IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent

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Abstract

Objective. The mechanisms of ectopic calcification in inflammatory diseases are poorly understood. We investigated the effects of inflammatory cytokines on the mechanisms of calcification in human adipose tissue-derived mesenchymal stem cells (hADSCs).

Methods. The effects of inflammatory cytokines were evaluated using hADSCs cultured in osteoblast induction medium. mRNA expression was measured by real-time PCR and protein levels were measured by western blotting. Cell mineralization was evaluated by Alizarin Red S staining.

Results. In hADSCs, administration of IL-6/soluble IL-6 receptor (sIL-6R), TNF or IL-1β accelerated calcification through enhanced expression of an osteoblast differentiation marker, runt-related transcription factor 2 (RUNX2). IL-6/sIL-6R had the greatest effect. The transcription of mRNA for receptor tyrosine kinase-like orphan receptor 2 (ROR2), involved in the non-canonical wingless-type (WNT) MMTV integration site pathway, was increased, while β-catenin expression, an essential factor in the canonical WNT signalling pathway for osteoblast differentiation, did not change. Suppression of signal transducer and activator of transcription 3 (STAT3), but not STAT1, by small interfering RNA (siRNA) exerted a strong inhibitory effect on RUNX2 and ROR2 expression, and inhibited accelerated calcification.

Conclusion. IL-6/sIL-6R stimulation accelerated the ROR2/WNT5A pathway in hADSCs in a STAT3-dependent manner, resulting in augmented calcification. These results suggest that the mechanisms of ectopic calcification accelerated by IL-6 in hADSCs may be involved in chronic inflammatory tissues and that IL-6 inhibitors may be beneficial in the treatment of ectopic calcification in inflammatory diseases.

Key words: IL-6, ROR2, ADSCs, STAT3, WNT5A, ectopic calcification.

Introduction

Physiological calcification in living organisms involves the deposition of minerals such as calcium and phosphorus in the bone matrix during bone formation. During the development of bone, osteoblasts derived from mesenchymal stem cells (MSCs) become embedded in the bone matrix and differentiate into bone cells. In addition to physiological calcification, ectopic calcification can also occur in vivo. A typical example is arteriosclerosis-related vascular wall calcification, which is caused by the simple deposition of calcium in vascular walls. However, recent studies have suggested that transformation of vascular smooth muscle cells into osteoblasts may be responsible for vascular wall calcification [1].

The finding that bone matrix proteins, including osteopontin, osteonectin and bone sialoprotein (BSP), are present in calcified tissues suggests that calcification may occur via osteoblast-like differentiation [2, 3]. Fat cells,
hematopoietic cells and mesenchymal cells exist in large numbers in subcutaneous adipose tissue. However, these cells are terminally differentiated and are unlikely to acquire osteoblast-specific traits or cause calcification. Alternatively, immature and undifferentiated MSCs can be induced to differentiate into osteoblasts by inflammatory cytokines and are more likely to be involved in subcutaneous calcification. Multipotent adipose tissue-derived MSCs (ADSCs) are present in subcutaneous adipose tissue. ADSCs are multipotent cells that can differentiate into MSC-specific adipocytes, chondrocytes and osteoblasts [4, 5]. MSCs isolated from patients with autoimmune diseases, such as SLE or other inflammatory diseases, are reportedly associated with abnormal function or differentiation, unlike MSCs from healthy individuals, suggesting that MSCs are involved in disease pathogenesis [6].

Multiple factors, including inflammation and oxidative stress, that are associated with diabetes, renal failure and ageing are involved in osteoblast-like differentiation [7]. In addition, autoimmune diseases are associated with inflammation-related ectopic calcification. In particular, scleroderma and JDM are complicated by ectopic subcutaneous calcification. Between 4% and 25% of patients with scleroderma and ~30% of patients with JDM develop ectopic calcification complications [8]. Scleroderma and JDM are autoimmune diseases that affect the skin and muscle tissues, respectively. These diseases are thought to be mediated by inflammatory cells such as lymphocytes and macrophages that infiltrate subcutaneous adipose tissue and muscle tissue. These cells secrete inflammatory cytokines, such as IL-6, TNF and IL-1β, which may be involved in calcification [9, 10]. This mechanism of immune-mediated calcification was supported by a study in which the administration of TNF inhibitors reduced ectopic calcification [11]. We recently demonstrated that inflammatory cytokines stimulate the differentiation of MSCs into osteoblasts by activating the non-canonical wingless-type (WNT) signalling pathway [12].

These findings suggest that inflammatory cytokines may be involved in ectopic calcification observed in the pathology of autoimmune diseases by stimulating the differentiation of ADSCs into osteoblasts. The aim of this study was to determine the ability of inflammatory cytokines to accelerate the differentiation of human ADSCs (hADSCs) into osteoblast-like cells in vitro.

**Methods**

**Cells**

hADSCs were purchased from Cytori (San Diego, CA, USA). ADSCs were cultured in ADSC basal medium supplemented with l-glutamine, gentamicin, amphotericin and 10% fetal bovine serum (FBS) [ADSC growth medium (ADSCGM), Lonza, Walkersville, MD, USA] at 37°C in a 5% CO₂ atmosphere and were subcultured every 6–7 days. ADSCs from passages 2–10 were used in this study. The multipotency of ADSCs was assessed by their ability to differentiate into osteoblasts, chondrocytes and adipocytes. ADSCs (1 × 10⁵ cells) were seeded into 24-well plates (Coming, Corning, NY, USA) and cultured in osteoblast induction medium (OIM; 50 µM ascorbic acid, 10 mM β-glycerophosphoric acid and 0.1 µM dexamethasone), adipocyte induction medium [minimum essential medium (MEM) α, hydrocortisone, isobutyl-methylxanthine and indomethacin] or seeded into a conical tube three-dimensional (3D) culture and cultured in chondrocyte induction medium (D-MEM, insulin, transferrin, selenious acid, BSA, linoleic acid, ascorbic acid, proline, pyruvate, TGF-β3 and dexamethasone) at 37°C in a 5% CO₂ atmosphere. All media were obtained from Lonza. Ethics approval for the experiments using hADSCs was granted by the Ethics Committee of the University of Occupational and Environmental Health, Japan.

**ADSCs-conditioned medium assay**

ADSCs (1 × 10⁵ cells) were seeded into 24-well plates and cultured in OIM at 37°C in a 5% CO₂ atmosphere. Recombinant human TNF (R&D Systems, Minneapolis, MN, USA), human IL-1β (RELIAtech, Wolfenbüttel, Germany) or human IL-6 (Miltenyi Biotec, Bergisch Gladbach, Germany) with human soluble IL-6 receptor (sIL-6R) (R&D Systems) were added to OIM. The medium was replaced every 2–3 days throughout the experiments. These experiments were performed with and without 1-h cycloheximide (5 µg/ml) pretreatment of hADSCs.

**Measurement of mRNA expression**

Total mRNA was collected with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was obtained by reverse transcription according to the manufacturer’s instructions. Real-time PCR was performed using primers specific for runt-related transcription factor 2 (RUNX2) (Hs-01047978-m1), receptor tyrosine kinase-like orphan receptor 2 (ROR2) (Hs-00171695-m1) and WNT MMTV integration site family member 5A (WNT5A) (Hs_00998537m1), WNT7A (Hs-01055707-m1), WNT7B (Hs-00536497-m1) and WNT10B (Hs-00559664-m1) (Applied Biosystems, Foster City, CA, USA). RUNX2, ROR2 and WNT5A mRNA expression levels were normalized to the levels of β-actin (TaqMan probe Hs_99999903_m1) as an endogenous control and calculated using the ΔΔCT method.

**Mineralization assay**

Cell mineralization was evaluated by Alizarin Red S (ARS) staining (Sigma-Aldrich, St Louis, MO, USA). Briefly, cells were cultured in the indicated conditions in a 24-well plate and fixed with 10% formaldehyde for 15 min and rinsed with deionized water before adding 350 µl of 1% ARS solution (pH 4.1) per well. After incubation at room temperature for 15 min, the cells were washed with deionized water.

**Western blotting**

Cells were washed twice with cold PBS and dissolved with lysis buffer containing 50 mM Tris-HCl (pH 8.0),
150 mM NaCl, protease inhibitor and 10% NP-40. Equal amounts of proteins (20 μg) were electrophoresed by SDS-PAGE, transferred onto nitrocellulose membranes and blotted with antibodies against β-catenin, signal transducer and activator of transcription 1 (STAT1), STAT3, phosphorylated (p)-STAT1, p-STAT3 (Cell Signaling Technology, Beverly, MA, USA) or β-actin (Sigma-Aldrich), followed by incubation with secondary antibodies (GE Healthcare, Chalfont St Giles, UK).

Small interfering RNA
The following small interfering RNA (siRNAs) were purchased from Invitrogen (Carlsbad, CA, USA): STAT1 siRNA-1 (5'-GGAGAGCACAGCAGCGCCUGUAUU-3'), STAT1 siRNA-2 (5'-CCUGUCACAGCGGAGGAUCAAU-3'), STAT3 siRNA-1 (5'-GCCAAUGUGAUGCUUC CCUGAUUG-3'), STAT3 siRNA-2 (5'-UGGCCCAA UGGAUCAUGCUACAGCA-3') and negative control siRNA (low GC: 12935-200; medium GC: 12935-112; high GC: 12935-400). Transfection was performed using lipofectamine RNAiMAX (Invitrogen). In brief, hADSCs (1 × 10^4 cells) were plated on a 24-well plastic plate in 500 μl of antibiotic-free ADSCGM 1 day before transfection. On the next day, transfection reagents containing 6 pmol siRNA and 1 μl lipofectamine RNAiMAX in a final volume of 100 μl with Opti-MEM I (Invitrogen) was added to each well and incubated for 24 h before adding OIM supplemented with IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) for 3 or 24 h.

Immunohistochemistry
Ethics approval was obtained from the Ethics Committee of the University of Occupational and Environmental Health, Japan for the use of patient tissue. Formalin-fixed, paraffin-embedded sections (3-μm thick) from a DM patient were used for immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei, Tokyo, Japan) with mouse anti-human RUNX2 (ABNOVA, Taipei, Taiwan) and mouse anti-human FIG. 1 Inflammatory cytokines accelerated mineralization and RUNX2 mRNA expression

(A) hADSCs were cultured in OIM supplemented with TNF, IL-1β or IL-6/sIL-6R (0.01, 0.1, 1.0, 10 and 100 ng/ml) and Alizarin Red S staining was performed on day 8. Data are representative of three experiments with similar findings. (B, C) hADSCs were cultured in OIM supplemented with TNF (1.0 ng/ml), IL-1β (0.1 ng/ml) or IL-6/sIL-6R (100 ng/ml). Total RNA was isolated at 24 h (B) and on day 8 (C) and RUNX2 mRNA expression was determined by real-time PCR. Data are shown as the mean (S.E.M.) values of three experiments. *P < 0.05, **P < 0.01 vs without OIM and cytokines by analysis of variance and post hoc Dunnett’s test.
IL-6 (R&D Systems, Minneapolis, MN, USA). Briefly, endogenous peroxidase was inactivated in a 3% hydrogen peroxide (H₂O₂) solution after proteolytic digestion using proteinase K (Dako, Glostrup, Denmark). These sections were then blocked with serum-free protein block (Dako, Glostrup, Denmark), followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min and the colour was developed by incubating the sections in 3,3’-diaminobenzidine and H₂O₂ for 10 min, followed by counterstaining with haematoxylin solution. Negative control sections were treated with isotype-matched mouse IgG1.

Statistical analysis

Data are expressed as mean (s.e.m.). Differences between two groups were tested for statistical significance using Student’s t-test. Analysis of variance (ANOVA) was used to compare three or more groups. If the ANOVA was significant, Dunnett’s multiple comparison test was used as a post hoc test. In all analyses, a P-value < 0.05 was considered significant.

Results

Inflammatory cytokines accelerated mineralization and RUNX2 mRNA expression in hADSCs

We first confirmed the multipotency of hADSCs, as they could differentiate into adipocytes, chondrocytes and osteoblast-like cells in vitro (data not shown). Previous studies demonstrated that inflammatory cytokines might be involved in osteoblast differentiation and calcification [13, 14]. Therefore we investigated the effects of different inflammatory cytokines on osteoblast-like differentiation of hADSCs. In vitro, hADSCs were cultured in OIM supplemented with TNF, IL-1β or IL-6/sIL-6R (0.01, 0.1, 1.0, 10 and 100 ng/ml). On day 8 of culture, calcification was assessed by ARS staining. TNF, IL-1β and IL-6/sIL-6R at concentrations of 1.0 ng/ml, 0.01 ng/ml and 100 ng/ml, respectively, accelerated the calcification compared with OIM alone (Fig. 1A). All three cytokines augmented RUNX2 mRNA expression, an osteoblast differentiation marker, within 1 day of culture (Fig. 1B). On day 8, the enhanced expression of RUNX2 mRNA persisted in all cytokine-supplemented cultures relative to OIM alone, with IL-6/sIL-6R showing the greatest effect (Fig. 1C). The addition of IL-6 alone did not enhance RUNX2 mRNA expression, suggesting that functional IL-6 receptors were not expressed by hADSCs (data not shown). These results demonstrated the acceleratory effect of IL-6/sIL-6R on calcification by stimulating osteoblast-like differentiation. This indicated that the IL-6 signalling pathway had the most potent effect on the differentiation of hADSCs.

IL-6 increased ROR2 mRNA expression without affecting β-catenin expression in osteoblast-like differentiation

We then studied the role of the WNT signalling pathways (canonical and non-canonical) in osteoblast-like differentiation. To determine the expression level of β-catenin, a vital component of the canonical WNT pathway, hADSCs were cultured in OIM supplemented with 100 ng/ml of IL-6/sIL-6R and western blotting analysis was performed 4 days later. However, β-catenin expression was not significantly different in cultures with or without IL-6/sIL-6R (Fig. 2A).

Next, an RT-PCR assay was performed to evaluate the expression of WNT3A, WNT5A, WNT7B and WNT10B mRNAs and assess their involvement in osteoblast-like differentiation in cultured hADSCs. mRNAs for WNT3A, WNT7B and WNT10B were not detected on days 0, 8 or 11 of culture. In contrast, WNT5A mRNA, which encodes a representative Wnt protein and is involved in the non-canonical pathway, was constantly expressed at high
levels on days 0, 8 and 11 (Fig. 2B). The addition of IL-6/sIL-6R did not enhance this expression (data not shown). hADSC expression of ROR2 mRNA, which encodes a cognate receptor for WNT5A, was enhanced 24 h after the addition of IL-6/sIL-6R compared with untreated hADSCs or hADSCs cultured in OIM alone. Pretreatment of hADSCs with cycloheximide significantly inhibited the ability of IL-6/sIL-6R to induce ROR2 mRNA expression ($P=0.0495$) (Fig. 2C). Taken together, these results suggest that IL-6 accelerates calcification by indirectly induc
ing the transcription of ROR2 mRNAs in hADSCs.

**STAT3 is involved in IL-6-mediated ROR2 mRNA expression in hADSCs**

As STAT1 and STAT3 play important roles in the IL-6 signalling pathway, we investigated their involvement in IL-6-induced ROR2 mRNA expression in hADSCs. Phosphorylated STAT1 and STAT3 were not detected in OIM cultures. However, the administration of IL-6/sIL-6R to hADSC cultures stimulated the nuclear transport of both STAT1 and STAT3 (Fig. 3A). We then examined the effects of inhibiting STAT1 and STAT3 expression on the IL-6 signalling pathway. The expression of STAT1 and STAT3 was effectively suppressed using two different siRNA sequences for each protein (STAT1: siSTAT1#1 and siSTAT1#2; STAT3: siSTAT3#1 and siSTAT3#2). siSTAT3 did not affect IL-6/sIL-6R-induced STAT1 phosphorylation and siSTAT1 did not affect STAT3 phosphorylation (Fig. 3B). In this experiment, hADSCs were cultured in OIM for 8 days after transduction with these siRNAs and protein expression levels were assessed by western blotting. Treatment with either sequence markedly reduced the protein levels of STAT1 and STAT3 compared with administration of the control sequence (Fig. 3C). There were no apparent changes in WNT5A mRNA and ROR2 mRNA expression levels following the suppression of either STATs under OIM conditions (Fig. 3E).

Next, hADSCs were cultured in OIM supplemented with 100 ng/ml of IL-6/sIL-6R after siRNA and ROR2 mRNA expression was measured 24 h later. While inhibition of

**Fig. 3** STAT3 plays an important role in IL-6-mediated ROR2 mRNA expression

(A) Nuclear and cytoplasmic extract from hADSCs stimulated with IL-6/sIL-6R for 30 min were collected. Expression and phosphorylation of STAT1 and STAT3 were detected by western blotting. (B, C) hADSCs were transfected with STAT1 (siSTAT1#1, 2), STAT3 (siSTAT3#1, 2) or control siRNAs (siCon) for 2 days. hADSCs stimulated with IL-6/sIL-6R within 30 min (B) or cultured with OIM alone for 8 days (C) were collected. Expression and phosphorylation of STAT1 and STAT3 were detected by western blotting. (D, E) Transfected hADSCs were cultured in OIM with or without IL-6/sIL-6R (100 ng/ml). Total RNA was isolated from transfected hADSCs cultured in OIM with IL-6/IL-6R at 24 h and ROR2 (D) and WNT5A (E) mRNA expression was determined by real-time PCR. Values are the mean (s.e.m.) of three independent experiments. *$P<0.05$ by t-test. n.s: not significant.
STAT1 protein expression did not show any effect, inhibition of STAT3 protein expression reduced the ability of IL-6/sIL-6R to induce ROR2 mRNA expression (Fig. 3D). Conversely, inhibition of STAT1 or STAT3 did not affect WNT5A mRNA expression (Fig. 3E). These findings suggest that the nuclear transport of pSTAT3 in the IL-6 signalling pathway leads to the enhanced expression of ROR2 mRNA.

STAT3 is essential for IL-6/sIL-6R-induced osteoblast-like differentiation and mineralization

We examined the possible involvement of STAT1 and STAT3 in the acceleration of calcification in hADSCs by IL-6/sIL-6R. hADSCs were cultured in OIM in the absence or presence of IL-6/sIL-6R and incubated with control or STAT1/3 siRNAs. hADSCs were then cultured in OIM for 8 days. Mild calcification was observed in hADSCs cultured in OIM and the addition of IL-6/sIL-6R markedly accelerated calcification (Fig. 4A). Inhibition of STAT1 protein expression in cultured hADSCs using the two different siRNA sequences hardly affected calcification after 8 days of culture in the presence or absence of IL-6/sIL-6R (Fig. 4A). In contrast, inhibition of STAT3 protein expression markedly inhibited the accelerated calcification of hADSCs induced by IL-6/sIL-6R (Fig. 4B). Although STAT3 inhibition reduced calcification of IL-6/sIL-6R-treated hADSCs to levels close to those of controls, it hardly affected the calcification of hADSCs grown in OIM alone.

Next, we measured RUNX2 mRNA expression in hADSCs cultured in OIM in the absence or presence of IL-6/sIL-6R following transfection with control or STAT1/3 siRNAs. Incubation in OIM supplemented with IL-6/sIL-6R significantly up-regulated RUNX2 mRNA expression compared with hADSCs cultured in OIM alone (Fig. 4C). Inhibition of STAT1 protein expression did not alter RUNX2 mRNA expression, whereas inhibition of STAT3 reduced IL-6/sIL-6R-induced RUNX2 mRNA expression in hADSCs (Fig. 4C). These results suggest that STAT3 is involved in calcification and osteoblast-like differentiation through a pathway independent of OIM.

IL-6 and RUNX2 are expressed in the subcutaneous adipose tissue from a patient with DM and ectopic calcification

Finally, we examined a biopsy of a calcified lesion from a 26-year-old female suffering from DM with multiple calcifications in the subcutaneous adipose tissue. The patient also had numerous subcutaneous and/or intramuscular calcifications in the extremities, abdomen and buttocks. The biopsy sample was obtained from an osteoid mass in the left thigh. Polarization microscopy was performed after immunostaining of the epidermal, dermal and adipose tissues with anti-IL-6 (Fig. 5A and B) and anti-RUNX2 antibodies (Fig. 5D and E). Inflammatory changes were noted in the dermal and adipose tissues, and irregular calcium deposits were detected around collagen fibres in the dermis. Immunostaining revealed IL-6-positive cells in collagen fibres in the dermis and RUNX2-positive cells around these cells. Immunohistochemical staining revealed that IL-6 was produced by CD4 T cells and presumably fibroblasts (supplementary Fig. S1, available at Rheumatology Online). Although hADSCs could not be observed because of a technical restriction, the cellular effects of IL-6-induced osteoblast-like differentiation were observed in the patient’s tissues.

Discussion

The cause and mechanisms of ectopic calcification in inflammatory diseases are poorly understood. The current study investigated the effects of inflammatory cytokines on the mechanisms of calcification in hADSCs. To the best of our knowledge, this is the first study to demonstrate that inflammatory cytokines can accelerate calcification in hADSCs. Of the cytokines tested, IL-6 enhanced calcification to the greatest degree. In addition, IL-6 expression was detected in the calcified subcutaneous tissues obtained from a patient with DM and ectopic calcification, suggesting that IL-6 is involved in the pathology of the disease.
The canonical and non-canonical WNT pathways play important roles in osteoblast differentiation [15-17]. The current study suggests that IL-6 stimulation of hADSCs could also activate the non-canonical WNT pathway [18, 19]. WNT5A is constitutively expressed in hADSCs, and IL-6 stimulation did not increase WNT5A mRNA expression further. The enhanced expression of ROR2 mRNA was detected 24 h after OIM/IL-6 administration, but not after stimulation with OIM alone, or stimulation with OIM supplemented with TNF or IL-1β (Fig. 2 and data not shown). However, pretreatment of the cells with cycloheximide suppressed the enhancement of ROR2 mRNA. These findings suggest that indirectly enhanced ROR2 mRNA expression through IL-6-mediated signalling may induce osteoblast-like differentiation. Furthermore, luciferase assay with the ROR2 promoter revealed that IL-6 did not directly induce ROR2 transcription after a short period of IL-6 stimulation (data not shown).

Of all the inflammatory cytokines tested, IL-6 had the most potent effect on calcification in hADSCs. Previous studies reported that the nuclear transport of phosphorylated STAT3 is critical in IL-6-stimulated osteoblast-like differentiation [14, 20-23]. Consistent with these findings, we observed that stimulation of hADSCs with IL-6 triggered the nuclear transport of STAT3. Furthermore, STAT3-specific siRNA, but not STAT1, blocked the acceleratory effects of IL-6 on osteoblast-like differentiation of hADSCs. The pro-calcifying effects of OIM were not inhibited by STAT3 inhibition, suggesting that the induction of osteoblast-like differentiation by OIM and IL-6 may occur through different signalling pathways. Interestingly, stimulation of hADSCs with IL-6 alone did not induce...
calcification, indicating that an as yet unidentified factor is required for IL-6-induced calcification. IL-6 may directly regulate the transcription of this factor, as STAT3-specific siRNA blocked IL-6-induced ROR2 mRNA expression. Furthermore, a GAS motif, the putative cis element for STAT3, transcription factors activated downstream of IL-6, is present in the promoter region of ROR2. IL-6-induced intracellular signalling can be promoted from its receptor components, gp130 and IL-6Rα or sIL-6R [24, 25]. Of these, IL-6 receptor components, only gp130 expression was observed in hADSCs. However, in the subcutaneous adipose tissues in vivo, sIL-6R may be present because of the shedding action of monocytes, which in turn activates IL-6 [26].

RUNX2 is a master transcription factor indispensable for the differentiation of progenitor cells into osteoblasts [27]. Treatment of hADSCs with OIM alone induced their differentiation into osteoblasts, but calcification only occurred after RUNX2 mRNA expression. In the presence of inflammatory cytokines, calcification was accelerated. Notably this was accompanied by enhanced RUNX2 mRNA expression, suggesting that cytokine-induced calcification involves osteoblast-like differentiation. However, in the current study, receptor activator of nuclear factor xB ligand, a late-stage osteoblast differentiation marker, and osteocalcin were not expressed by hADSCs (data not shown). This suggests that normal osteoblast differentiation did not occur in our in vitro culture system. This finding could be explained by the observation that peroxisome proliferator-activated receptor gamma, a master transcription factor for adipocyte differentiation, is expressed at higher levels in hADSCs (i.e. adipocyte progenitor cells) than in MSCs derived from other tissues. Thus this effect would counteract osteoblast-like differentiation [28–30]. It is important to note that the hADSCs used in the current study originated from healthy individuals and that hADSCs are largely resistant to osteoblast differentiation [31]. However, hADSCs isolated from patients with complications of ectopic calcification might have defective mechanisms of calcification inhibition.

Finally, we assessed IL-6 and RUNX2 expression in the subcutaneous adipose tissues, providing evidence of ectopic calcification from a patient with DM. IL-6-producing cells were frequently observed and cells expressing RUNX2 were prominent around the IL-6-producing cells. The relevance of serum IL-6 levels to vascular endothelial damage followed by calcification has been reported [32] and the involvement of IL-6 in the pathological processes of atherosclerosis has been reviewed [33]. However, the effect of blocking IL-6 on the pathogenesis of atherosclerosis is unknown. As some patients with JDM do not develop ectopic calcification, it seems likely that the causal factors of calcification are not only inflammatory cytokines. A recent study detected abnormal function and differentiation of MSCs in patients with SLE. Therefore abnormalities in hADSCs may also be involved in the pathogenesis of JDM. Future research is required to examine hADSCs in patients with JDM showing ectopic calcification.

In conclusion, we have developed an OIM-based system to induce osteoblast-like differentiation and observed that activation of STAT3 by IL-6/sIL-6R induced ROR2 mRNA expression in hADSCs. Thus the expression of ROR2 and WNT5A may induce activation of the non-canonical WNT pathway, leading to acceleration of calcification. IL-6-producing cells and surrounding RUNX2-expressing cells were detected in the subcutaneous adipose tissues from a patient with DM and ectopic calcification. These results suggest that inflammatory cytokine-induced ectopic calcification in hADSCs may also occur in chronic inflammatory diseases associated with other autoimmune diseases and arteriosclerosis. Therefore the development of IL-6 inhibitors may be an effective strategy for treating ectopic calcification in JDM and other inflammatory disorders.

**Rheumatology key messages**

- IL-6 accelerated mineralization, RUNX2 mRNA and ROR2 expression in adipose tissue derived-mesenchymal stem cells (ADSCs).
- IL-6 and RUNX2 were expressed in calcified subcutaneous adipose tissues from a DM patient.
- IL-6 acts on ADSCs and plays a role in ectopic calcification.

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**Supplementary data**

Supplementary data are available at Rheumatology Online.
References


