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The Effect of Recombinant Human Interleukin-6 on Osteogenic Differentiation and YKL-40 Expression in Human, Bone Marrow–Derived Mesenchymal Stem Cells

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Abstract

Human mesenchymal stem cells are an attractive cell source for tissue engineering and regenerative medicine applications, especially because of their differentiation potential toward the mesenchymal lineage. Furthermore, this cell type participates in the regeneration of tissue damage and plays an important role in immunity. Similarly, chitinase-like proteins have been proposed to aid in tissue remodeling, inflammation, and differentiation processes. The chitinase-like protein YKL-40 in particular is indicated in preventing damage to the extracellular matrix in response to proinflammatory cytokines, even though its biological function remains speculative. Finally, interleukin (IL)-6, a pleiotropic acute phase protein, participates in the regulation of bone turnover and immunoregulation. The physiological role of IL-6 in bone homeostasis is complex, exerting different effects on osteoblasts and osteoclasts depending on their differentiation stage. The aim of this study was to determine the effect of recombinant human IL-6 (5 ng/mL) on *YKL-40* expression and osteogenic differentiation of human mesenchymal stem cells. Recombinant human IL-6 induced a donor-dependent change in mineralization and significantly promoted YKL-40 protein secretion. However, *YKL-40* gene expression remained unaffected, and no statistically significant differences in the expression of osteogenic marker genes could be observed.

Key words: chitinase-like protein YKL-40; mesenchymal stem cells; osteogenic differentiation; recombinant human interleukin-6

Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells, residing as a heterogeneous cell population in the bone marrow stroma, adipose tissue, umbilical cord blood, and other tissues.[1] MSCs participate in the regeneration of tissue damage by migrating to sites of inflammation in response to damage-associated chemokines and cytokines, and they attract immune cells to the location of injury.[2] In addition, MSCs play an important role in immunity and can modulate immune responses by cell contact–dependent mechanisms and secretion of cytokines and growth factors, affecting regeneration and inflammation at the sites of tissue injury.[3] Osteoblasts are the major bone-forming cells and originate from MSCs via tightly regulated expression of bone-specific transcription factors and matrix proteins.[4]

The mechanism of osteogenic differentiation can be divided into two stages: initiation and maturation phases.[5] During initiation of osteogenic differentiation, cells slowly proliferate, express several osteogenic marker genes, and secrete collagen type I, which is the main structural component of bone extracellular matrix (ECM).[6] Mineralization of the matrix by deposition of calcium phosphate substituted hydroxypatite characterizes the maturation phase, commonly initiated 2–3 weeks after induction of the differentiation process.[7] Successful osteogenic differentiation *in vitro* is accompanied by the sequential expression of the functional matrix proteins collagen type I, osteopontin, and osteocalcin.[4,6] Collagen type I is secreted during the initiation phase of the differentiation process and is an essential part of the final mineralized ECM.[6] Osteopontin, an intermediate stage marker expressed by immature osteoblasts, is believed to have functions in the stabilization of the matrix, whereas osteocalcin, a late-stage osteogenesis marker, may participate in the final mineralization of the ECM.[6] Bone is a complex tissue defined by constant turnover and remodeling in

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response to environmental and endogenous stimuli.[8] In addition to maintaining the rigid structure of the skeletal system to allow movement and loading, bone needs to be both light and flexible.[9] The sophisticated and tightly controlled interplay of several cell types is the basis for the regulation of this intricate process, providing appropriate cues for the maintenance of bone structure, function, and remodeling.[9]

The chitinase-like protein YKL-40 (CHI3L1, HCgp39) is a secreted, 40-kDa mammalian glycoprotein, expressed by articular chondrocytes, differentiated macrophages, synoviocytes, and osteoblasts.[10,11] *In vivo* biological function remains controversial, but crystallographic analysis shows that binding of a putative ligand can induce conformational changes in the protein, indicating a potential signaling role.[12] Upregulation of YKL-40 in several inflammatory and degenerative diseases (e.g., rheumatoid arthritis, osteoarthritis, and certain cancers) implies that YKL-40 in particular might prevent damage to the ECM by reducing the deleterious effects of proinflammatory cytokines.[13,14] Furthermore, since YKL-40 is expressed in normal bone marrow, a connection between the host response to inflammation and the process of tissue repair seems plausible.[15,16]

Interleukin (IL)-6 belongs to the gp130 cytokine family of receptor subunit signal transducers and is generally considered an acute phase protein with pleiotropic activity.[17,18] The physiological role of IL-6 is manifold, ranging from immunoregulation and participation in hematopoiesis, to the regulation of bone turnover and angiogenesis.[18] The IL-6–mediated *in vivo* effects on bone homeostasis are generally considered to be shifted towards bone resorption rather than bone formation. Since the bone environment is naturally rich in IL-6, mesenchymal precursor cells are known to express low levels of IL-6 receptor, which is essential for IL-6 signal transduction. MSC-derived osteoblast precursor cells are believed to either gain expression of IL-6 receptor during homing to sites of bone remodeling or may require the presence of soluble IL-6 receptor for complete activation.[17]

We have recently showed that low levels of lipopolysaccharide (10 ng/mL) improve osteogenesis, increase cytokine secretion (especially IL-6), and strongly promote YKL-40 gene expression and protein secretion.[11] In the present study, we determined whether recombinant human (rh) IL-6 (5 ng/mL) may be responsible for the effects seen on osteogenic differentiation and YKL-40 expression in human, bone marrow–derived MSCs.

Materials and Methods

Cell culture and proliferation

Cell culture experiments were carried out with human, bone marrow–derived MSCs (Lonza, Basel, Switzerland) in Dulbecco's modified Eagle's medium/F12 media (Gibco/Life Technologies, Carlsbad, CA) supplemented with penicillin/streptomycin (Invitrogen/Life Technologies, Carlsbad, CA) and 10% MSC-approved fetal calf serum (Stemcell Technologies, Vancouver, Canada) at 37°C, 5% CO_2, and 95% humidity. To induce osteogenic differentiation, basal expansion media was switched to Differentiation Basal Medium Osteogenic (Lonza) supplemented with dexamethasone, ascorbate, L-glutamine, streptomycin/penicillin, β-glycerophosphate, and MSC growth supplement, and 4000 cells/cm^2 were seeded. Three independent donors were used to determine the effect of the continuous presence of 5 ng/mL rhIL-6 (ORF Genetics, Kopavogur, Iceland) in the culture media on osteogenic differentiation of human (h)MSCs. Proliferation and viability were assessed with MTT proliferation assay (LGC Standards AB, ATCC Bioproducts, Boras, Sweden) following standard protocols.

Analysis of gene expression

RNA isolation was performed using Qiagen BioRobot workstation (Qiagen, Hilden, Germany) and the EZ-1 RNA Cell Mini Kit (Qiagen) following manufacturer's instructions. Samples were homogenized in a FastPrep 24-instrument (MP Biomedicals, Santa Ana, CA) using Lysing Matrix D tubes (MP Biomedicals) containing 1.4-mm ceramic spheres before RNA isolation. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies, Carlsbad, CA) was used for transcription of RNA with a master mix containing 2.0 μL of reverse transcription (RT) buffer, 0.8 μL of 25 $\times$dNTP (100mM), 2.0 μL of 10 $\times$ Random Primers RT, 1.0 μL of Multiscribe reverse transcriptase, 1.0 μL of RNase inhibitor, and 3.2 μL of nuclease-free H_2O per sample. Cycling conditions were as following: 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, and then 4°C. Samples were stored at −20°C before quantitative polymerase chain reaction (qPCR) analysis.

Gene expression of selected genes was quantified using the 7500 Real Time PCR System (Applied Biosystems). Ten microliters of ready-made Taqman master mix (Applied Biosystems), 1 μL of Taqman assay (Applied Biosystems), and 9 μL of 1:10 diluted sample cDNA were prepared per sample. Samples were analyzed at least in duplicate for each of the three donors. Taqman assays used during this study were: *YKL-40* (Hs00609691_m1), *COL1A2* (collagen type I; Hs01028970_m1), *ALP* (alkaline phosphatase; Hs01029141_g1), and *RUNX-2* (runt-related transcription factor 2; Hs00231692_m1). Data analysis of qPCR results was performed using GenEX 5.3.2.13 software (MultiD, Göteborg, Sweden). Missing data were handled with RT-PCR replicates and outliers determined and deleted using Grubb's test. Gene expression results were globally normalized with a cut-off at Ct = 34. In the next step, technical repeats were averaged and relative quantities were calculated from the average of control samples from three independent donors. The logarithm was taken and different data sets were prepared for Ct values, mean centering, and auto-scaling of samples, and after transposition as well to analyze the genes.

Validation of osteogenic differentiation

To determine ALP activity, cell lysates were incubated for 30 min in a solution of 1 mg/mL p-nitrophenyl phosphate in 0.2 M Tris buffer (Sigma Aldrich Inc., St. Louis, MO) in distilled water (dH_2O) at 37°C. Optical density was measured at 405 nm in a MultiSkan spectrum spectrometer. Alkaline phosphatase activity was calculated as nanomoles of p-nitrophenol per minute following the general Beer-Lambert law.

For specific staining of calcification during osteogenic differentiation, medium was removed and cell layers washed with phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde. Next, cells were washed with dH_2O and then stained with a 2% Alizarin Red solution (Sigma Aldrich

Inc.) in dH_2O at pH 4.1 for 20 min. Cell layers were then washed with dH_2O, and pictures were taken in an inverted microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany) with Infinity Capture 5.0.2 software. For quantification, stained and dried cell layers were hydrated overnight in dH_2O, then incubated in a 10 % cetyl-pyridinium chloride (Sigma Aldrich Inc.) solution in dH_2O; optical density was measured at 562 nm in a MultiSkan spectrum spectrometer.

To validate Alizarin Red staining of calcification, von Kossa staining of mineralization was performed. Cells were washed with PBS and fixed in 4% paraformaldehyde. Standard protocols were used for von Kossa staining and images were taken in a fluorescent microscope (Olympus BX51, Olympus, Center Valley, PA) using Cell A Imaging Software (Olympus).

Polystyrene beads cytokine assay

Secretion of eight cytokines into medium supernatants was detected using a custom-designed Luminex 8Plex Human Cytokine assay (Panomics/Affymetrix, Santa Clara, CA) with a detection limit of 1 pg/mL. Supernatant aliquots were stored at −80°C and were analyzed in duplicate. The assay was performed according to manufacturer's instructions. Cytokines determined were IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p(40), RANTES, and tumor necrosis factor (TNF)-α.

YKL-40 enzyme-linked immunosorbent assay

Levels of YKL-40 in the media supernatants of cells after 21 days of osteogenic differentiation were determined using a sandwich enzyme-linked immunosorbent assay (ELISA; MicroVue YKL-40, Quidel, San Diego, CA) with a detection limit of 5.4 ng/mL. Supernatant aliquots were stored at −80°C and were analyzed in duplicates. ELISA was performed according to manufacturer's instructions and concentration of YKL-40 determined from a standard curve as nanograms per milliliter.

Statistical analysis

Data are presented as mean plus or minus standard error. Statistical analysis was performed using Prism 5.01 software (GraphPad Software Inc., San Diego, CA) and for qPCR analysis using GenEx 5.3.2.13 software. *T*-test was used to evaluate the effect of IL-6 during osteogenic differentiation of hMSCs; $p < 0.05$ was considered statistically significant. Three independent donors were used in all experiments.

Results

Proliferation

The presence of rhIL-6 (5 ng/mL) during short-term (single passage) expansion of hMSCs did not result in any changes in attachment, cell morphology, or proliferation (Fig. 1). Cells grew with typical spindle-shaped morphology and proliferation followed a latent exponential growth curve, as traditionally observed for this cell type.[19] During osteogenic differentiation, morphological changes in cell shape were observed and calcium hydroxyapatite crystals were deposited. The rhIL-6 did not affect osteoblastic cell morphology, yet a visual increase in collagen deposition could be observed, causing the contraction of cell layers and finally detachment of the complete cell sheet after 24 days in culture (visual observations, data not shown).

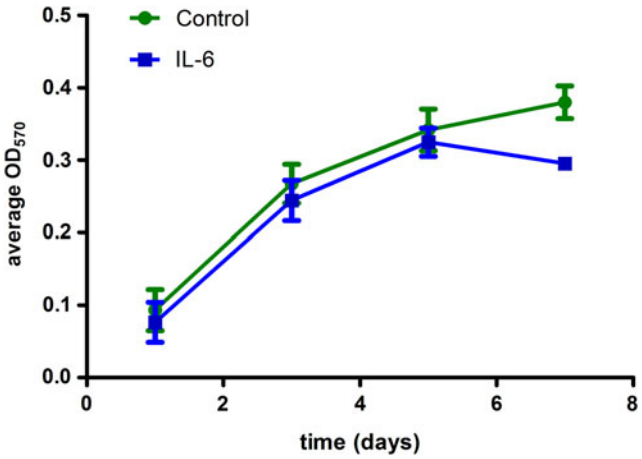

FIG. 1. Proliferation of human mesenchymal stem cells (hMSCs) during short-term expansion. Proliferation was determined using MTT proliferation assay at days 1, 3, 5, and 7. Error bars are standard error ($n = 15$).

Osteogenic gene expression

The presence of rhIL-6 did not significantly alter the expression of any of the genes studied (Fig. 2). The lack of significance during statistical analysis is due to the very high standard error introduced by the use of three independent donors, resulting in donor variation. In comparison to the control group, rhIL-6 stimulation increased the expression of *ALP* by 12.60 ± 11.75 fold, the expression of *RUNX-2* by 2.51 ± 1.95 fold, and the expression of *COL1A2* by 13.74 ± 12.73 fold, respectively.

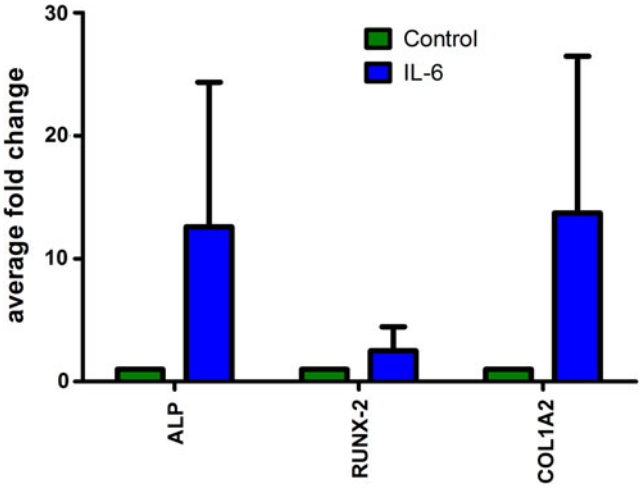

FIG. 2. Effect of recombinant human interleukin (rhIL)-6 on osteogenic gene expression. Expression of alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX-2*), and collagen I (*COL1A2*), determined after 21 days of osteogenic differentiation. Error bars are standard errors ($n = 6$).

Validation of osteogenic differentiation

After 18 days of osteogenic differentiation, the activity of ALP was not statistically different between control samples (2.42 ± 0.71) and rhIL-6–stimulated samples (2.73 ± 0.40) (Fig. 3A). The deposition of calcium hydroxyapatite crystals was determined by von Kossa staining and Alizarin Red staining, with the latter being specific for calcification and quantifiable with the aid of cetyl-pyridinium chloride. Visual examination of von Kossa stainings showed dark brown deposits, in the presence of rhIL-6, which were indicative of mineralization, whereas no signs of mineralization were observed in the control samples after 21 days of osteogenic differentiation (Fig. 3B). The equivalent staining by Alizarin Red strongly visualized the effects of donor variation on the deposition of calcium hydroxyapatite (Fig. 3C). One donor showed minimal calcification in the control samples, while the presence of rhIL-6 was accompanied by the complete absence of calcium-induced retention of the Alizarin Red stain (Fig. 3C, upper panel). A second donor displayed stronger overall calcification, with an increased number of calcified modules in the presence of rhIL-6 (Fig. 3C, lower panel). The quantitation of the staining in the two previously mentioned donors compensated for the donor variation and ultimately showed no difference in the calcification state between the control samples (0.1135 ± 0.003) and IL-6–stimulated samples (0.1299 ± 0.011; Fig. 3D).

Cytokine secretion into media supernatants

The secretion of several pro- and anti-inflammatory cytokines was determined after 20 days of osteogenic differentiation to evaluate whether the presence of rhIL-6 affected overall cytokine secretion into media supernatants. No secretion of IL-1β, IL-10, or RANTES was observed in control cells or rhIL-6–stimulated cells. Minimal secretion of IL-4, IL-12p(40), and TNF-α was seen, but no difference between the groups could be identified (data not shown). As expected, the levels of IL-6 in media supernatants were significantly higher in rhIL-6–stimulated cultures (543.825 ± 55.843 pg/mL in rhIL-6 samples versus 7.990 ± 0.418 pg/mL in control samples; $p < 0.001$; Fig. 4). The expression of the proinflammatory cytokine IL-8 was high, both in control samples (104.082 ± 2.904 pg/mL) and rhIL-6–stimulated samples (95.992 ± 10.167 pg/mL), but no statistical difference was observed.

YKL-40 gene expression and protein secretion

The effect of rhIL-6 on the chitinase-like protein YKL-40 was evaluated both on the gene and the protein level.

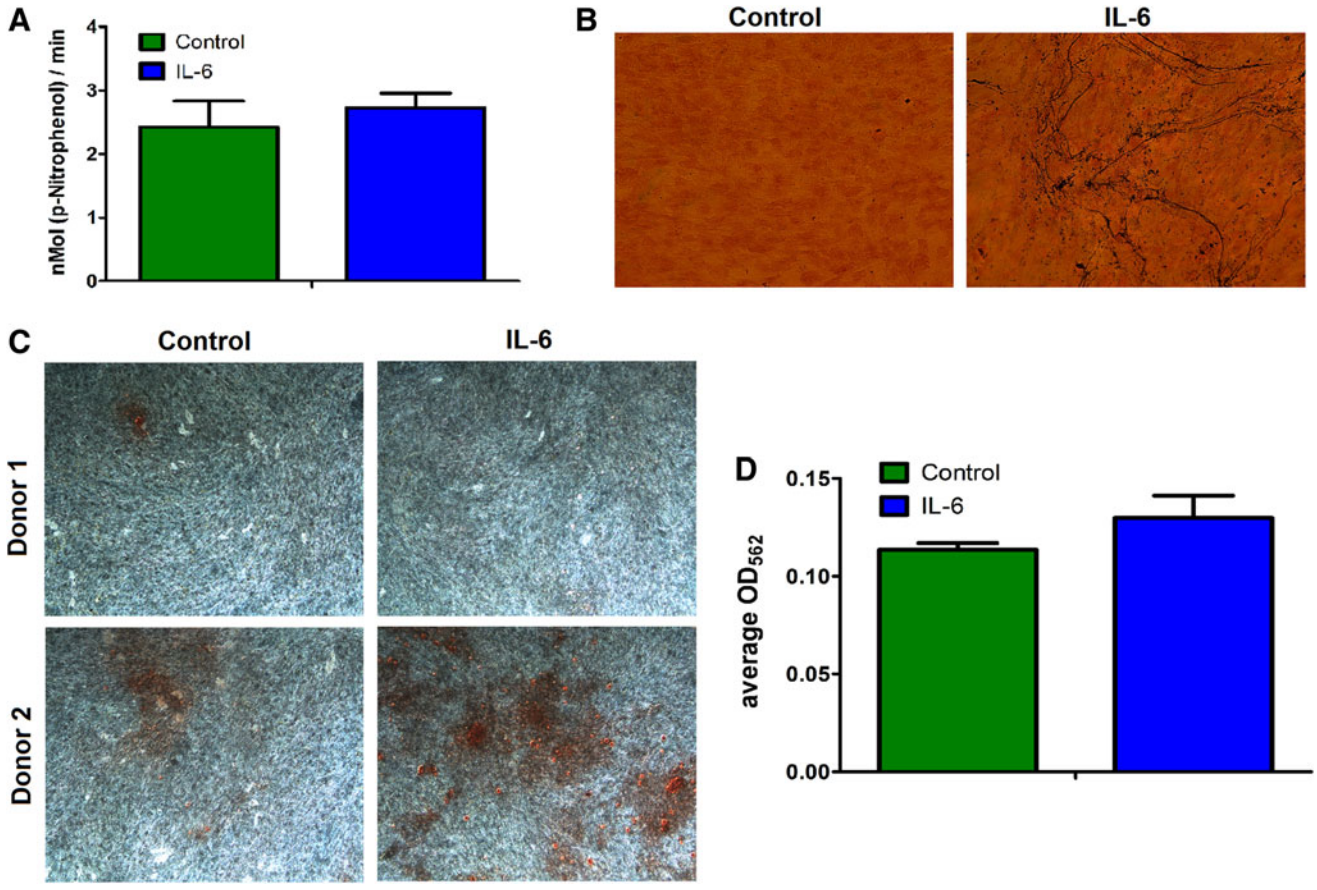

FIG. 3. Quality of osteogenic differentiation. **(A)** Alkaline phosphatase (ALP) activity at 18 days during osteogenic differentiation. Error bars are standard errors ($n = 3$). **(B)** von Kossa Staining of mineral deposition during osteogenic differentiation. Brown color depicts mineralization. Pictures are representative from three donors. **(C)** Alizarin Red staining of calcification at 21 days of osteogenic differentiation. Red staining is specific for calcium deposition. Pictures of two independent donors are shown to visualize donor variation in calcification. **(D)** Alizarin Red quantitation after 21 days of osteogenic differentiation. Error bars are standard errors ($n = 3$).

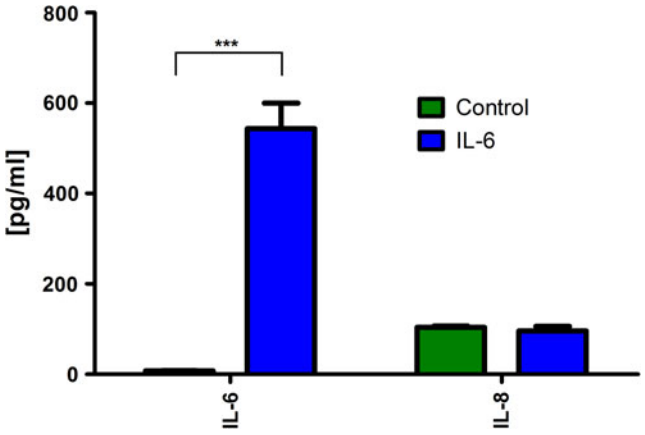

FIG. 4. Effect of rhIL-6 on cytokine secretion during osteogenic differentiation. Secretion of interleukin (IL)-6 and IL-8 at 20 days of osteogenic differentiation. Error bars are standard errors ($n = 6$); ***$p < 0.001$.

Following 21 days of osteogenic differentiation, no difference in the expression of the YKL-40 gene was observed between the groups (1.2 ± 0.22 fold increase in rhIL-6 versus control; Fig. 5A). However, the secretion of YKL-40 protein was significantly higher in samples stimulated with rhIL-6 compared to control samples (24.43 ± 3.14 ng/mL in control samples versus 40.40 ± 3.70 ng/mL in rhIL-6 samples; $p < 0.01$; Fig. 5B).

Discussion

In this study, we report the effect of rhIL-6 (5 ng/mL) on expression and secretion of YKL-40, as well as on osteogenic differentiation of hMSCs. The proinflammatory cytokine IL-6 is known for its role in bone turnover and promoting committed osteoprogenitor cells towards differentiation.[17] The hMSCs used in the present study were derived from the bone marrow environment, which is naturally rich in IL-6.[17] Therefore, the hypothesis that the hMSC osteoblastic phenotype is influenced by the proinflammatory cytokine IL-6 has long been abolished.[17,20] Here, we confirm this notion by the absence of any statistically significant response during the evaluation of the quality of osteogenic differentiation. However, we were able to show a donor-dependent increase in mineralization in the presence of rhIL-6, accompanied by a significantly higher secretion of YKL-40 protein after 21 days of osteogenesis.

In the presence of an osteogenic stimulus, hMSCs differentiated along the osteogenic lineage, as judged by the expression of osteogenic marker genes and mineralization of the ECM. rhIL-6 stimulation did not affect the quality of osteogenic differentiation, except for a nonsignificant increase in collagen production (visual observation) and a donor-dependent difference in calcification. Osteogenic differentiation of bone marrow–derived hMSCs and murine MSCs was previously shown to remain unaffected by rhIL-6 when soluble IL-6 receptor was absent.[17,21]

We have recently shown that low amounts of lipopolysaccharide can strongly promote osteogenic differentiation of hMSCs as seen in increased marker gene expression, ALP activity, and mineralization. This increase in differentiation was accompanied by significantly increased cytokine secretion, namely IL-6, IL-8, and RANTES, as well as higher levels of YKL-40 gene expression and protein secretion.[11] rhIL-6 has been linked to increased plasma YKL-40 levels in a human model of sepsis.[16] On the other hand, Recklies et al.[16] showed that rhIL-6 alone was not sufficient to induce *YKL-40* gene expression in a murine primary chondrocyte model. There was no mention of the concentration that was applied, but their results correlate very well with ours because we also did not see *YKL-40* gene expression induced by rhIL-6.[16] However, we did see an increase in YKL-40 protein secretion after 21 days of osteogenic differentiation in the presence of rhIL-6. It is possible that *YKL-40* gene expression was affected at intermediate stages during the differentiation process because we only determined gene expression at the end of the differentiation period (21 days) and the aforementioned study focused on early time points (24 and 48 h)[16]. In addition, rhIL-6 may affect the posttranslational regulation of YKL-40, independent of transcriptional regulation of gene expression. This potential effect of rhIL-6 on YKL-40 protein should be determined in further studies.

Conclusion

In this study, we showed the effect of rhIL-6 on the expression of YKL-40 and the biology of osteogenic differentiation of human, bone marrow–derived MSCs. rhIL-6 did not affect the quality of osteogenic differentiation after 21 days in culture, yet a donor variation–dependent increase

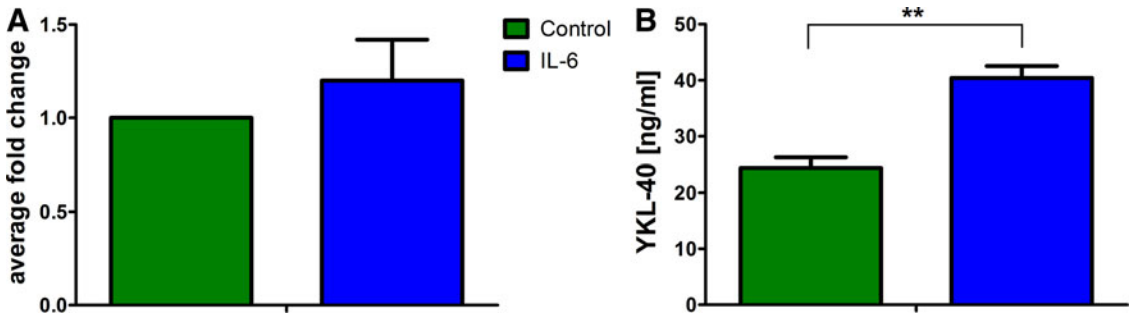

FIG. 5. Effect of rhIL-6 on the chitinase-like protein YKL-40. **(A)** Expression of *YKL-40*, determined after 21 days of osteogenic differentiation. Error bars are standard errors ($n = 6$). **(B)** YKL-40 protein secretion during osteogenic differentiation of hMSCs. Error bars are standard errors ($n = 6$); **$p < 0.01$.

in mineralization could be observed. The expression of *YKL-40* in the presence of rhIL-6 was comparable to control samples, whereas the secretion of YKL-40 protein was significantly increased in three independent donors. The determination of how rhIL-6 is involved in the posttranscriptional regulation of YKL-40 could aid in better understanding the physiological role of YKL-40 and narrow the search for potential endogenous ligands.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ALP = alkaline phosphatase
COL1 = collagen type I
dH_2O = distilled water
ECM = extracellular matrix
ELISA = enzyme-linked immunosorbent assay
hMSC = human mesenchymal stem cell
IL = interleukin
MSC = mesenchymal stem cell
PBS = phosphate-buffered saline
qPCR = quantitative polymerase chain reaction
rh = recombinant human
RT = reverse transcription
RUNX-2 = runt-related transcription factor 2
TNF = tumor necrosis factor