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Prostanoid Pattern and iNOS Expression During Chondrogenic Differentiation of Human Mesenchymal Stem Cells

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Abstract Availability of human chondrocytes is a major limiting factor regarding drug discovery projects and tissue replacement therapies. As an alternative human mesenchymal stem cells (hMSCs) from bone marrow are taken into consideration as they can differentiate along the chondrogenic lineage. However, it remains to be shown whether they could form a valid model for primary chondrocytes with regards to inflammatory mediator production, like nitric oxide (NO) and prostanoids. We therefore investigated the production of NO and prostanoids in hMSCs over the course of chondrogenic differentiation and in response to IL-1β using primary OA chondrocytes as reference. Chondrogenic differentiation was monitored over 28 days using collagen I, collagen II, and collagen X expression levels. Expression levels of inducible nitric oxide synthase (iNOS), levels of NO, and prostanoids were assessed using PCR, Griess assay, and GC/MS/MS, respectively. The hMSCs collagen expression profile during course of differentiation was consistent with a chondrocytic phenotype. Contrary to undifferentiated cells, differentiated hMSCs expressed iNOS and produced NO following stimulation with IL-1β. Moreover, this induction of iNOS expression was corticosteroid insensitive. The spectrum of prostanoid production in differentiated hMSCs showed similarities to that of OA chondrocytes, with PGE_2 as predominant product. We provide the first detailed characterization of NO and prostanoid production in hMSCs in the course of chondrogenic differentiation. Our results suggest that differentiated hMSCs form a valid model for chondrocytes concerning inflammatory mediator production. Furthermore, we propose that IL-1β stimulation, leading to corticosteroid-insensitive NO synthesis, can be used as a sensitive marker of chondrogenesis. J. Cell. Biochem. 98: 798–809, 2006.

Key words: nitric oxide; prostanoids; chondrocytes; mesenchymal stem cells; chondrogenic differentiation; osteoarthritis

Chondrocytes are critical for the development, growth, and maintenance of articular cartilage. However, this cell type is also responsible for the cartilage degradation seen in osteoarthritis (OA). This is the result of a loss of balance between the anabolic and catabolic activities with the latter becoming more prominent. This tipping of the scales towards a catabolic phenotype is induced by inflammatory cytokines, for example, interleukin-1β (IL-1β). However, the effect of such inflammatory cytokines relies on the production of additional inflammatory mediators [Goldring and Berenbaum, 2004].

One major effect of IL-1β stimulation on chondrocytes is the induction of inducible nitric oxide synthase (iNOS or NOS-2) and cyclooxygenase-2 (COX-2) expression. These enzymes lead to the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), two potent inflammatory mediators to which chondrocytes are themselves sensitive. In OA, high local concentrations of NO and PGE_2 are present in cartilage thus influencing chondrocyte physiology [Amin et al., 2000; Goldring and Berenbaum, 2004].

It has been suggested that high concentrations of NO or its derivatives contribute to the development of OA by inhibiting the synthesis and promoting the degradation of cartilage extra-cellular matrix (ECM) [Abramson et al.,

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2001]. NO has many effects on chondrocytes, among others it inhibits their response to the anabolic growth factor IGF-1 [Studer et al., 2000; Studer, 2004]. NO promotes joint inflammation by upregulation of IL-1-converting enzyme (ICE) and proinflammatory cytokine IL-18 synthesis while decreasing the level of the ICE inhibitor: protease inhibitor-9 [Boileau et al., 2002]. Furthermore, exposure to high levels of intracellular NO induces chondrocyte apoptosis [Clancy et al., 2001; Oliver et al., 2004]. However, NO levels in synovial fluid failed to correlate with the clinical classification of OA knees [Brenner et al., 2004]. Previous studies have shown that NO itself is not cytotoxic to chondrocytes but may be when combined with O_2^- [Del Carlo, 2002].

Very low concentrations of IL-1 are sufficient to stimulate NO production in chondrocytes. This contrasts with most other cell types where multiple stimuli are required for iNOS induction. Notably, the synthesis of NO in human OA cartilage derives from a glucocorticoid-insensitive induction of iNOS expression [Vuolteenaho et al., 2001].

PGE_2 can exert both catabolic or anabolic effects in chondrocytes depending on the microenvironment and which of the four receptor subtypes are present. Indeed, PGE_2 has also been shown to be involved in the development of OA. PGE_2 modulates proteoglycan and collagen synthesis [Goldring et al., 1996; Abramson, 1999], stimulates matrix metalloproteinase-2 expression [Choi et al., 2004] and enhances matrix metalloproteinase-3 production [Amin et al., 2000].

In addition, PGE_2 inhibits chondrocyte proliferation [Blanco and Lotz, 1995] and induces chondrocyte apoptosis [Amin et al., 1997; Notoya et al., 2000]. Strikingly, PGE_2 was also the only parameter, which we recently found to correlate with the WOMAC-index scores of patients with knee OA [Brenner et al., 2004].

The overproduction and role of PGE_2 in OA cartilage have been reported several times. However, COX-2 overexpression in OA not only leads to the production of PGE_2 but to a variety of prostanoid endproducts that have not been exactly characterized.

The availability of human chondrocytes is limited and they are difficult to expand in vitro. This is obviously a limiting factor for investigations on the regulation of the above-mentioned mediators. Bone marrow human mesenchymal stem cells (hMSCs) can be differentiated into chondrocytes and are able to synthesize cartilage matrix [Yoo et al., 1998; Neumann et al., 2002]. What makes hMSCs such an attractive alternative to primary chondrocytes is that they can be relatively easily expanded and harvested [Solchaga et al., 2004]. hMSCs differentiated into chondrocytes could thus provide a solution for drug development projects and tissue replacement therapies targeting OA. Indeed, in recent years there has been increasing interest in hMSCs as an alternative to primary chondrocytes for drug discovery projects and tissue transplant therapies [Redman et al., 2005]. However it is known that currently used protocols are not capable to induce homogenous differentiation leading to uniform hyaline cartilage, but rather to a phenotype more similar to OA cartilage or as recently reported intervertebral disc-like cells [Winter et al., 2003; Steck et al., 2005].

Still, most studies were focussed on the expression of ECM-markers such as collagen subtypes. Beside this, there was only limited interest in the inflammatory mediator production by hMSCs during and following chondrogenesis, although production of high levels of NO and PGE_2 after stimulation of IL-1β is specific for chondrocytes.

The aim of our study was to generate a detailed characterization of NO and prostanoid production by hMSCs undergoing chondrogenic differentiation. Collagen mRNA levels were used as controls for the quality of the chondrogenic differentiation process of hMSCs. Furthermore, we investigated whether hMSCs having undergone chondrogenic differentiation responded to stimulation by IL-1β in similar manner to chondrocytes, that is, glucocorticoid-insensitive induction of iNOS expression.

MATERIALS AND METHODS

Cartilage Processing

Cartilage specimens were obtained with institutional approval from OA patients undergoing total knee or hip joint replacement in a local orthopedic hospital. The cartilage was dissected from the underlying bone and fibrocartilaginous areas were discarded. The cartilage surfaces were rinsed several times with DMEM/F12 (Gibco Life Technologies, Eggenstain, Germany), with gentamicin (Gibco). Scalpels were used to cut cartilage in sections

3 mm apart. These tissue pieces were then digested with pronase (Calbiochem, Bad Soden, Germany) 4 mg/ml over 90 min and collagenase P (Roche Biochemicals, Mannheim, Germany) 1 mg/ml overnight in DMEM/F12 supplemented with 5% FBS (Gibco) and gentamicin (50 μg/ml) to isolate chondrocytes. The released chondrocytes were washed with HBSS (Gibco) and filtered through a 70-μ nylon membrane.

Alginate Culture

Chondrocytes were encapsulated in alginate beads immediately after isolation according to the method of Häuselmann et al. [1994]. Briefly, cells were suspended in 1.2% sodium alginate (Sigma-Aldrich Co., Taufkirchen, Germany) in 150 mM NaCl (4×10^6 cells/ml alginate solution). The chondrocyte suspension was passed drop-wise through a 22-gauge needle into a 102 mM $CaCl_2$ solution under constant stirring. Following 10 min polymerization, beads were washed with 150 mM NaCl and DMEM/F12.

Chondrocytes in alginate beads were cultured in DMEM/F12 supplemented with 20% FBS, 1 mM L-cysteine (Fluka Biochemicals, Buchs, Switzerland), 25 μg/ml ascorbate (Fluka), and 50 μg/ml gentamicin. Chondrocytes were incubated at 37°C in a humidified gas mixture containing 5% CO_2.

Bone Marrow Human Mesenchymal Stem Cells (hMSCs)

Frozen hMSCs from healthy donors (age 18–30 years) were purchased from Cambrex (Cambrex Bio Science, Verviers, Belgium). Cells were plated in 75 or 150 cm^2 flasks and cultured in Mesenchymal Stem Cells Growth Medium, a low glucose Dulbecco's modified Eagle's medium supplemented with L-glutamine, penicillin, streptomycin, and fetal calf serum (all from Cambrex). Cells were fed every 3–4 days. When the cells reached 80% confluency they were passaged.

The cells used for chondrogenic differentiation procedure were derived from passage 5.

Chondrogenic Differentiation

For chondrogenic differentiation 25×10^4 hMSCs were placed in a 15-ml conical polypropylene tube, washed with Incomplete Chondrogenesis Induction Medium (ICIM) consisting of Differentiation Basal Medium Chondrogenic (Cambrex) supplemented with 1 mM sodium pyruvate, 0.17 mM ascorbic acid—sodium salt, proline, glutamine, 100 nM dexamethasone, 1% ITS + Premix, and antibiotics: Pen/Strep (supplementation available from Cambrex as Chondrogenic SingleQuots). After washing with ICIM cells were resuspended in 0.5-ml complete chondrogenic medium (CCM) consisting of supplemented ICIM and 0,01 μg/ml TGF-β3 (R&D Systems, Wiesbaden, Germany). Cells were centrifuged at 1,000 rpm for 5 min at RT. The pellets were maintained in culture with 1 pellet/tube and 0.5 ml CCM/tube. Medium was changed every 2–3 days.

NO and Eicosanoid Generation

To determine IL-1β-induced generation of NO and eicosanoids chondrocytes in alginate beads and chondrogenic pellets were placed in 24-well dishes.

Cells were stimulated with human 1 nM IL-1β (R&D Systems) for 24 h.; where necessary cells were preincubated with dexamethasone, 10 μM (Sigma), for 30 min and then stimulated for next 24 h with IL-1. The concentration of IL-1β used in these experiments was well above the concentration of IL-1β that induced maximum NO synthesis by human chondrocytes (data not shown). After 24 h generated NO was measured as accumulated nitrite using the Griess assay. Cell culture supernatant (150 μl) was mixed with 10 μl 1% sulphanilamide (Sigma) in 0.1 N HCl and 10 μl 0.1% *N*-(1naphthyl)-ethylenediamine (Sigma). Absorbance was read at 544 nm in reference to 690 nm in a microplate reader. Nitrite (NO_2^-) concentrations were calculated by using a $NaNO_2$ standard curve (0–35 μM) in cell culture medium.

In parallel concentrations of COX-products in cell culture supernatants were determined using specific gas chromatography/triple stage quadrupole mass spectrometry (GC/MS/MS) as described by Schweer et al. [1994].

RNA Extraction and TaqMan PCR

RNA was prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, cell pellets were harvested and homogenized in RLT Buffer (Qiagen). Total RNA was extracted from the homogenate using manufacturer's instructions. DNA contaminations were removed with DNA-free kit (Ambion, Dresden, Germany). Finally RNA was eluted with 50 μl distilled water (Ambion) and used as a template for cDNA synthesis. To generate cDNA, RNA was

reversed-transcribed using RAV2-reverse polymerase (Amersham, Freiburg, Germany) and random hexanucleotide primers (Roche Diagnostics, Mannheim, Germany). Equal portions of the first strand synthesis reaction were used for the following quantitative PCR-analysis.

Real-time PCR, using the ABI SDS 7900 (Applied Biosystems, Foster City, CA), was performed in a total volume of 25 μl in a 96-well plate. For all genes the final reaction mix contained: TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primers at final concentrations of 0.9 μM for each primer, the corresponding probe at the final concentration of 0.2 μM. The primers and probes for collagens, iNOS, and 18 s were designed using Primer Express (for nucleotide sequence see Table I). For all PCRs 10 ng cDNA was added to the reaction mix. A no template control (NTC) that contained all the above reagents except for cDNA was also included to detect the presence of contaminating DNA. All experiments were performed in triplicates or quadruplicates. Amplification and fluorescence detection was conducted with a standard program of 40 cycles. A result was found negative where no amplification occurred, that is, the threshold cycle (Ct) value was greater than 40 cycles. For standardization of the gene expression levels determined by TaqMan analysis mRNA derived cDNA signal in each sample was calculated relative to 18-s ratio as an internal control.

TABLE I. Real-Time PCR Amplicons

Gene (accession no.)	Forward primer	Taqman-probe	Reverse primer
iNOS (NM_000625)	GGCTCGTGCAGGACTCACA	(FAM) ACCTCAGCAAAGCCCTCAGCAGCAT (TAMRA)	GAGCCTCATGGTGAACACGTT
Collagen 1A1 (NM_000088)	ATGGCTGCACGAGTCAACCGG	(FAM) TGGTTTTGTATTCAATCACTGTCTTG (TAMRA)	GCTTCACCTAGAGCGTCACTGT
Collagen 2A1 (NM_001844)	GGCAATAGCAGGTTCACGTACAC	(FAM) CCTGAAGGATGGCTGCACGAAACATACC (TAMRA)	GATAACAGTCTTGCCCCACTTACC
Collagen 10A1 (NM_000493))	ACCCAACACCAAAGACACAGTTCT	(FAM) ATTCCCTACACCATAAAGA (MGB)	CTTGCTCTCCTCTTACTGCTATACCTTT
18S (X03205)	CGGCTACCACATCCAAGGAA	(VIC) TGCTGGCACCAGACTTGCCCTC (TAMRA)	GCTGGAATTACCGCGGCT

RESULTS

Collagen Subtype Expression During the Course of Chondrogenic Differentiation

Collagen I expression. The TGF-β3 induced differentiation of hMSCs into chondrocytes led to a decrease of collagen I gene transcript levels in the first week of differentiation compared to undifferentiated stem cells in monolayer. Afterwards, the level of collagen I mRNA was stable during the rest of the course of chondrogenic differentiation (Fig. 1a).

Collagen II expression. The differentiation of hMSCs led to an expression of cartilage-specific collagen II. Collagen II transcripts were present after 7 and 14 days of differentiation, however expression of collagen II reached highest levels in the third and fourth week of differentiation (Fig. 1b).

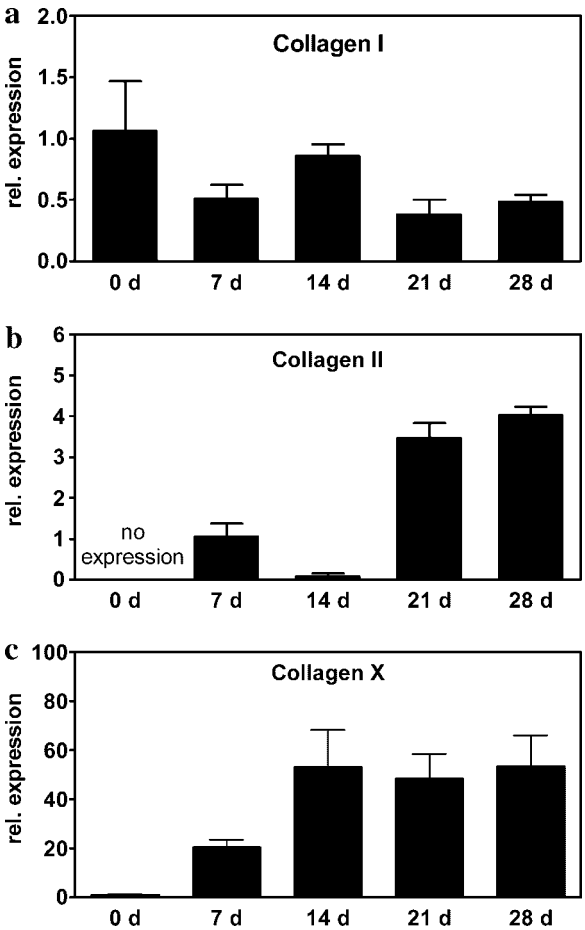

Fig. 1. Relative gene expression levels of collagen I (**a**), collagen II (**b**), collagen X (**c**) during chondrogenic differentiation. The expression level of undifferentiated hMSC or hMSC after 7 days of differentiation (collagen II) was set as one and fold-exchange was calculated. PCR was performed in triplicate (collagen II) or quadruplicate. For standardization of the gene expression levels determined by TaqMan analysis mRNA derived cDNA signal in each sample was calculated relative to 18-s ratio as an internal control. Data are presented as mean ± SD (n = 4).

Collagen X expression. An increase in collagen X mRNA levels was observed already after 7 days of chondrogenic differentiation. Further increase of collagen X gene expression was observed after 14 days and this expression level remained constant for the next 2 weeks (Fig. 1c).

Eicosanoid Production

Figure 2a shows the spectrum of prostanoid production by human chondrocytes from OA cartilage cultured in alginate beads. Human chondrocytes release a broad spectrum of prostanoids: PGE_2, PGI_2 (shown as 6-keto-$PGF_{1\alpha}$ the stable product of prostacycline hydrolysis), TxB_2, PGD_2, $PGF_{2\alpha}$, F-isoprostanes and 8-epi- $PGF_{2\alpha}$ (for exact concentrations see Table IIa). Under basal conditions PGE_2, TxB_2, and isoprostanes are the major COX-products released by human chondrocytes and are present in equal amounts.

IL-1β treatment resulted in 14-fold induction of PGE_2 synthesis in comparison to control. The production of 6-keto-$PGF_{1\alpha}$, PGD_2, $PGF_{2\alpha}$, and 8-epi$PGF_{2\alpha}$ was also upregulated (1.6–4-fold) whereas synthesis of TxB_2 and isoprostanes remained unchanged. The total amount of other prostanoids was several-fold smaller than the amount of PGE_2. Enhanced production of prostanoids after IL-1β stimulation was inhibited by dexamethasone.

The spectrum of eicosanoid production by hMSC differentiated into chondrocytes in the pellet culture system has shown similarities to that of primary chondrocytes, with PGE_2 as predominant product (Fig. 2b). Besides hMSCs released PGE_2, PGI_2, TxB_2, PGD_2, and $PGF_{2\alpha}$ during chondrogenic differentiation (for exact concentrations see Table IIb). We did not detect

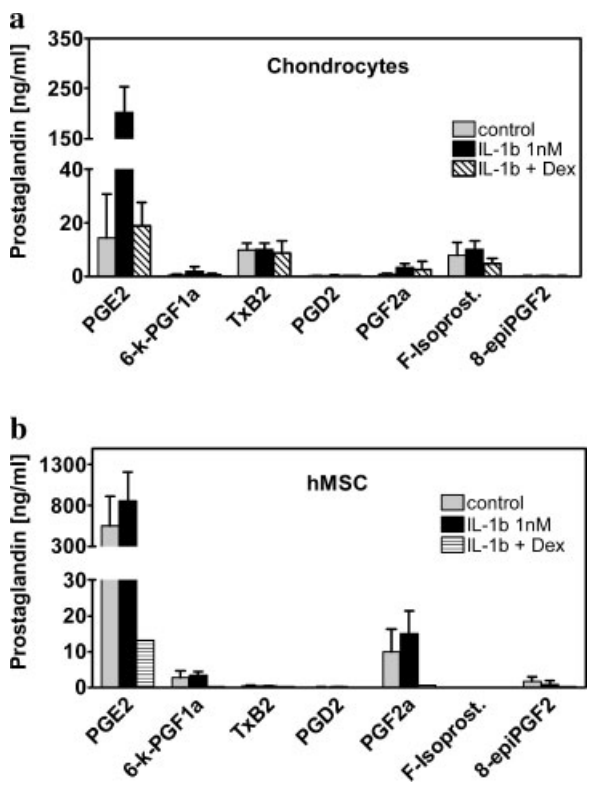

Fig. 2. Prostanoids content of cell culture supernatants of articular chondrocytes, ca. 150.000 cells (**a**) and chondrogenic pellets, ca. 500.000 cells after 6 days of differentiation (**b**). The cells were stimulated with IL-1β 1 nM for 24 h in the absence or presence of 10 μM dexamethasone. Values given are means ± SEM (a, n = 4; b, n = 3).

TABLE II. Prostanoids Content (ng/ml) of Culture Supernatants of Articular Chondrocytes (a) and Chondrogenic Pellets After 6 Days of Differentiation (b)

	Control	IL-1β 1 nM	IL-1b 1 nM + 10 μM Dex
(a): Chondrocytes			
PGE2	14.36 ± 16.51	202 ± 52	18.81 ± 8.81
6-k-PGF1a	0.54 ± 0.26	2.05 ± 1.6	0.64 ± 0.5
TxB2	9.79 ± 2.59	10.06 ± 2.32	8.81 ± 4.29
PGD2	0.15 ± 0.03	0.29 ± 0.13	0.18 ± 0.13
PGF2a	0.81 ± 0.3	3.32 ± 1.52	2.39 ± 3.09
F-Isoprost.	7.74 ± 4.87	10.27 ± 3.02	4.73 ± 1.98
8-epiPGF2	0.09 ± 0.05	0.15 ± 0.08	0.09 ± 0.01
(b): hMSC			
PGE2	546 ± 363	862 ± 355	13.02 ± 10.15
6-k-PGF1a	2.69 ± 1.98	3.39 ± 1	0.26 ± 0.26
TxB2	0.31 ± 0.21	0.32 ± 0.14	0.12 ± 0.12
PGD2	0.07 ± 0.05	0.11 ± 0.07	0.02 ± 0.00
PGF2a	9.86 ± 6.48	15 ± 6.34	0.54 ± 0.1
F-Isoprost.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8-epiPGF2	1.72 ± 1.14	0.72 ± 1.17	0.16 ± 0.01

The cells were stimulated with IL-1β 1 nM for 24 h in the absence or presence of 10 μM dexamethasone. Values given are means ± SEM (a, n = 4; b, n = 3).

F-isoprostanes except 8-epi $PGF_{2\alpha}$. Interestingly spontaneous release of PGE_2 was much higher in pellets at the beginning of differentiation than in chondrocytes. Additionally, there was a big difference in spontaneous PGE_2 release between chondrogenic pellets using hMSCs originating from different bone marrow donors. Both spontaneous and IL-1β-induced PGE_2 production was inhibited by dexamethasone. Chondrogenic pellets under basal and stimulated conditions released higher amounts of $PGF_{2\alpha}$ than chondrocytes, while production of TxB_2 was higher in chondrocytes.

Dexamethasone does not Inhibit IL-1β Induced NO Formation in Human OA Chondrocytes or in hMSCs Undergoing Chondrogenic Differentiation

Human chondrocytes from OA cartilage cultured in alginate beads and chondrogenic pellets of differentiated hMSCs generated significant amounts of NO_2^- after stimulation with 1 nM IL-1β (Fig. 3a).

OA chondrocytes spontaneously released low levels of NO_2^- in the culture medium (0.5 μM). IL-1β enhanced NO_2^- synthesis 30-fold in these cells. Addition of dexamethasone had no inhibitory effect on NO_2^- levels.

hMSCs spontaneously released small amounts of NO_2^- (0.3 μM) after having undergone 14 days of chondrogenic differentiation. IL-1β induced a 10-fold increase in NO_2^- levels. Again, dexamethasone had no inhibitory effect on this induction of NO_2^- production (Fig. 3b).

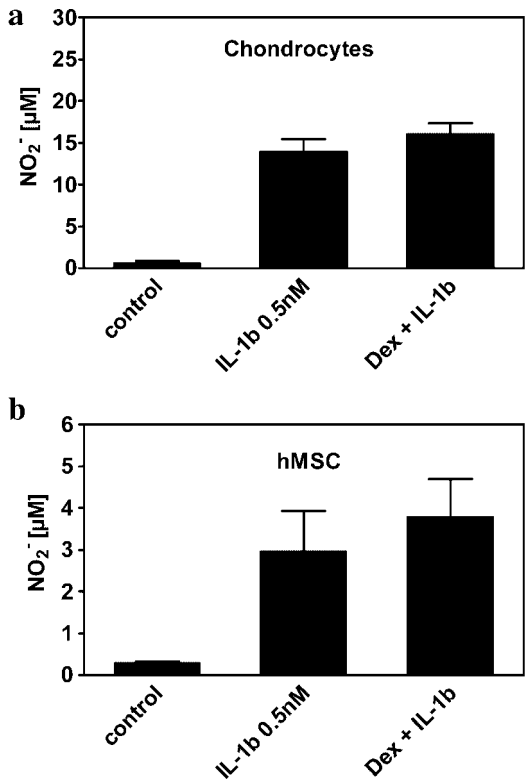

Fig. 3. NO_2^- content of culture supernatants of articular chondrocytes (**a**) and chondrogenic pellets after 14 days of differentiation (**b**). The cells were stimulated with IL-1β 1 nM for 24 h in the absence or presence of 10 μM dexamethasone. Values given are means ± SD (a, n = 4; b, n = 3).

Nitric Oxide Production and iNOS Gene Expression During the Course of Chondrogenic Differentiation

Undifferentiated hMSCs did not produce NO spontaneously or in response to IL-1β (Fig. 4a, day "0"). On the contrary, chondrogenic pellets released low levels of NO_2^- spontaneously already after 7 days of differentiation and enhanced production of this mediator was observed after stimulation with IL-1β (twofold change). Moreover, IL-1β-induced NO_2^- release increased during the course of chondrogenesis and reached highest levels in the third week of differentiation. IL-1β enhanced the synthesis of NO_2^- 16-fold in these cells (Fig. 4a, day 18). The subsequent decline in the rate of NO_2^- synthesis after IL-1β stimulation occurred from 18th day of pellet culture despite the same culture conditions. The reason might be reduction of cellular viability. Results obtained by Sekiya indicate a progressive loss of cells as hMSCs differentiate into chondrocytes in the pellet system. This group observed decrease in the DNA content during the course of chondrogenic differentiation. The total DNA content on the day 21 was only about 40% of the initial amount of DNA on the day 0 [Sekiya et al., 2002].

We observed also decrease of NO_2^- production by human chondrocytes in beads if they are for a long time in culture as well (data not shown). The level of spontaneously generated NO_2^- in control pellets was stable during the differentiation course (0.2–0.33 μM).

IL-1β-induced iNOS gene expression during chondrogenic differentiation correlates with NO_2^- generation (Griess assay). We found no iNOS expression in undifferentiated hMSCs in monolayer under basal conditions or after stimulation with IL-1. However, during chondrogenic differentiation iNOS expression increased in response to IL-1β and reached highest levels in the third week of differentiation (Fig. 4b).

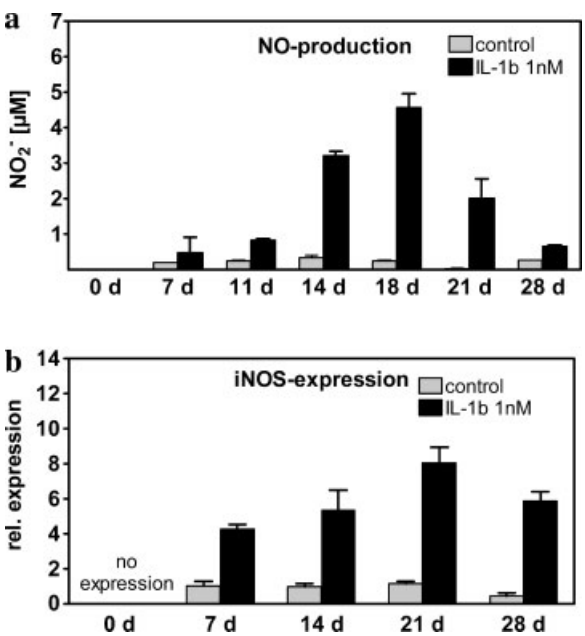

Fig. 4. Induction of NO_2^- production (**a**) and iNOS gene-expression (**b**) by IL-1 during chondrogenic differentiation (undifferentiated hMSC in monolayer are given as 0 d, than 7, 11, 14, 18, 21, 28 days of differentiation with TGF-β3). Cells were stimulated with IL-1β [1 nM] for 24 h. Nitrite accumulation in culture supernatants was measured using Griess assay. Results are given as the mean ± SD (n = 2). The expression level of hMSC after 7 days of differentiation was set as one and fold-exchange was calculated. PCR was performed in quadruplicates and data is presented as mean ± SD.

DISCUSSION

OA is characterized by defective repair of cartilage in affected joints. In such joints, the chondrocytes undergo changes leading them to produce altered cartilage matrix. This is thought to occur because chondrocytes are under the influence of multiple mediators, among others prostaglandins, NO and reactive oxygen species. High local concentrations of these mediators in OA cartilage modulate chondrocyte metabolism. Interfering with the production of such mediators would be a key goal for the treatment of OA.

The principle chondrogenic differentiation capacity of hMSCs has been previously demonstrated, however it has been also recognized that chondrogenesis of hMSCs leads to the formation of "cartilage-like tissue," which phenotypically differs from hyaline cartilage. Still, little is known concerning hMSCs physiology other than that they are able to synthesize extracellular matrix molecules. We therefore studied inflammatory mediator production in two different, but well established cell culture models: pelleted hMSCs under conditions promoting chondrogenesis and in OA chondrocytes cultivated in alginate beads as reference.

Our first step was to validate the extent and quality of hMSCs chondrogenic differentiation by studying relevant marker genes. Cartilage development is initiated by mesenchymal cell condensation, followed by chondrocyte maturation. This differentiation and maturation process is asso-

ciated with the expression of specific genes. These genes include extracellular matrix components such as collagen type II, which is a major extracellular protein in cartilage and essential for normal cartilage structure and function [Tanaka et al., 2000; Sandell and Aigner, 2001]. Collagen type II is a marker for activated functional chondrocytes. Hypertrophic chondrocytes are characterized by the expression of type X collagen [Kirsch et al., 1992; von der Mark et al., 1992; Sandell and Aigner, 2001]. Expression of collagen type X is also a hallmark for chondrocytes from OA cartilage [Nah et al., 2001]. The expression of collagen type I is typical for fibroblastic cells and dedifferentiated chondrocytes [Sandell and Aigner, 2001].

We first characterized the expression of collagen type II, collagen type I, and collagen X in hMSCs put under conditions promoting chondrogenesis to determine the extent of differentiation.

Expression of collagen II occurred late in the differentiation process: The highest levels of expression were reached only after 4 weeks under conditions promoting differentiation. The early pattern of expression, that is, a rapid upregulation after 7 days of differentiation was consistent with previous reports. Yang et al. [2004] observed a rapid increase in collagen II mRNA levels after 7 days of hMSCs differentiation in pellet culture system. Hering et al. [2004] and Barry et al. [2001] made similar observations and concluded that expression of collagen type II is low in immature cartilage and increases with maturation.

Undifferentiated hMSCs expressed high levels of collagen I, fitting well with the fibroblastic morphology of these cells in primary monolayer culture [Yoo et al., 1998]. Collagen type I was expressed during the whole differentiation process but to a lower extent than in monolayer. Interestingly, it was shown in animal models and human biopsy samples that while MSC migrating into a site of injured cartilage do indeed differentiate into chondrocytes, they synthesize fibrocartilage rather than hyaline cartilage [Yoo et al., 1998].

In the present study, collagen X expression was observed already after 7 days of chondrogenic differentiation. Detection of collagen X in chondrogenic aggregates is suggestive of a hypertrophic phenotype. Presence of collagen X during in vitro chondrogenic differentiation was also reported by Yoo et al. [1998] and Winter et al. [2003] in similar cell culture models. Collagen X transcripts reached high levels already in the second week of differentiation and remained stable thereafter. Our findings are consistent with results obtained by Topping et al. [1994] regarding fracture repair. This group detected collagen X in the ECM shortly after collagen II. Unfortunately most differentiation studies were conducted for periods of only 2 or 3 weeks limiting the extent to which comparisons can be made.

In summary, current differentiation protocols are not able to generate healthy articular cartilage using hMSCs. Indeed, cells in our experiments entered the chondrogenic differentiation, but as characterized by expression of collagen X rapidly progressed to the hypertrophic state.

The issue of COX-2 induction in chondrocytes is very complex because PGE_2 is far from being the only prostanoid produced by chondrocytes. These different eicosanoids act via different receptors and there are many signaling pathways involved, which can exert multiple and divergent effects on chondrocyte metabolism. There are many reports dealing with PGE_2 in cartilage pathophysiology but only a few concerning other COX-products.

We provide the first detailed characterization of prostanoid production in human chondrocytes and hMSC undergoing chondrogenic differentiation.

In our experimental settings we used IL-1β as an OA-relevant stimulus to induce COX-2 expression.

IL-1β-induced production of prostanoids in chondrocytes and hMSC was inhibited by dexamethasone, which implicates involvement of glucocorticoid sensitive COX-2 [Masferrer et al., 1992]. In our study, the failure of dexamethasone to reduce spontaneous PGE_2 and TxB_2 release in chondrocytes suggests involvement of constitutively expressed COX-1 [Masferrer and Seibert, 1994].

PGE_2 was by far the predominant COX-product in IL-1β-stimulated chondrocytes. Predominance of PGE_2 over other prostanoids could be related to the upregulation of microsomal prostaglandin E synthase1 (mPGES-1), the final enzyme in the PGE_2 biosynthesis pathway, which was recently reported to be overexpressed in OA cartilage and in human chondrocytes stimulated with IL-1β [Masuko-Hongo et al., 2004]. Interestingly, hMSCs spontaneously

released large amounts of PGE_2 at the 6th day of differentiation. This was due to induced COX-2 activity as indicated by dexamethasone inhibition. There were also big individual differences in the spontaneous PGE_2 release between cells from different bone marrow donors. Spontaneous PGE_2 production declined at later time points of differentiation (data not shown). Production of very high levels of PGE_2 at the beginning of chondrogenic differentiation suggest importance of this prostaglandin in cell differentiation. COX-2 and PGE_2 have been shown to be involved in the differentiation of multiple cell types, however the role of PGE_2 in chondrogenic differentiation is not quite clear. There have been reports on a negative influence of PGE_2 on chondrocyte differentiation [Jacob et al., 2004]. On the other hand Clark et al. [2005] have recently shown that EP2 and EP4 receptor activation of cAMP metabolism may represent a central axis of events that facilitate the impact of PGE_2 on the processes of hMSC commitment to chondrogenesis and ultimate chondrocyte maturation. Furumatsu et al. [2004] have shown that cAMP-response element binding protein (CBP/p300) acts as important SOX-9 co-activator during chondrogenesis. This transcription factor is necessary for chondrogenic differentiation by providing transcriptional signals for expression of collagen II gene. These observations indicate that PGE_2 exerts positive effects on collagen synthesis and chondrogenic differentiation [Goldring et al., 1990; Schwartz et al., 1998]. However, in addition to its modulatory role on chondrocyte homeostasis, PGE_2 is also known to play an important role in synovial inflammation indicating an additional indirect role in the pathogenesis of arthritis. High concentrations of PGE_2 produced by OA tissue might have a role in the degradation of bone and cartilage associated with OA [Hardy et al., 2002].

6-keto-$PGF_{1\alpha}$, TxB_2, $PGF_{2\alpha}$, and in very low levels of PGD_2 and 8-epi-$PGF_{2\alpha}$ are other eicosanoids released by human chondrocytes and hMSCs during chondrogenic differentiation. Jacob et al. [2004] reported that PGD_2 and $PGF_{2\alpha}$ enhanced chondrogenic differentiation and hyaline cartilage matrix deposition (collagen II and glycosaminoglycans) of dedifferentiated articular chondrocytes.

Isoprostanes were under detection limit in hMSCs, but human chondrocytes under basal conditions released significant amounts of isoprostanes. Interestingly 8-epi-$PGF_{2\alpha}$ and other isoprostanes were not only a product of non-enzymatic arachidonic acid oxidation, but seemed to be to large extent generated by COX-2 as their formation could be inhibited by dexamethasone. The phenomenon of COX-2-dependent formation of isoprostanes was also observed in other biological systems, which limits their use as a marker of free radical generation [Pratico et al., 1995; Klein et al., 2001].

We provided here a detailed characterization of prostanoid production in chondrocytes and hMSCs during chondrogenic differentiation revealing that PGE_2 is the predominant COX-product in both culture systems.

Nitric oxide was synthesized by human chondrocytes in alginat beads in micromolar amounts after exposure to IL-1β. Chondrogenic pellets developed the capability to produce NO after stimulation with IL-1β as a result of chondrogenic differentiation. Undifferentiated hMSCs and hMSCs having undergone adipogenic and osteogenic differentiation did not produce NO after IL-1β stimulation (data not shown).

The subsequent increase in NO production after IL-1β stimulation during the differentiation course of hMSCs is consistent with other studies showing that IL-1β effects are dependent on the differentiation status of the mesenchymal cells [Blanco and Lotz, 1995]. In chondrocytes it has been shown that IL-1β responsiveness depends upon expression of the differentiated chondrocyte phenotype [Goldring and Berenbaum, 1999].

Insensitivity of NO production to corticosteroids differentiates the regulation of iNOS in chondrocytes from other cell types, such as macrophages and hepatocytes [Amin et al., 2000].

Interestingly iNOS expression and NO production was dexamethasone-insensitive not only in human chondrocytes but also in hMSCs during chondrogenic differentiation. We observed that undifferentiated hMSCs and hMSC after adipogenic and osteogenic differentiation did not produce NO after IL-1β stimulation. In addition, dedifferentiated chondrocytes lose their ability to release NO after IL-1β stimulation (data not shown). We therefore postulate that the phenomenon of NO production after IL-1β stimulation and corticosteroid insensitive iNOS expression can be used as marker of chondrogenic differentiation.

The influence of NO on the chondrocytic phenotype and therefore on the pathology of OA remains to be fully elucidated. Earlier studies on the expression of iNOS and NO production in the joint diseases have focused on its potential toxicity and on the role in the inflammatory process. However, recently it has been reported that NO by itself is not cytotoxic to cultured chondrocytes and can even be protective under the conditions of oxidative stress [Del Carlo, 2002]. Furthermore, in recent years opinions have changed concerning the role of iNOS in inflammation. Results are accumulating on a protective and regulatory role of iNOS [Tatemichi et al., 2003; Darmani et al., 2004; Suschek et al., 2004]. Interestingly, potential beneficial effects of NO have been reported in environments other than cartilage. NO has been shown to have positive effects in wound healing [Luo and Chen, 2005], NO has also been shown to positively influence the differentiation processes of cardiomyocytes [Kanno et al., 2004] and neurons [Gibbs, 2003]. Still, it remains to be clarified whether NO can contribute to chondrogenic differentiation.

In the present study we have demonstrated corticosteroid-insensitive iNOS expression and NO production in hMSCs during chondrogenic differentiation. We have also provided a detailed characterization of prostanoid production during chondrogenesis. Our investigations suggest that hMSCs undergoing chondrogenic differentiation could be used to investigate the regulation of the production of these inflammatory mediators in a cell system relevant to OA chondrocytes. In addition, while hMSCs chondrogenic differentiation was classically categorized via gene expression of cartilage matrix proteins, we demonstrate that corticosteroid-insensitive NO production in response to IL-1β stimulation can be used as additional markers for differentiation status of human chondrocytes.

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