA). Total RNA (500 ng) was reverse transcribed with the QuantiTec Reverse Transcription (Qiagen Kit) into cDNA. PCR amplification was performed on a Light Cycler (Roche Diagnostics, Switzerland) using Fast-Start™ DNA Master SYBR Green I real-time PCR kit (Roche Molecular Biochemicals, Switzerland). The expression of the genes was normalized to the expression of human cyclophilin B (CPB) (5′tgtggtgtttggcaaagttc3′; 3′ gtttatcccggctgtctgtc5′) (Qiagen). The list of primers (Qiagen) is as follows: BMP2 (5′ccaccatgaagaatctttgga3′; 3′gagttggctgttgcaggttt5′), RUNX2 (5′gtggacgaggcaagagttt3′; 3′tggggtctgtaatctgactc5′), Shn-3 (5 ccctgagccataaccctgaa ′3′; 3′ gtaggacttggcgttggtgt 5′), Wnt5a (5′ attcttggtggtcgctaggt3′; 3′accttcgatgtcggaattgat5′), DKK1 (5′ ccttggatgggtattccaga3′; 3′tccatgagagccttttctcc5′).

1.6. Statistical analysis

To study the effect of treatment conditions and that of culture duration, a two-way analysis of variance for repeated measures was performed using Graphpad Prism software. *P* values were determined for every analysis. *P* values of <0.05 were considered significant.

2. Results

2.1. TNF-α and IL-17A potentiated calcium deposits and extracellular matrix mineralization

Mineralization of the extracellular matrix is one of the key markers of bone formation. FLS from H, OA and RA were cultured for 17 days in osteogenic medium with and without TNF-α and/or IL-17A. Alizarin red staining was used to visualize mineralization. As shown in Fig. 1A, column 2, culture of FLS with osteogenic factors alone induced a weak matrix mineralization, which appeared at day 17, whatever the cell origin. In H-FLS, only the combination of IL-17 and TNF-α increased significantly mineralization at day 17 (Fig. 1A, column 5; 1B. In OA-FLS and RA-FLS, both TNF-α and the combination of IL-17 and TNF-α enhanced matrix mineralization (Fig. 1A, column 3 and 5; 1C, 1D). Importantly, the combination of TNF-α and IL-17A highly enhanced the mineralization in all 3 cell types (Fig. 1A, column 5). Thus, TNF-α and IL-17A alone could enhance bone mineralization, with a potentiation when combined.

2.2. TNF-α and IL-17A increased alkaline phosphatase activity

Alkaline phosphatase activity (ALP) is an enzyme essential for bone mineralization and its activity is used as a marker of osteogenic differentiation in vitro and in patients [20]. ALP activity was measured at days 5, 7 and 14 (Fig. 2). In all 3 cell types, osteogenic medium alone induced a very modest increase in ALP activity starting at day 5 compared to control medium. In H-FLS, there was no significant difference in ALP activity between cells cultured in osteogenic medium alone or with pro-inflammatory cytokines, whatever the time point. In OA-FLS, only the combination of IL-17A and TNF-α at day 7 had a significant effect on ALP activity when compared to osteogenic medium alone (Fig. 2B, ** $p < 0.01$). In RA-FLS, both IL-17A and TNF-α alone or in combination induced a significant increase in ALP activity at early time-points (day 5 and 7) as compared to osteogenic medium alone (Fig. 2C, * $p < 0.05$; ** $p < 0.01$). Moreover, this effect was maintained at day 14 for cells treated with the combination of IL-17A and TNF-α. ALP activity reached higher levels in RA-FLS than in H- or OA-FLS. Therefore, these results support those of the alizarin red staining, by showing that the combined action of TNF-α and IL-17A increased and accelerated osteogenesis, specifically for RA-FLS.

2.3. TNF-α and IL-17A increased Wnt5a and decreased DKK1 mRNA levels

Having established an net effect of TNF-α and IL-17A on calcified matrix formation during the differentiation of FLS, levels of key genes