Inhibition of TAK1 and/or JAK Can Rescue Impaired Chondrogenic Differentiation of Human Mesenchymal Stem Cells in Osteoarthritis-Like Conditions

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Objective: To rescue chondrogenic differentiation of human mesenchymal stem cells (hMSCs) in osteoarthritic conditions by inhibition of protein kinases.

Methods: hMSCs were cultured in pellets. During early chondrogenic differentiation, these were exposed to osteoarthritic synovium-conditioned medium (OAS-CM), combined with the Janus kinase (JAK)-inhibitor tofacitinib and/or the transforming growth factor β -activated kinase 1 (TAK1)-inhibitor oxozaenol. To evaluate effects on chondrogenesis, the glycosaminoglycan (GAG) content of the pellets was measured at the time that chondrogenesis was manifest in control cultures. Moreover, mRNA levels of matrix molecules and enzymes were measured during this process, using real-time polymerase chain reaction (RT-PCR). Initial experiments were performed with hMSCs from a fetal donor, and results of these studies were confirmed with hMSCs from adult donors.

Results: Exposure to OAS-CM resulted in pellets with a much lower GAG content, reflecting inhibited chondrogenic differentiation. This was accompanied by decreased mRNA levels of aggrecan, type II collagen, and Sox9, and increased levels of matrix metalloproteinase (MMP)1, MMP3, MMP13, ADAMTS4, and ADAMTS5. Both tofacitinib (JAK-inhibitor) and oxozaenol (TAK1 inhibitor) significantly increased the GAG content of the pellets in osteoarthritis (OA)-like conditions. The combination of both protein kinase inhibitors showed an additive effect on GAG content. In agreement with this, in the presence of OAS-CM, both tofacitinib and oxozaenol increased mRNA expression of sox9. The expression of aggrecan and type II collagen was also up-regulated, but this only reached significance for aggrecan after TAK1 inhibition. Both inhibitors decreased the mRNA levels of MMP1, 3, and 13 in the presence of OAS-CM. Moreover, oxozaenol also significantly down-regulated the mRNA levels of aggrecanases ADAMTS4 and ADAMTS5. When combined, the inhibitors caused additive reduction of OA-induced MMP1 mRNA expression. Counteraction of OAS-CM-induced inhibition of chondrogenesis by these protein kinase inhibitors was confirmed with hMSCs of two different adult donors. Both tofacitinib and oxozaenol significantly improved GAG content in cell pellets from these adult donors.

Conclusions: Tofacitinib and oxozaenol partially prevent the inhibition of chondrogenesis by factors secreted by OA synovium. Their effects are additive. This indicates that these protein kinase inhibitors can potentially be used to improve cartilage formation under the conditions occurring in osteoarthritic, or otherwise inflamed, joints.

Introduction

ARTICULAR CARTILAGE IS non-vascularized and non-innervated and has a limited capacity to repair itself, thereby presenting a major clinical problem. Many efforts are made to tissue engineer cartilage or manipulate the joint to circumvent the incapability of natural repair. For tissue engineering purposes, stem cells are placed in a cartilage defect or stem cell recruitment from the bone marrow is

stimulated by penetrating the subchondral bone plate. However, cartilage requiring repair is generally located in a diseased joint and not in a healthy joint. This diseased joint will contain a mixture of factors that potentially will not benefit the chondrogenesis of the mesenchymal stem cells (MSCs) in the defect. Several studies showed that synovial fluid obtained from knees of patients with a traumatic chondral defect can inhibit chondrogenic redifferentiation of monolayer expanded human chondrocytes.^{1,2} However, it

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should be noted that these studies were performed with differentiated cells rather than true progenitor cells. Krüger *et al.*³ showed that chondrogenic differentiation of human subchondral progenitor cells is affected by synovial fluid from donors with osteoarthritis (OA) or rheumatoid arthritis (RA). Previously, we have shown that factors secreted by the synovial membrane of OA joints inhibit chondrogenic differentiation of human mesenchymal stem cells (hMSCs),⁴ thereby impairing successful tissue engineering. We could not discern a clear relationship between the levels of our primary suspects interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) in the osteoarthritic synovium-conditioned medium (OAS-CM) and the strength of inhibition of chondrogenesis.⁴ So, the exact catabolic factors and pathways responsible for the inhibition of chondrogenesis remain to be identified. Since specific knowledge on the nature of these factors is lacking, we aimed at modulating this effect by targeting common signaling pathways.

In the present study, we, therefore, inhibited protein kinases to block catabolic intracellular pathways in hMSCs. We focused on the inhibition of transforming growth factor β -activated kinase 1 (TAK1) and Janus kinases (JAKs), as TAK1 and JAKs are common routes of several specific, but different, cytokine signaling pathways. The TAK1 inhibitor oxozaenol was used in our model of OA-induced inhibition of chondrogenesis, due to the central role of TAK1 in the intracellular signaling of a number of important inflammatory cytokines and growth factors.^{5,6} In response to IL-1, TNF- α , toll-like receptor (TLR) agonists, and transforming growth factor β (TGF- β)/bone morphogenetic protein (BMP), it mediates the activation of the nuclear factor κ B (NF- κ B), c-Jun N-terminal kinase (JNK), and p38 pathways. TAK1 is now defined as an activating kinase for the I κ B kinase (IKK) complex, composed of IKK α , IKK β , and NF- κ B essential modulator, via association with TNF receptor-associated factor 2 (TRAF2) and TRAF6 in TNF- α and IL-1–TLR signaling pathways, respectively.^{7,8} We also used the JAK inhibitor tofacitinib (also known as CP-690,550) in our model of OA-induced inhibition of chondrogenesis. Tofacitinib primarily inhibits JAK1 and JAK3 and, to a lesser extent, JAK2. Most of the STAT-activating cytokine receptors (i.e., type I and type II cytokine receptors) do not have tyrosine kinase activity and instead require JAKs to initiate intercellular signaling. The JAK family of proteins are tyrosine kinases and constitute four members (JAK1, JAK2, JAK3, and TYK2) in mammals.^{9,10} The JAK/STAT signaling pathway is activated by numerous growth factors and cytokines, including members of the interferon (IFN) family, such as IFN γ and IL-10; of the gp130 family, such as IL-6, oncostatin M, and leukemia inhibitory factor; of the γ C family, such as IL-2; and of the single chain family, such as erythropoietin.^{11–15} Since JAK activation is needed for signaling through the receptors for cytokines that are integral to lymphocyte function,^{16,17} tofacitinib may be able to modulate many aspects of the immune response. Tofacitinib is in clinical development for the indications of RA, psoriasis, renal transplant prevention, inflammatory bowel disease, and dry eye. Phase III studies in RA show its efficacy and safety,^{18,19} and tofacitinib is recently approved by the FDA for the treatment of RA.

In the present study, we aimed at rescuing chondrogenic differentiation of hMSCs in OA-like conditions by blocking

the JAK/STAT or/and the TAK1 intracellular pathways. We showed that both protein kinase inhibitors can rescue the chondrogenesis that was impaired by synovial factors, and that jointly they can do more than separately.

Materials and Methods

Culture of hMSCs

Initial experiments were performed with hMSCs from a fetal donor (ScienCell Research Laboratories, Carlsbad, CA). Passage 2 bone marrow-derived hMSCs were expanded for 12 days, diluting these cells thrice during each passage. This was performed in Mesenchymal Stem Cell Growth Medium (Lonza, Verviers, Belgium). The hMSCs were stored in liquid nitrogen at passage 5. These passage 5 cells were expanded and passaged for another approximate 12 days before using these cells in the experiment at passage 7. At this passage, the cells retained their multilineage potential as determined by chondrogenic, osteogenic, and adipogenic differentiation experiments (data not shown).

Besides these fetal cells, we used hMSCs of two adult donors. Bone marrow was aspirated from patients undergoing total hip replacement surgery after informed consent was obtained. The procedures were approved by the local ethics committee of the Erasmus MC, University Medical Center Rotterdam (MEC 2004-142). hMSCs were isolated based on their plastic adherence. The heparinized bone marrow aspirates were seeded at a density of $2\text{--}5 \times 10^5$ cells/cm² in hMSC expansion medium consisting of Minimum Essential Medium- α (Gibco, Carlsbad, CA) that was supplemented with 10% fetal bovine serum (Lonza), 50 μ g/mL gentamicin (Gibco), 1.5 μ g/mL fungizone (Gibco), 1 ng/mL fibroblast growth factor 2 (AbD Serotec, Kidlington, United Kingdom), and 25 μ g/mL ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO). Non-adherent cells were washed off after 24 h, and adherent cells were further expanded. At subconfluence, hMSCs were trypsinized and replated at a density of 2300 cells/cm². MSC expansion medium was refreshed twice per week. Passage 3 cells were used for experiments.

Chondrogenic differentiation of hMSCs

hMSCs from fetal origin or from adult donors were chondrogenically differentiated by culturing in high cell density through pelletation (0.25×10^6 cells/pellet) in 0.5 mL of serum free chondrogenic differentiation medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with insulin (6.25 mg/mL), transferrin (6.25 mg/mL), sodium selenite (6.25 ng/mL), prolin (0.4 mg/mL), sodium pyruvate (1 mg/mL), linoleic acid (5.35 mg/mL), ascorbic acid (50 mg/mL), bovine serum albumin (BSA; 1 mg/mL), and dexamethasone (10^{-7} M) (Sigma-Aldrich). This serum-free chondrogenic differentiation medium was supplemented with TGF- β 1 (10 ng/mL; R&D Systems, Minneapolis, MN) and BMP2 (50 ng/mL; R&D Systems).²⁰ After pelletation, the medium was refreshed thrice a week, adding fresh growth factors each time.

Conditioned medium derived from OA synovium

Synovium was obtained from OA patients undergoing total hip replacement. Pieces of the joint capsule, with the synovial membrane on it, were used to produce OA

synovium-conditioned medium. In comparison to using monolayers of isolated synoviocytes, this method seemed more optimal, because it yields products of all cell types of the OA synovium, still embedded in the relatively intact synovial membrane. The synovium was divided into pieces and cultured in a six-well plate in DMEM with 0.1% BSA (0.3 g tissue/mL). The supernatant was collected after 24 h. Debris was removed by centrifugation, after which the medium was stored at -20°C . Two different pools of OAS-CM were used, each consisting of pooled OAS-CM from seven different OA patients. This was stored in small aliquots at -20°C until further use. The mean age of OA patients was 72.4 (SD 9.1) for pool 1 and 65.7 (SD 3.7) for pool 2.

Blocking of chondrogenesis by OAS-CM and use of protein kinase inhibitors to counteract this

hMSC of fetal donor. At day 3 after pelletation, 10% OAS-CM and protein kinase inhibitors were added. (5Z)-7-Oxozaenol (Tocris Bioscience, Bristol, United Kingdom) and tofacitinib (LC Laboratories, Woburn, MA) were used at concentrations of $1\text{ }\mu\text{g/mL}$ and 800 ng/mL , respectively. These concentrations were found to be at plateau level in initial dose-response studies based on glycosaminoglycan (GAG) content of pellets (data not shown). Pellets were preincubated with the inhibitors (or the solvent dimethyl sulfoxide [DMSO] only) for 10 min before the addition of the OAS-CM. The final concentration of all components of the chondrogenic differentiation medium, including the growth factors, was kept equal in all conditions. The media were replaced by fresh medium $\pm$ OAS-CM and/or inhibitors at day 5 after pelletation. After 24 or 72 h of exposure to OAS-CM and/or the inhibitors (days 4 and 6 respectively), pellets were harvested for mRNA isolation. At day 7 after pelletation, pellets were harvested for measurement of GAG content.

hMSCs of adult donors. Since it is known that the progression of chondrogenesis in MSCs from adult donors is slower than the chondrogenesis of MSCs from fetal donors, the timing of the administration of OAS-CM and protein kinase inhibitors was adjusted. To ensure proper timing of administration, treatment with OAS-CM and protein kinase inhibitors was started at different time points: from day 7, 14, or 21 on, the chondrogenic differentiation medium was supplemented with 10% OAS-CM (1:1 mix of pools 1 and 2) and 800 ng/mL tofacitinib, $1\text{ }\mu\text{g/mL}$ oxozaenol, or a combination of both inhibitors. The final concentration of all

components of the chondrogenic differentiation medium was maintained equal in all conditions. Pellets cultured in chondrogenic differentiation medium with or without 10 ng/mL TGF- β 1 and 50 ng/mL BMP2 served, respectively, as positive and negative controls. Since both inhibitors were dissolved in DMSO, levels of DMSO were equalized over all conditions to which OAS-CM, tofacitinib, or oxozaenol were added. To test whether DMSO had a negative effect on the chondrogenesis of hMSCs from adult donors, conditions in which pellets were cultured in chondrogenic medium with TGF- β 1 and BMP2 with additional DMSO were included. Medium was refreshed thrice per week. At days 7, 14, and 21 (time points at which treatment of pellets was started), pellets were harvested to evaluate chondrogenesis. The remaining pellets were cultured for 28 days and harvested for GAG quantification.

Measurement of GAG content in cell pellets

For measurements of the GAG content, each individual cell pellet was first digested overnight with 0.1% papain (Sigma-Aldrich) in digestion buffer (200 mM NaPO_4 , 100 mM NaAc , 5 mM cysteine HCl, 10 mM EDTA, and pH 6.4) at 60°C . Then, the GAG concentration of the digest was measured using the Farndale assay.²¹ This method is based on a shift in the maximum absorbance wavelength of the dye 1,9-dimethylmethylene blue after binding to multiple sulfate groups (metachromasia). The absorbance of dimethylmethylene blue after binding with the sulfate groups in samples of the digest was measured at 535 nm . Each experimental group consisted of three cell pellets.

RNA isolation and quantitative RT-PCR

At 24 and 72 h after the start of exposure to OAS-CM and protein kinase inhibitors, pellets were harvested for analysis of mRNA levels. Total RNA was extracted from the cell pellets (two pellets for each sample) using TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol. Isolated RNA was DNase treated and reverse transcribed. Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA). Reverse transcriptase (RT) primer nucleotide sequences are listed in Table 1. Real-time quantitative polymerase chain reaction (PCR) was performed using a StepOnePlus sequence detection system (Applied Biosystems). Messenger RNA levels were normalized to the housekeeping

TABLE 1. HUMAN PRIMERS USED FOR RT-qPCR

Gene	Fwd primer (5' $\rightarrow$ 3')	Rev primer (5' $\rightarrow$ 3')
Ribosomal protein S27a	GTTAAGCTGGCTGTCTGAAA	CATCAGAAGGGCACTCTCG
Aggrecan	GCCTGCGCTCCAATGACT	ATGGAACACGATGCCTTTTCAC
Type II collagen	CACGTACACTGCCCTGAAGGA	CGATAACAGTCTTGCCCCACTT
Sox9	TGGGCAAGCTCTGGAGACTT	CCCGTTCTTCACCGACTTCCT
MMP1	ACTGCCAAATGGGCTTGAAG	TTCCCTTTGAAAAACCGGACTT
MMP3	GAGGCATCCACACCCTAGTT	TCAGAAATGGCTGCATCGATT
MMP13	ATTAAGGAGCATGGCGACTTCT	CCCAGGAGGAAAAGCATGAG
ADAMTS4	AATTCAGGTACGGATACAACAATGTG	GCAGCTTCAGGGCCAAGTAG
ADAMTS5	GCTCACGAAATCGGACATTTACTT	ACCAAAGGTCTCTTCACAGAATTTG

RT-qPCR, real-time quantitative RT-PCR.

gene ribosomal protein S27a (RPS27a) levels, and all conditions and genes were expressed relative to the non-catabolic control condition (ddCt).

Histological analysis

Pellets were fixed in phosphate-buffered formalin for 7 days, followed by dehydration using an automated tissue-processing apparatus (Tissue Tek VIP; Sakura, Alphen aan den Rijn, The Netherlands). Next, the pellets were embedded in paraffin, and 7 mm tissue sections were prepared. Sections were mounted on Superfrost plus glass (Thermo Scientific, Waltham, MA), stained with Safranin O and Fast Green, and mounted with Permount.

Statistical analysis

Normality was verified with Kolmogorov–Smirnov and Shapiro–Willk tests using SPSS 15.0. When necessary, logarithmic transformation was performed to obtain normally distributed data. For unpaired data, the Student's *t*-test was performed. For normally distributed paired data, a generalized estimated equations model was used. For non-normally distributed paired data, a Kruskal–Wallis test was performed, followed by a Mann–Whitney *U* test. Correction for multiple testing was performed using Bonferroni correction. *p*-Values < 0.05 (two-tailed) were regarded as statistically significant.

Results

Time course of chondrogenic differentiation of hMSCs from fetal donors

To induce chondrogenesis of hMSCs from fetal donors, cell pellets were cultured in chondrogenic differentiation medium, including TGF- β 1 and BMP2, during 7 days. Histological sections (Fig. 1) show that at day 3 after pelletation the pellets were still small and did not stain with Safranin O. At day 4 after pelletation, the first signs of chondrogenic differentiation were visible. At day 7, Safranin O-stained histological sections showed that the pellets were strongly enlarged and stained positive for proteoglycans. The exposure to 10% OAS-CM during days 3–7 caused suppression of chondrogenic differentiation (Fig. 1).

The TAK1 inhibitor oxozeaenol improves chondrogenesis of hMSCs in OA-like conditions

GAG content of pellets at day 7 after pelletation of hMSCs from fetal origin was used as a measure for chondrogenic differentiation. Exposure to 10% OAS-CM from day 3 onward caused a decrease of more than 50% in the GAG content of the pellets at day 7 (Fig. 2A). Both pools of OAS-CM that were used, each consisting of OAS-CM from seven different OA patients, suppressed chondrogenesis, albeit stronger with pool 2 than pool 1. Supplementation with 1 μ g/mL oxozeaenol from day 3 onward significantly enhanced the GAG content of the pellets exposed to OAS-CM as compared with OAS-CM with only the solvent. The same pattern was found for the two different pools of OAS-CM.

For the measurement of mRNA levels using RT-PCR, cell pellets were harvested at day 4, which was 24 h after the start of exposure to OAS-CM $\pm$ inhibitor. The mRNA expressions of the cartilage-specific extracellular matrix (ECM) molecules aggrecan and type II collagen and of the transcription factor sox9, which is involved in chondrocyte differentiation and cartilage formation, were significantly decreased by the OAS-CM (Fig. 2B). Treatment with oxozeaenol counteracted this, although only for sox9 this counteraction reached statistical significance (Fig. 2B). mRNA expressions of all three matrix metalloproteinases (MMPs) and both aggrecanases studied were highly up-regulated by the OAS-CM at day 4 (Fig. 2C, D). About 1 μ g/mL oxozeaenol was able to significantly counteract this effect of the OAS-CM on all these enzymes, except for MMP13. At a later time point (day 6), when cartilage formation is much more advanced under control conditions, the same patterns were found (data not shown).

The JAK inhibitor tofacitinib improves chondrogenesis of hMSC in OA-like conditions

Supplementation with 800 ng/mL tofacitinib from day 3 onward significantly enhanced the GAG content of the pellets exposed to OAS-CM from pool 1 as compared with OAS-CM with only the solvent, where this was not significant for pool 2 (Fig. 3A). In this experiment, OAS-CM from pool 2 inhibited chondrogenesis relatively strong, and this could have hindered counteraction by tofacitinib. At day 4, which was 24 h after the start of exposure to OAS-

FIG. 1. Histological sections of human mesenchymal stem cell (hMSC) from fetal origin in pellet culture, at different stages of chondrogenic differentiation (days after pelletation). At day 7, the effect of exposure to osteoarthritic synovium-conditioned medium (OAS-CM) from the two different pools is shown. Sections were stained with Safranin O for proteoglycan staining and counterstained with Fast Green. Original magnification 50 $\times$. Color images available online at www.liebertpub.com/tea

FIG. 2. Transforming growth factor β -activated kinase 1 (TAK1) inhibition by oxozeaenol partially counteracts the effect of OAS-CM on fetal hMSCs. Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 1 μ M oxozeaenol from day 3 after pelletation. (A) Glycosaminoglycan (GAG) content of pellets at day 7 after pelletation. Values represent mean $\pm$ SD ($n=3$). (B–D) mRNA expression (ddCT) was measured at 24 h after the addition of 10% OAS-CM and/or 1 μ M oxozeaenol from day 3 after pelletation of hMSCs. Values represent mean $\pm$ SD ($n=6$). Generalized estimating equations model with correction for multiple testing. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

CM $\pm$ inhibitor, the mRNA expressions of aggrecan, type II collagen, and sox9 were significantly decreased by the OAS-CM, and treatment with tofacitinib significantly counteracted this, except for type II collagen (Fig. 3B). mRNA expressions of all three MMPs and both aggrecanases were highly up-regulated by the OAS-CM at day 4 (Fig. 3C, D).

About 800 ng/mL of tofacitinib was able to counteract this effect of the OAS-CM on all three MMPs (Fig. 3C). In contrast to TAK1 inhibition, JAK inhibition by tofacitinib had no significant effect on the enhanced levels of the aggrecanases ADAMTS4 and 5 in OA-like conditions (Fig. 3D). At a later time point (day 6), when cartilage

FIG. 3. Janus kinase (JAK) inhibition by tofacitinib partially counteracts the effects of OAS-CM on fetal hMSCs. Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 800 ng/mL tofacitinib from day 3 after pelletation. (A) GAG content of pellets at day 7 after pelletation. Values represent mean $\pm$ SD ($n=3$). (B–D) mRNA expression (ddCT) was measured at 24 h after the addition of 10% OAS-CM and/or 800 ng/mL tofacitinib from day 3 after pelletation of hMSCs. Values represent mean $\pm$ SD ($n=6$). Generalized estimating equations model with correction for multiple testing. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

formation is much more advanced under control conditions, the same patterns were found, including clear insensitivity of aggrecanase mRNA levels to tofacitinib (data not shown).

TAK1 and JAK inhibition contribute additively to the rescue of chondrogenesis of hMSC in OA conditions

Combinations of oxozaenol and tofacitinib were applied, to study whether this combination has more effect than the addition of a single inhibitor.

In this experiment (Fig. 4A), the GAG content of pellets at day 7 was again severely decreased after treatment with OAS-CM from day 3 onward. The effect of OAS-CM from both pools was significantly decreased by 1 μ M oxozaenol and by 800 ng/mL tofacitinib added from day 3 on. Further counteraction of OAS-CM was demonstrated when both inhibitors were combined. GAG content after the combined treatment was significantly increased compared with oxozaenol or tofacitinib alone. The same pattern was found for the two different pools of OAS-CM. mRNA levels at day 4 were measured using RT-PCR. In Figure 4C–E, it was tested whether the combination of the two inhibitors significantly counteracted OAS-CM and whether the effect of the combination was significantly stronger than that of the single inhibitors. In this experiment, up-regulation of the mRNA levels of aggrecan, type II collagen, and sox9 at day

4 (Fig. 4E) did not reach statistical significance, even when both inhibitors were combined. The effects on MMP expression were more clear. OAS-CM induced high mRNA levels of all three MMPs, and these were significantly decreased by the combination of both inhibitors. Only for MMP1, the combination showed stronger counteraction than each of the single inhibitors, indicating that their effects were additive in this regard (Fig. 4C). Down-regulation of MMP3 expression by oxozaenol alone appeared to be equivalent to the combined effect of both inhibitors. On the other hand, tofacitinib suppressed MMP13 mRNA levels equally well, as compared with the combination of both inhibitors. Overall (also at the later time point), in this experiment, tofacitinib had the strongest effect on MMP13 expression; while oxozaenol more efficiently blocked MMP1 and MMP3 expression, as compared with tofacitinib. These data confirm the experiments with single inhibitors (Figs. 2C and 3C) that also showed this relative difference in the regulation of MMP1 and MMP3 versus MMP13. OAS-CM-induced mRNA expression of aggrecanases (Fig. 4D) was significantly counteracted by the combination of both inhibitors. Additive effects were not found here. In fact, oxozaenol significantly counteracted OAS-CM-induced aggrecanase expression, while tofacitinib effects did not reach significance. This is in line with the experiments shown in Figures 2D and 3D, where tofacitinib was less

FIG. 4. TAK1 and JAK inhibitors are additive in counteraction of the effect of OAS-CM on fetal hMSCs. Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 1 μ M oxozaenol and/or 800 ng/mL tofacitinib from day 3 after pelletation. (A) GAG content of pellets at day 7 after pelletation. Values represent mean $\pm$ SD ($n=3$). (B) Histological sections of hMSC from fetal origin in pellet culture, showing the counteraction of the effect of OAS-CM by the combination of 1 μ M oxozaenol and 800 ng/mL tofacitinib. Sections were stained with Safranin O and Fast Green. Original magnification 50 $\times$. (C–E) mRNA expression (ddCT) was measured at 24 h after the addition of 10% OAS-CM $\pm$ 1 μ M oxozaenol and/or 800 ng/mL tofacitinib from day 3 after pelletation of hMSC. Values represent the mean $\pm$ SD ($n=6$). Generalized estimating equations model with correction for multiple testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Color images available online at www.liebertpub.com/tea

potent in the down-regulation of aggrecanases as compared with oxozaenol.

Effects of protein kinase inhibitors on cartilage formation by adult MSC

MSCs from two adult donors were cultured as cell pellets for 28 days in chondrogenic medium with TGF- β and BMP2. After 7, 14, and 21 days, administration of 10% OAS-CM, oxozaenol, tofacitinib, or combinations of both was started and pellets were cultured for a total of 28 days. MSCs from both donors deposited significant amounts of GAGs in cell pellets from day 14 onward (Fig. 5A). The OA-like conditions simulated by the OAS-CM inhibited chondrogenesis of hMSCs strongly: When the addition of OAS-CM was started after 7 days of culture, hardly any GAGs were measured after 28 days of culture. The addition of OAS-CM from day 14 or 21 not only inhibited further chondrogenesis, but also previously deposited GAGs were degraded, as indicated by GAG measurement in pellets that were harvested after 14 or 21 days. This indicates a strong effect of the OAS-CM. In both hMSC donors, the administration of oxozaenol that had been started at day 7 or 14 did not result in prevention or counteraction of the effect of OAS-CM, of which the administration was started at the same time as the oxozaenol. The administration of OAS-CM and oxozaenol from day 21 onward resulted in a counteraction of the effect of OAS-CM. The administration of tofacitinib and OAS-CM from day 14 or 21 onward significantly prevented the OAS-CM-induced loss of GAGs (Fig. 5B). This effect was strongest for hMSCs from donor 1.

In hMSCs from both donors, the administration of OAS-CM and a combination of both inhibitors from day 14 or 21 onward resulted in a significant rescue of chondrogenesis. In donor 1, oxozaenol had no additional effect. In donor 2, the combination of OAS-CM and both inhibitors resulted in the deposition of more GAGs than when pellets were treated with OAS-CM and the single inhibitors. These results obtained in experiments with adult hMSCs confirmed those from the

experiments with fetal hMSCs. When OAS-CM and inhibitors were added before chondrogenesis was initiated, no relevant levels of GAGs were detected. When OAS-CM and inhibitors were added after the initiation of chondrogenesis, tofacitinib, or the combination of both, oxozaenol and tofacitinib were able to partially rescue chondrogenesis.

Discussion

Previously, we have demonstrated that factors produced by OA synovium inhibit chondrogenic differentiation of hMSCs.⁴ In the present study, we investigated the rescue of chondrogenetic differentiation in OA-like conditions by the inhibition of protein kinases. Our studies indicate for the first time that both the JAK inhibitor tofacitinib and the TAK1 inhibitor oxozaenol can improve chondrogenesis of both fetal and adult hMSCs in the presence of OAS-CM. Measurement of GAG content also showed that the effects of TAK1 and JAK inhibition on chondrogenesis were additive. At the mRNA level, both inhibitors counteracted the OAS-CM-induced down-regulation of expression of the master gene of chondrogenesis, *sox9*, and of the cartilage-specific ECM molecules aggrecan and type II collagen. Moreover, both inhibitors counteracted OAS-CM-induced up-regulation of expression of MMPs.

Our findings in a model for chondrogenic differentiation of hMSCs under catabolic, OA-like, conditions are in line with studies in adult articular chondrocytes, where MAP kinase activation has been associated with increased expression of MMPs and aggrecanases and with decreased expression of aggrecan/proteoglycans.²² Although in both situations genes for ECM molecules are down-regulated and those for cartilage-degrading enzymes are up-regulated, this does not predict that the same pathways are used in hMSCs during early chondrogenesis and in adult articular chondrocytes.

Besides inflammatory cytokines, the growth factor TGF- β also can signal via TAK1. This growth factor is an important stimulator of cartilage-related ECM production. We found

FIG. 5. The TAK1 inhibitor and the combination of the TAK1 and JAK inhibitor partially counteract the effect of OAS-CM in adult hMSCs. Adult hMSCs from two different donors were cultured for 28 days in chondrogenic medium and exposed to 10% OAS-CM, and/or 1 μ M oxozaenol and/or 800 ng/mL tofacitinib from day 7, 14, or 21. OAS-CM from the two different pools was pooled together for this experiment. (A) GAG content as percentage of positive control pellets cultured in the presence of dimethyl sulfoxide (DMSO). Negative control pellets cultured for 28 days without transforming growth factor β (TGF- β) and bone morphogenetic protein 2 (BMP2), positive control pellets cultured with TGF- β and BMP2 for 7, 14, 21, or 28 days and pellets cultured for 28 days and treated with OAS-CM from day 7, 14, or 21 ($n=3$). Generalized estimating equations model with correction for multiple testing, *** $p<0.001$. (B) GAG content as fold increase versus pellets cultured in the presence of OAS-CM and DMSO for 28 days. Treatment with OAS-CM and/or oxozaenol and/or tofacitinib was started after 7, 14, or 21 days ($n=3$). Generalized estimating equations model with correction for multiple testing, * $p<0.05$ versus pellets cultured in the presence of OAS-CM and DMSO.

that the blocking of its intracellular signaling via the non-canonical, TAK1-dependent, pathway did not have much effect on proteoglycan content in cell pellets after chondrogenic differentiation in anabolic conditions (Fig 2A). This is in line with studies in adult articular chondrocytes, indicating that ECM synthesis is mainly regulated via the activation of Smads.²³ These two signaling routes, however, are not completely separate, as interactions between TAK1 and the Smad proteins have been described.^{24,25}

In catabolic conditions (+OAS-CM), we found effects of inhibition of TAK1. Improvement of chondrogenesis by the inhibition of TAK1 was found in these conditions. It has been suggested, from *in vitro* data, that BMP and TGF- β signaling via TAK1 can regulate chondrogenesis, hypertrophic differentiation, and chondrocyte proliferation.^{26–31} Moreover, the deletion of TAK1 in chondrocytes resulted in cartilage defects during embryonic development.³² There are indications that TAK1 is especially involved in the earliest phase of chondrogenesis.³² In our experiments with fetal hMSCs, we started TAK1 inhibition at 3 days after pelletation, which may be after this critical phase. The results of the time-course experiment with adult hMSCs stress the importance of adequate timing of the inhibition, as an early start with OAS-CM and TAK1 inhibition did not result in significant levels of GAG production. In addition, inhibition of the signaling via TAK1 of factors in the OAS-CM that impair chondrogenesis might outweigh the possible negative effects of TAK1 inhibition on the same process.

It has been shown that in adult human articular chondrocytes, MMP levels can be down-regulated by inhibiting JAK3³³ or TAK1.³⁴ In the present study, we found the same in fetal hMSCs during early chondrogenic differentiation in OA-like circumstances. The involvement of both pathways, which are used by cytokines signaling via totally different receptors, indicates that multiple cytokines in OAS-CM jointly determine the expression of MMPs. The effect of treatment with one of the two inhibitors would then be dependent on the relative contribution of these cytokines. In line with this, tofacitinib was more potent in down-regulating OA-induced MMP13 expression as compared with oxozaenol, while it was equal to or less potent in the down-regulation of MMP1 and MMP3 expression.

The effects of both inhibitors were additive in the regulation of MMP1 but not in the regulation of the other MMPs, the aggrecanases, and the cartilage ECM molecules. This suggests that in some aspects, both inhibitors block the same pathways, and in others they do not. Interestingly, oxozaenol significantly counteracted the OAS-CM-induced up-regulation of mRNA expression of the aggrecanases ADAMTS4 and ADAMTS5, while tofacitinib was less potent in this regard. The effect of the TAK1 inhibitor is in agreement with the fact that aggrecanase expression is induced by IL-1 and TNF- α ,^{35,36} which signal intracellularly via TAK1. On the other hand, JAK inhibition had no obvious effect on aggrecanase levels, although aggrecanases can be induced by IL-6 and oncostatin M,^{36,37} that need JAKs for intracellular signaling. This could implicate that IL-6 and oncostatin M levels in OAS-CM are too low to induce aggrecanases, or that relevant receptors and co-receptors of these cytokines are expressed at a low level in hMSCs.

hMSC of two adult donors were used to verify the findings in hMSCs of a fetal donor. Several factors such as

donor age are known to affect the chondrogenic differentiation capacity of adult hMSCs. As expected, these hMSCs showed much slower chondrogenic differentiation. In addition, in these experiments, tofacitinib and, to a lesser degree, oxozaenol partially blocked the effects of OAS-CM. The smaller effects of oxozaenol on hMSCs of these two adult donors can not only reflect differences between donors, but also the OAS-CM caused much stronger inhibition of chondrogenic differentiation compared with the studies with cells of the fetal donor. This is in line with clinical findings that microfracture treatment, which is based on the formation of cartilaginous repair tissue by hMSCs, is known to have better clinical results for younger patients.³⁸ Since the experiments with adult hMSCs are more representative for the patient population presenting with cartilage lesions or OA, this validation of the results obtained with the fetal hMSCs is essential. Studies with hMSC from more donors and lower concentrations of the OAS-CM are needed to explore the therapeutic window of TAK1 and JAK inhibition.

In summary, we showed that both the JAK inhibitor tofacitinib and the TAK1 inhibitor oxozaenol can improve chondrogenesis in terms of GAG content of cell pellets, which is impaired by the presence of OAS-CM. Inhibition of ECM synthesis and stimulation of ECM degradation by OAS-CM appeared to be counteracted by both inhibitors. Our findings could have major implications for therapies that are currently being applied or developed to heal cartilage damage. Many of these therapies depend in some manner on tissue repair and chondrogenesis by hMSCs, such as abrasion arthroplasty (in conjunction with debridement), Pridie drilling, and microfracture. These therapies, in essence, mimic a full thickness defect.^{38–41} These therapies depending on the chondrogenesis of precursor cells are likely to fail in a diseased joint environment. Especially in these conditions, JAK- and TAK1-inhibitors can rescue chondrogenic differentiation, thereby promoting cartilage regeneration by mesenchymal progenitor cells. The presence of an inflammatory milieu that impairs chondrogenic differentiation is not only confined to the OA joint, but will also be present in joints with a cartilage defect, most times accompanied by ACL lesions or a torn meniscus. Therefore, blocking the deleterious effect of an inflammatory milieu on chondrogenesis by the use of protein kinase inhibitors might be considered an attractive option to boost cartilage repair in both joints with traumatic defects and joint diseases with a clear inflammatory component.

Acknowledgments

The research leading to these results has received funding from the Netherlands Institute of Regenerative Medicine (NIRM) and from the European Union's 7th Framework Program under grant agreement no. NMP3-SL-2010-245993.

Disclosure Statement

No competing financial interests exist.

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Received: September 4, 2013

Accepted: January 31, 2014

Online Publication Date: March 21, 2014