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Hypoxia reduces the inhibitory effect of IL-1β on chondrogenic differentiation of FCS-free expanded MSC

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Summary

Objective: Mesenchymal stromal cells (MSC) are a promising tool for tissue engineering of the intervertebral disc (ID). The IDs are characterized by hypoxia and, after degeneration, by an inflammatory environment as well. We therefore investigated the effects of inflammation induced with interleukin (IL)-1β and of hypoxia (2% O_2) on the chondrogenic differentiation of MSC.

Methods: Bone-marrow-derived MSC (bmMSC) were cultured in a fetal-calf-serum-free medium and characterized according to the minimal criteria for multipotent MSC. Chondrogenic differentiation of MSC was induced following standard protocols, under hypoxic conditions, with or without IL-1β supplementation. After 28 days of differentiation, micromasses were analyzed by histochemical staining and immunohistochemistry and by determining the mRNA level of chondrogenic marker genes utilizing quantitative RT-PCR.

Results: Micromasses differentiated under IL-1β supplementation are smaller and express less extracellular matrix (ECM) protein. Micromasses differentiated under hypoxia appear larger in size, display a denser ECM and express marker genes comparable to controls. The combination of hypoxia and IL-1β supplementation improved chondrogenesis compared to IL-1β supplementation alone. Micromasses differentiated under standard conditions served as controls.

Conclusion: Inflammatory processes inhibit the chondrogenic differentiation of MSC. This may lessen the regenerative potential of MSC *in situ*. Thus, for the cell therapy of IDs using MSC to be effective it will be necessary to manage the inflammatory conditions *in situ*. In contrast, hypoxic conditions exert beneficial effects on chondrogenesis and phenotype stability of transplanted MSC, and may improve the quality of the generated ECM.

Key words: Mesenchymal stem cells, FCS-free expansion, Chondrogenic differentiation, Hypoxia, Inflammation.

Introduction

Degeneration of the intervertebral disc (ID) is one of the most widespread health problems in the industrialized world, causing substantial economic damage[1,2]. Histologically, a healthy ID is characterized by low cell density with a high proportion of extracellular matrix (ECM) and is without blood and lymphatic vessels or innervation[3]. Avascular discs have no access to regenerative cells in case of damage, and the low intrinsic regenerative capacity further exacerbates degeneration of the ID. This process is accompanied by a progressive loss of cells and proteoglycans[4,5] in the nucleus pulposus (NP), an increased risk for disruptions of the annulus fibrosus (AF) and changes in the biomechanical properties of the ID.

Current therapeutic options to prevent further degeneration of traumatized IDs include reduction of inflammation and pain by drugs, physical therapy, removal of extruded tissue or implantation of artificial discs. Most of these therapies focus on the treatment of symptoms[6]. Cell-based therapies for the biological reconstruction of the injured tissue are largely in a developmental stage. Cells suitable for use in a cell therapy include autologous cells of the ID[1] and adult mesenchymal stromal cells (MSC)[7]. In animal models, a retardation of disc degeneration was reported after implantation of cells from the NP into the ID[8,9]. However, a major disadvantage of regimens based on transplantation of autologous cells into a degenerated or injured ID is the fact that only low numbers of suitable cells can be isolated, and the harvested cells often turn out to proliferate at low rates. Compensating for the low numbers of NP-cells through expansion *in vitro* over long periods of time would

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increase the risk of dedifferentiation of the chondrocytes into a fibroblast-like phenotype[10]. Such cells regenerate a fibrous, scar-like tissue rather than the proteoglycan-rich tissue needed for regeneration of the NP. In addition, the regenerative power of autologous cells isolated from a degenerated disc would probably not be sufficient. Because of these problems, adult MSC are attracting increasing interest as an alternative strategy for cellular regeneration[11].

MSC are characterized by a high proliferation capacity and the ability to differentiate into several lineages. MSC differentiated along the chondrogenic lineage display a phenotype similar to that of cells in the ID[12]. In particular, the gene expression of important ECM components of the ID, such as type II collagen or aggrecan, is significantly increased in chondrogenically differentiated MSC[12]. Furthermore, co-cultures of human MSC and NP-cells with cell–cell contact facilitated the differentiation of MSC into an NP-cell like phenotype[13]. Again, one limiting factor for the translation of such a strategy into clinical success could be the quality and numbers of autologous NP-cells available from affected discs. We therefore focused this study on improving the chondrogenic differentiation of MSC only.

During expansion *in vitro* up to the point of injection into the NP, MSC are exposed to unphysiological conditions. Once *in situ*, they are likely to face a hypoxic environment (as low as 1% O_2)[14–17] and in some cases, an inflammatory one as well. In the degenerated ID the expression of matrix-degrading proteases such as matrix metalloproteinase (MMP)-1, -3, and -13 was noted in the NP and in the inner AF[18]. This would certainly have an impact on the chondrogenic capacity of any transplanted cells. To achieve adequate survival rates of the transplanted MSC and sufficient chondrogenic capacity, it is necessary to analyze the influences to which the MSC are exposed *in situ* in order to develop strategies to prepare the cells for those conditions. Inflammatory mediators such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α have unfavorable effects on differentiated chondrocytes or MSC. The gene expression of key components of the ECM in ID, i.e., type II collagen and aggrecan, was particularly affected[19,20]. In contrast, hypoxia has been shown to have beneficial effects on phenotype stability of bovine articular chondrocytes[21]. Hypoxia facilitated chondrogenesis of rat MSC[22] and human adipose-derived stem cells[23,24]. In this study we investigated the influences of inflammatory and hypoxic conditions separately and in combination on the chondrogenic differentiation of human bone-marrow-derived MSC (bmMSC).

In order to reach the ultimate goal of establishing cell therapies for patients, it is important to develop Good Manufacturing Practice (GMP)-compatible protocols for the isolation, expansion and differentiation of the cells. The use of fetal calf serum (FCS) in cell cultures represents a fundamental problem. Its use in tissue engineering is associated with many risks, including viral and prion transmission or disadvantageous immune responses, making FCS unsuitable for therapeutic applications[25–27]. In order to meet GMP requirements, it has been suggested that human serum should be used in place of FCS[28–30]. In this study we used a GMP-compatible expansion medium containing human plasma and platelet concentrate[29].

Materials and methods

ISOLATION OF HUMAN MSC AND CELL CULTURE

Bone-marrow aspirates were obtained from the BGU Center for Traumatology, Tübingen from the femur shaft of 28 patients undergoing total hip replacement after approval by the ethics committee of the University of Tübingen. The age of the donors ranged from 45 to 83 years. Human MSC were isolated using Ficoll density gradient fractionation (Ficoll-paque plus, Amersham Biosciences, 400 g, 30 min, room temperature, RT). Mononucleated cells in the interphase were collected, washed with phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) and seeded in 75 cm^2 cell culture flasks (BD Bioscience, Heidelberg, Germany), at a density of 2×10^5 cells/cm^2. After 24 h of incubation at 37°C, 5% CO_2 in humidified atmosphere, non-adherent cells were removed. MSC were expanded in medium as described by Mueller *et al.*[29]. This fresh frozen plasma platelet (FFPP) medium is composed of low-glucose DMEM (Dulbecco's Modified Eagle Medium, Lonza) supplemented with 5% human fresh frozen plasma (FFP), 10^8/ml platelets (University of Tübingen, Blood Donor Center), 2 mM glutamine (Lonza), 1000 IU heparin sodium (Roth, Karlsruhe, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany). The expansion medium was changed twice a week. When MSC reached 80% of confluence, the cells were detached with accutase (PAA, Pachingen, Austria) and seeded at an inoculation density of 3×10^3 cells/cm^2. A commercially available MSC medium served as control medium in some experiments (MSCGM, Lonza).

CHARACTERIZATION OF HUMAN MSC

MSC were characterized according to defined minimal criteria for multipotent MSC[31]. To determine the proliferation capacity of MSC in FFPP, MSC were expanded in FFPP and compared to MSC expanded in commercially available medium (MSCGM, Lonza) over four consecutive passages (4 weeks' total), followed by induction of differentiation into the adipogenic, osteogenic and chondrogenic lineages.

For adipogenic differentiation 5×10^4 cells/well were seeded in 6-well plates (Greiner Bio One, Essen, Germany) and cultured in adipogenic induction medium containing DMEM high glucose (Invitrogen, Karlsruhe, Germany), 10% FCS (Biochrom, Berlin, Germany), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 0.2 mM indomethacin (Calbiochem, San Diego, USA), 0.01 mg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutylxanthine (both SIGMA-Aldrich, Steinheim, Germany). After 28 days of incubation in adipogenic induction medium, the monolayer cultures were stained with Oil Red O solution (SIGMA-Aldrich) and examined by light microscopy.

For osteogenic differentiation 5×10^4 cells/well were seeded in 6-well plates and cultured in osteogenic induction medium containing DMEM high glucose 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM β-glycerophosphate (Merck, Darmstadt, Germany), 1 μM dexamethasone and 0.17 mM ascorbic acid 2-phosphate (SIGMA-Aldrich). After 28 days in osteogenic induction medium, the monolayer cultures were stained with alizarin red S (SIGMA-Aldrich) and using the von Kossa staining protocol[32].

Micromass cultures were used for chondrogenic differentiation. In this procedure, 4×10^5 cells were seeded in 20 μl chondrogenic induction medium containing DMEM high glucose, 0.17 mM ascorbic acid 2-phosphate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 μM dexamethasone, an insulin, transferrin, sodium selenite, and linoleic acid, 1× ITS + 1 supplement (SIGMA-Aldrich), 0.35 mM L-proline (SIGMA-Aldrich) and 10 ng/ml transforming growth factor (TGF)-β3 (R&D Systems, Minneapolis, USA) in a round well of a 96-well plate (Greiner Bio One). After 2 h, 200 μl chondrogenic induction medium was added to the adherent cells. For chondrogenic differentiation under inflammatory conditions, the chondrogenic induction medium was supplemented with 2 ng/ml recombinant interleukin-1 beta (rhIL-1β, R&D Systems). Chondrogenic differentiation under hypoxia was performed using a reduced oxygen concentration (2% O_2, 37°C, humidified atmosphere, Hypoxia Workstation, Ruskinn, Bridgend, UK). After 28 days, micromass pellets were harvested and characterized by Alcian blue (SIGMA-Aldrich) and immunostaining of type I and type II collagens (monoclonal antibodies (mAB) for collagen type I and collagen type II, MP Biomedicals, Eschwege, Germany) on cryoslides. In addition, mRNA expression of relevant marker genes, especially collagen-1α2, collagen-2α1, collagen-10, aggrecan, BMP-2 (bone morphogenetic protein-2), IL-1β, CD-RAP (cartilage-derived retinoic acid-sensitive protein) as a regulator of chondrogenesis[33], SRY (sex determining region Y)-box 6 (SOX-6) and SOX-9, and MMP-1, -3 and -13 were analyzed by quantitative reverse transcription followed by polymerase chain reaction (qRT-PCR).

EXPRESSION OF CELL SURFACE ANTIGENS

The expression of CD90, CD14, CD11b (R&D Systems), CD105, CD73, CD45 and CD34 (BD Pharmingen, San Diego, USA) on MSC was analyzed by flow cytometry. Cells were detached gently using Accutase. Unspecific binding of antibodies *via* Fc-receptors was blocked with Gamunex (Talecris Biotherapeutics, Frankfurt, Germany). Then the MSC were washed twice with PFEA buffer (PBS, 2% FCS, 2 mM EDTA, 0.01% sodium azide) and incubated for 20 min at 4°C with phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated mAB. Unbound antibodies were washed away twice with PFEA buffer and the cells were analyzed by flow cytometry (BD LSRII, San Diego, USA). Unstained cells served as controls. Data were evaluated using the software FlowJo (Tree Star, Inc., Ashland, Oregon, USA).

TRANSCRIPT ANALYSIS

For RNA extraction micromass pellets were disrupted in mercaptoethanol solution (SIGMA-Aldrich) using a plastic pestle. RNA was isolated with the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription into cDNA was performed on 1 μg total RNA using the Advantage RT for PCR Kit (Clontech, Mountain View, USA). Gene-specific cDNA was enumerated by quantitative RT-PCR (qRT-PCR, LightCycler, Roche, Mannheim, Germany) utilizing commercially available primer pairs for collagen-1α2, collagen-2α1, collagen-10, aggrecan, BMP-2, IL-1β, CD-RAP, SOX-6, SOX-9, MMP-1, MMP-3 and MMP-13 (SearchLC, Heidelberg, Germany). Serial dilutions of recombinant DNA standards served as controls. β-actin served as the reference gene in each PCR is explained in qRT-PCR above.

STATISTICAL ANALYSIS

The mean values and standard deviations (SDs) were computed from the individual experiments utilizing MS Excel. A test for analysis of variance (ANOVA, Kruskal–Wallis, GraphPad InStat3) and post-hoc test were performed for statistical evaluation. P values below 0.05 were considered to be statistically significant. Data on mRNA expression represent the ratio to the reference gene β-actin and are expressed as means ± SD from individual experiments.

Results

CHARACTERIZATION OF MSC UPON EXPANSION IN FFPP MEDIUM

To characterize MSC expanded in FFPP medium, we compared their morphology, proliferation rate, differentiation capacity and expression of surface antigens. MSC expanded either in FFPP medium or in MSCGM containing FCS were able to adhere to plastic but displayed morphological differences. MSC expanded in MSCGM appeared more spread out than MSC expanded in FFPP medium (not shown). Furthermore, the number of MSCs generated in FFPP medium (passage 4: mean $1.9 \times 10^7 \pm 1.91 \times 10^6$) was significantly higher than the number of MSCs after expansion in MSCGM (passage 4: median $4.8 \times 10^6 \pm 1.64 \times 10^6$; $P < 0.001$, Fig. 1). The MSC are classified by the expression of a number of cell surface antigens. On MSC expanded in FFPP medium the expression of CD73, CD90 and CD105 was detected by flow cytometry. At the same time the expression of CD11b, CD45 and CD34 in these cells was low or completely absent. A similar expression profile of surface antigens was found on MSC expanded in MSCGM (Fig. 2).

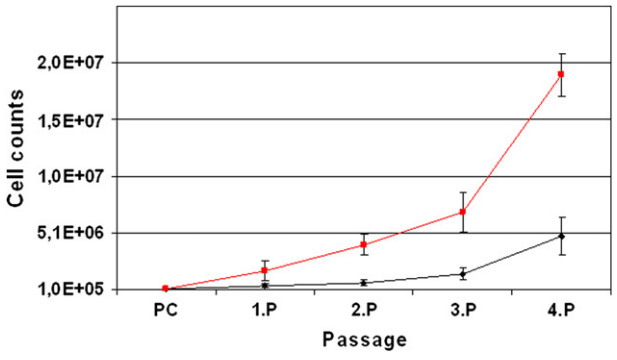

Fig. 1. **Proliferation of MSC**. Displayed are mean values and SD of numbers of MSC from three donors expanded over four passages either in FCS-free FFPP medium (red line) or in FCS-containing MSCGM medium (black line). MSC expanded in FCS-free FFPP medium are characterized by significantly higher proliferation rates than MSC expanded in FCS-containing MSCGM medium.

DIFFERENTIATION OF MSC *IN VITRO*

After 4 weeks of expansion, differentiation of MSC was induced and analyzed by cytochemical staining, histochemical staining and immunostaining. MSC expanded for 4 weeks in either MSCGM or FFPP medium maintained their ability to differentiate into the adipogenic, osteogenic and chondrogenic lineages. After 28 days in adipogenic induction medium MSC were filled with lipid vesicles and were positive for oil-red staining [Fig. 3(a) and (b)]. The accumulation of calcium in cells after 28 days of osteogenic induction was detected by von Kossa staining [Fig. 3(c) and (d)] and alizarin red (not shown). The deposition of proteoglycans during the chondrogenic differentiation was detected by Alcian blue staining [Fig. 3(e) and (f)]. Expansion of MSC in FFPP did not change their differentiation capacity *in vitro* compared to MSC expanded in MSCGM. We therefore continued our experiments with MSC expanded in the GMP-compatible FFPP medium only.

CHONDROGENIC DIFFERENTIATION UNDER INFLAMMATORY AND HYPOXIC CONDITIONS

Chondrogenic differentiation of MSC in the presence of IL-1β produced micromass pellets that were smaller [Fig. 4(b); ∅ mean 0.95 mm ± 0.13 mm] than those generated by standard differentiation [Fig. 4(a); ∅ mean 1.69 mm ± 0.26 mm]. Minute tissue fragments (∅ ≤ 100 μm) were observed in the presence of IL-1β and the tissue was frequently disrupted when microsections were prepared [Fig. 4(b)]. This indicated that the generated ECM was not of sufficient mechanical strength. Chondrogenic differentiation in the presence of IL-1β under hypoxia resulted in slightly increased dimensions of the pellets [Fig. 4(c); ∅ mean 1.05 mm ± 0.18 mm] when compared to differentiation in presence of IL-1β under normoxia [Fig. 4(b)]. In contrast, larger dimensions of cartilage micromasses were observed after differentiation of MSC under hypoxic conditions [Fig. 4(d); ∅ mean 2.13 mm ± 0.28 mm] when compared to differentiation according to the standard protocol [Fig. 4(a)].

The accumulation of proteoglycan and of type I and type II collagens in micromasses generated under different conditions was investigated by histological and immunological staining. The addition of IL-1β to differentiation media under normoxia generated micromasses with a weak Alcian blue staining, a sign of weak accumulation of ECM, especially of proteoglycans [Fig. 4(f)]. Differentiation in the presence of IL-1β under hypoxia resulted in more intense Alcian blue staining [Fig. 4(g)] than differentiation with IL-1β under normoxia [Fig. 4(f)], but less intense staining than that of the controls [Fig. 4(e)]. In samples differentiated under hypoxia we detected a more intense Alcian blue staining [Fig. 4(h)] compared to controls [Fig. 4(e)]. Similar staining patterns were achieved by immunohistochemistry with mAB to type I and type II collagens; after differentiation of MSC in the presence of IL-1β we did not detect any staining of type I and type II collagens [Fig. 4(j) and (n)]. Differentiation in the presence of IL-1β under hypoxia generated pellets that contained some type I collagen at the rim and a rather weak type II collagen staining all over the micromass [Fig. 4(k) and (o)]. Hypoxic differentiation of MSC yielded cells that produced type I and type II collagens throughout the micromass [Fig. 4(l) and (p)], and signal intensity was stronger than in the controls [Fig. 4(i) and (m)].

For a specific analysis of the influences of inflammatory and/or hypoxic conditions, we investigated the mRNA expression of chondrogenic marker genes (Table I). Under

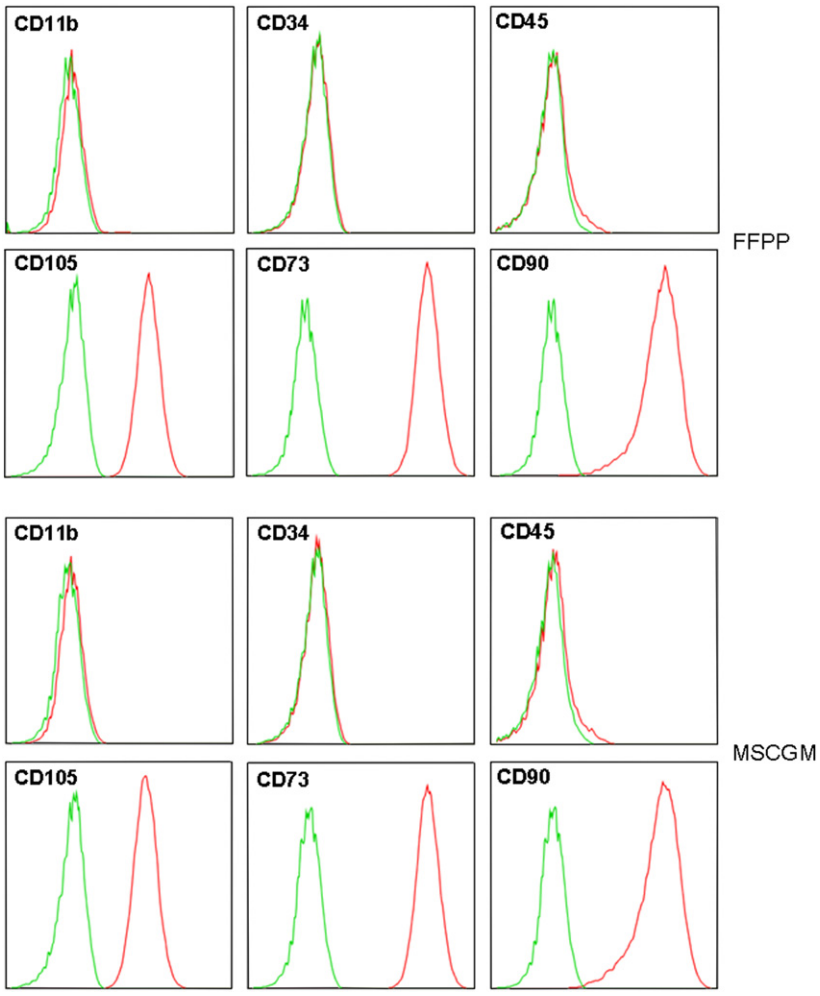

Fig. 2. **Immunophenotype**. Flow cytometric analysis of cell surface antigens of MSC expanded in FCS-free FFPP medium revealed expression of CD73, CD90, CD105 and a lack of expression of CD11b, CD34 and CD45. The histograms display the fluorescence profiles of MSC stained with the mAB (red lines) and the unstained controls (green lines).

standard conditions, the expression of type I collagen was significantly augmented ($P < 0.01$) after chondrogenic differentiation compared to MSC. Furthermore, expression of type II and type X collagens, aggrecan, and CD-RAP was raised 10^3- to 10^6-fold ($P < 0.001$; Table I). A robust, 10- to 100-fold increase was also observed for the expression of SOX-6 ($P < 0.01$), SOX-9 ($P < 0.001$) and BMP-2 ($P < 0.05$). Comparing transcript levels in cells after chondrogenic differentiation under standard conditions and in cells after differentiation under inflammatory conditions revealed a significant reduction of mRNAs encoding type I ($P < 0.01$), type II and type X collagens ($P < 0.05$), aggrecan ($P < 0.01$), SOX-6 ($P < 0.01$) and SOX-9 and CD-RAP ($P < 0.001$). Transcripts encoding BMP-2 remained high, whereas IL-1β was increased 10-fold (Table I). Chondrogenic differentiation under a combination of inflammatory and hypoxic conditions yielded a 10- to 100-fold increase of transcript levels of type II collagen ($P < 0.05$), aggrecan and CD-RAP ($P < 0.001$) compared to chondrogenic differentiation under inflammatory conditions alone. Transcript levels of type I and X collagens, BMP-2, IL-1β and SOX-6 did not reveal any significant variation. Under hypoxia, mRNA expression of most factors was not significantly changed compared to standard conditions.

Expression of IL-1β was detectable at low levels in MSC (five of eight donors) and in differentiated cells using the standard protocol (three of eight donors). A 10-fold increase in the IL-1β mRNA level was observed under inflammatory conditions alone and in combination with hypoxia (eight of eight donors). With chondrogenic differentiation under hypoxia alone, IL-1β mRNA expression was detectable in only two of eight donors (Table I). The mRNA expression of the proteinases MMP-1 and -3 was significantly increased by IL-1β (MMP-1: $P < 0.01$; MMP-3: $P < 0.05$), but this increase was ameliorated by hypoxia (Table I). Furthermore MMP-1 expression levels were lower under hypoxia than under normoxia. MMP-13 showed a significantly increased mRNA expression after chondrogenic differentiation ($P < 0.05$) but minor differences between the several differentiation conditions. The lowest expression of MMP-13 was found under hypoxia (Table I).

Discussion

For tissue engineering of the ID, adult MSC could be an alternative to autologous cells from the ID tissue itself[1,7]. The generation of sufficient numbers of cells for

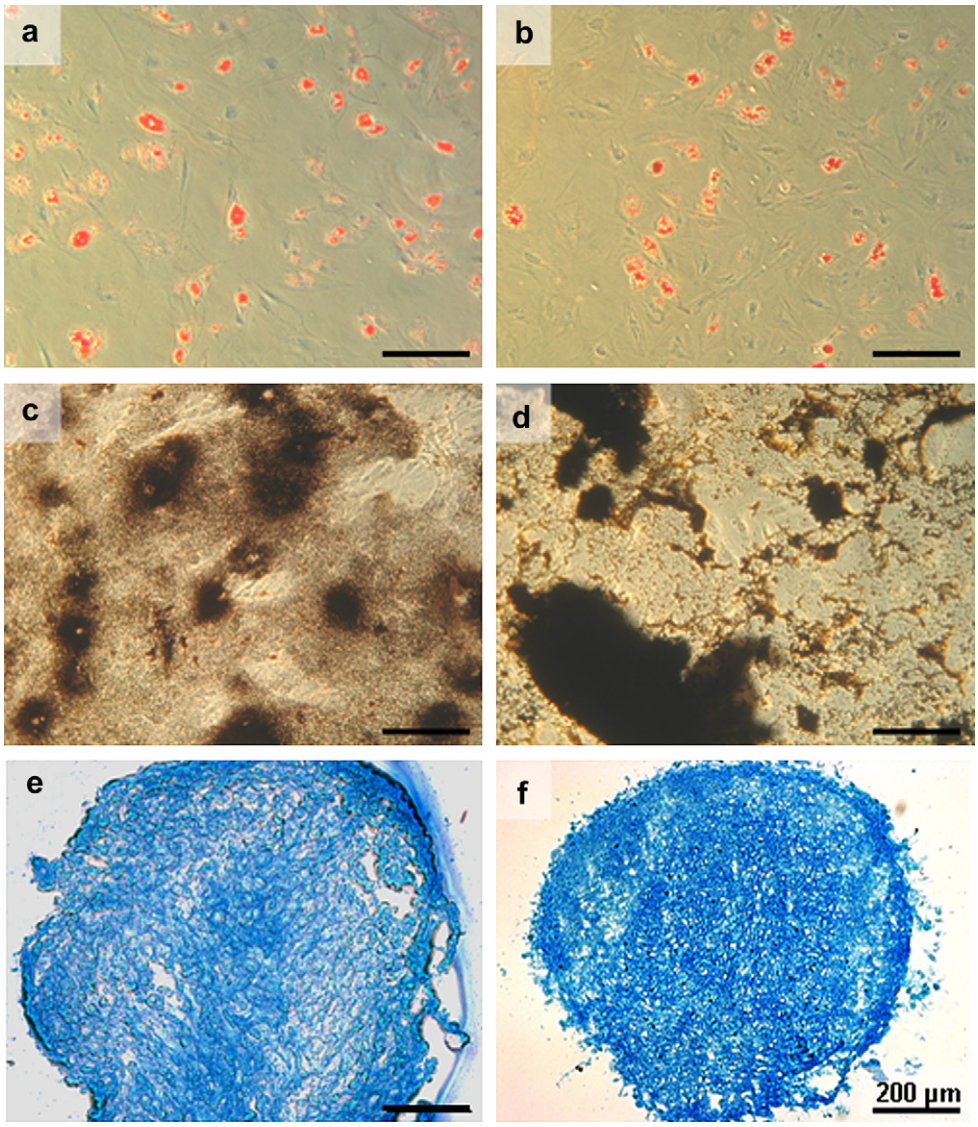

Fig. 3. **Differentiation capacity of MSC**. MSC were expanded in FFPP or MSCGM medium without induction of differentiation. On day 28 of expansion differentiation was induced. A similar differentiation into adipogenic (a, b), osteogenic (c, d) and chondrogenic (e, f) lineages was observed from MSC expanded in FCS-free FFPP medium (a, c, e) and FCS-containing MSCGM medium (b, d, f): Oil Red O staining (a, b) shows adipocytes filled with lipid vesicles. Von Kossa staining (c, d) indicates calcified areas generated by osteoblasts. Alcian blue staining (e, f) illustrates proteoglycan distribution of prepared 7 µm cryosections in micromasses. Scale bars = 200 µm.

transplantation is one of the main problems impeding the use of autologous cells for therapy. This problem could be solved by utilizing MSCs, which are characterized by high proliferation capacity. To investigate this possibility, we first examined a novel, FCS-free and GMP-compatible medium supplemented with pooled human plasma and platelet concentrate for the expansion of MSC[29]. Platelet-derived growth factors have been shown to have a positive effect on the proliferation of stromal cells[34]. We confirm that proliferation of MSC in FFPP medium is higher than in MSCGM. Accelerated growth of differentiation-competent MSC reduces the time span required to generate the quantities of MSC needed for clinical applications. In addition to faster availability, MSC can be utilized at lower passages, which has a beneficial effect on their differentiation and regenerative capabilities[35]. Applying the minimal criteria for defining MSC[31], we conclude that the GMP-compatible MSC expansion medium used in this study is a suitable replacement for media containing FCS.

Degeneration of IDs is frequently accompanied by inflammatory processes. Among other cytokines, the inflammatory cytokine IL-1β is involved in the destruction of cartilage matrix in chondrodestructive diseases[36] and is therefore in the focus of this study. IL-1β displays a clear effect on the chondrogenic differentiation of MSC. IL-1β induced its own expression, a significantly weaker mRNA expression of ECM components of IDs, and higher expression of ECM-degrading proteases. Decreased mRNA expression of SOX-9, Col2A1 and aggrecan was reported after IL-1β treatment of chondrogenic differentiated human multipotential mesenchymal cells[19], corroborating our results.

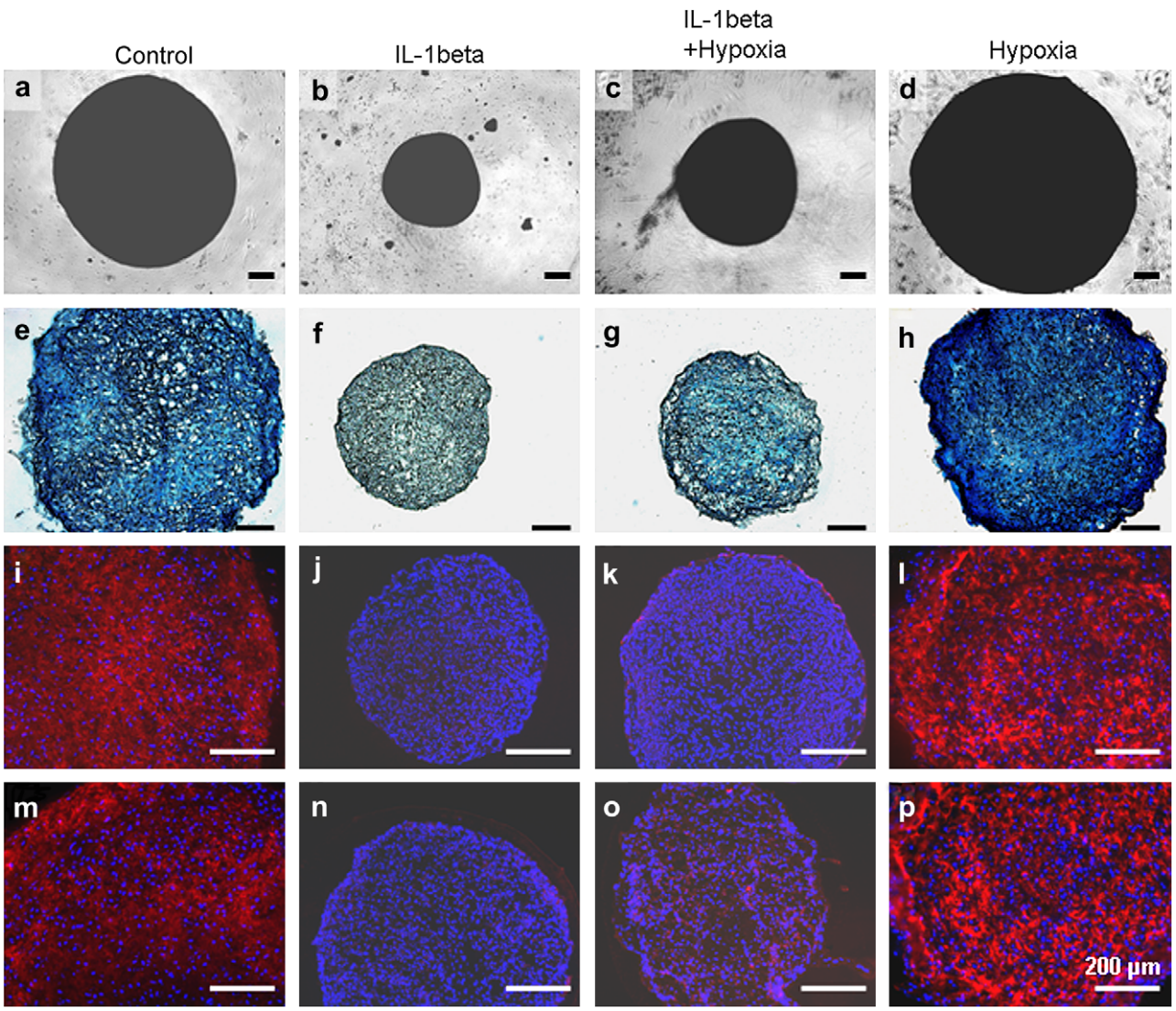

Fig. 4. **Characterization of chondrogenically differentiated micromass pellets under influence of IL-1β, under hypoxia in presence of IL-1β, and under hypoxia**. Morphology of generated micromass pellets. Pictures of micromasses were taken in cell culture wells after 4 weeks of chondrogenic differentiation (a–d; scale bars = 200 μm). The expression of proteoglycan in micromasses was detected in frozen sections by Alcian blue staining (e–h; scale bars = 200 μm). Note that IL-1β reduced Alcian blue staining intensity to a pale blue, indicating low aggrecan expression (f). The expression of type I (i–l) and type II (m–p) collagens was investigated in frozen sections by immunohistochemistry. Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). The figure presents the overlay of micrographs utilizing the PE and DAPI channels (i–p) (size bars = 200 μm).

The weaker mRNA expression of ECM components and the elevated expression of ECM-degrading proteases are mirrored by histochemical staining and immunostaining of micromasses: in the presence of IL-1β a weak accumulation of proteoglycans, type I and type II collagens was noted. As a consequence, micromass pellets remained smaller than controls and also displayed disruption of the ECM structure. In several cases the inner part of the pellet failed to completely regenerate an ECM (not shown). We conclude that the inflammatory processes have a negative effect on either the chondrogenic differentiation potential of MSC or on the matrix production of cells in this environment. For any cell therapy of the IDs using MSC to be effective, it will therefore be necessary to develop strategies to ensure the steady expression of ECM proteins by limiting inflammatory stimuli. Potential strategies for reducing IL-1β activity using, for example, IL-1 receptor antagonist, IL-1β mAB, the IL-1 Trap and inhibitors of IL-1β-converting enzyme are in progress[37,38].

In contrast to inflammatory processes, hypoxia, the second factor investigated in this study, elicits positive effects on the chondrogenic differentiation potential of MSC. Recent studies using different species have shown a hypoxia-induced enhancement of chondrogenesis by induction of gene expression of ECM components and chondrogenic transcription factors, as well as augmented deposition of type I and type II collagens and proteoglycan[22–24,39,40]. There is evidence that hypoxia-inducible factor (HIF)-1alpha plays a key role in the effects of hypoxia on chondrogenesis[22,24] and expression of HIF-1alpha could be stimulated by growth factors *in vitro*[41]. In addition to the effects of low oxygen on chondrogenic differentiation of MSC

Table I
mRNA expression of marker genes after chondrogenic differentiation under influence of IL-1β

	Collagen-1α2	Collagen-2α1	Collagen-10	Aggrecan	SOX-6	SOX-9
MSC	***1.92E+00***	***8.64E−07***	***6.99E−04***	***3.34E−04***	***7.10E−05***	***1.24E−03***
SD	*8.22E−01*	*1.20E−06*	*5.66E−04*	*4.78E−04*	*3.88E−05*	*1.01E−03*
N	***1.22E+01***	***2.59E+01***	***1.15E+00***	***8.88E−01***	***2.36E−03***	***7.97E−02***
SD	*9.92E+00*	*4.36E+01*	*9.78E−01*	*1.10E+00*	*1.79E−03*	*3.68E−02*
I	***2.26E+00***	***3.19E−04***	***2.04E−02***	***7.64E−04***	***7.71E−05***	***5.45E−03***
SD	*2.32E+00*	*3.59E−04*	*2.88E−02*	*6.84E−04*	*5.60E−05*	*2.27E−03*
HI	***4.26E+00***	***2.27E−02***	***2.45E−02***	***8.42E−03***	***1.38E−04***	***1.04E−02***
SD	*3.24E+00*	*3.63E−02*	*1.34E−02*	*4.78E−03*	*6.22E−05*	*2.19E−03*
H	***7.37E+00***	***8.68E+00***	***1.01E+00***	***3.95E−01***	***1.89E−03***	***9.78E−02***
SD	*3.08E+00*	*7.24E+00*	*1.33E+00*	*3.03E−01*	*1.89E−03*	*1.28E−01*
Comparison	***P* values**					
MSC vs N	$P = 0.0025$	$P = 0.0002$	$P = 0.0002$	$P = 0.0003$	$P = 0.0037$	$P = 0.0002$
MSC vs I	$P > 0.9999$	$P = 0.0578$	$P = 0.0504$	$P = 0.1206$	$P = 0.8169$	$P = 0.0548$
MSC vs HI	$P = 0.2788$	$P = 0.0512$	$P = 0.0512$	$P = 0.0516$	$P = 0.1833$	$P = 0.0509$
MSC vs H	$P = 0.0386$	$P = 0.0002$	$P = 0.0003$	$P = 0.0003$	$P = 0.0112$	$P = 0.0002$
N vs I	$P = 0.0011$	$P = 0.0239$	$P = 0.0263$	$P = 0.0027$	$P = 0.0026$	$P = 0.0201$
N vs HI	$P = 0.0848$	$P = 0.0521$	$P = 0.0598$	$P = 0.0512$	$P = 0.0524$	$P = 0.0523$
N vs H	$P = 0.2345$	$P = 0.3823$	$P = 0.0535$	$P = 0.3823$	$P = 0.3357$	$P = 0.4418$
I vs HI	$P = 0.3758$	$P = 0.0126$	$P = 0.3758$	$P = 0.0009$	$P = 0.1939$	$P = 0.0093$
I vs H	$P = 0.0195$	$P = 0.0002$	$P = 0.0503$	$P = 0.0241$	$P = 0.0391$	$P = 0.0121$
HI vs H	$P = 0.1939$	$P = 0.0512$	$P = 0.0167$	$P = 0.0601$	$P = 0.0516$	$P = 0.0610$
	BMP-2	CD-RAP	IL-1β	MMP-1	MMP-3	MMP-13
MSC	***2.97E−04***	***2.21E−05***	***7.89E−06***	***2.61E−04***	***5.01E−04***	***1.39E−04***
SD	*2.64E−04*	*2.79E−05*	*1.01E−05*	*2.86E−04*	*9.84E−04*	*7.28E−05*
N	***1.88E−02***	***5.49E−02***	***1.97E−05***	***1.17E−03***	***1.33E−02***	***4.56E−02***
SD	*7.60E−03*	*5.32E−02*	*3.23E−05*	*2.62E−03*	*7.95E−03*	*2.30E−02*
I	***4.57E−02***	***5.50E−04***	***1.80E−04***	***4.30E−02***	***2.74E−01***	***1.38E−01***
SD	*3.23E−02*	*5.14E−04*	*3.93E−04*	*3.76E−02*	*2.54E−01*	*1.08E−01*
HI	***3.12E−02***	***6.51E−03***	***2.91E−04***	***1.05E−02***	***1.24E−01***	***1.05E−01***
SD	*1.21E−02*	*2.63E−03*	*4.51E−04*	*6.83E−03*	*1.78E−01*	*5.35E−02*
H	***2.37E−02***	***6.48E−02***	***5.87E−06***	***2.84E−04***	***1.70E−02***	***1.87E−02***
SD	*1.38E−02*	*4.91E−02*	*1.65E−05*	*2.95E−04*	*1.85E−02*	*1.57E−02*
Comparison	***P* values**					
MSC vs N	$P = 0.0368$	$P = 0.0003$	$P = 0.9153$	$P = 0.6991$	$P = 0.0556$	$P = 0.0129$
MSC vs I	$P = 0.0003$	$P = 0.0513$	$P = 0.0507$	$P = 0.0225$	$P = 0.0128$	$P = 0.0043$
MSC vs HI	$P = 0.0667$	$P = 0.0567$	$P = 0.1333$	$P = 0.0538$	$P = 0.0538$	$P = 0.0097$
MSC vs H	$P = 0.0484$	$P = 0.0003$	$P = 0.2189$	$P = 0.6623$	$P = 0.0643$	$P = 0.0503$
N vs I	$P = 0.0830$	$P = 0.0535$	$P = 0.0501$	$P = 0.0043$	$P = 0.0226$	$P = 0.4286$
N vs HI	$P = 0.1939$	$P = 0.0532$	$P = 0.1343$	$P = 0.0714$	$P = 0.0514$	$P = 0.0952$
N vs H	$P = 0.8865$	$P = 0.5054$	$P = 0.5516$	$P = 0.4286$	$P = 0.9307$	$P = 0.0519$
I vs HI	$P = 0.4970$	$P = 0.0009$	$P = 0.7758$	$P = 0.1958$	$P = 0.2619$	$P > 0.9999$
I vs H	$P = 0.1893$	$P = 0.0212$	$P = 0.0193$	$P = 0.0403$	$P = 0.0173$	$P = 0.0952$
HI vs H	$P = 0.6667$	$P = 0.0586$	$P = 0.0542$	$P = 0.0557$	$P = 0.3929$	$P = 0.0557$

Transcript levels of marker genes including SD normalized to β-actin of bmMSC before (MSC) and after chondrogenic differentiation under standard conditions (N), in presence of IL-β (I), in presence of IL-β and under hypoxia (HI), or under hypoxia (H). The mean values of the normalized transcript levels (bold italics) ± SD (italics) were computed from the individual experiments, and an ANOVA test (Kruskal–Wallis) and post-hoc test were performed for statistical evaluation. *P* values below 0.05 were considered to be statistically significant (bold).

derived from different origins[22,24] hypoxia also supports the chondrogenic potential of mature articular chondrocytes during expansion[21].

A comparison of the chondrogenic differentiation at hypoxia vs normoxia reveals enhanced deposition of ECM components in micromasses under hypoxia. This did not correlate with an increased mRNA expression of ECM components or other chondrogenic factors. Instead, the chondrogenic benefits of hypoxia correlated with a slightly reduced expression of IL-1β, which in turn would block its autocrine regulation and effects on the expression of MMP-1 and MMP-3. In the presence of IL-1β, hypoxia did not significantly regulate the expression of IL-1β, but it did allay the IL-1β-associated reduction of type II collagen, aggrecan, SOX-9 and CD-RAP expression and in addition it lowered the expression of the cartilage-degrading proteases MMP-1 and MMP-3. The chondrogenic benefit of hypoxia in our experiments did not seem to be associated with an up-regulation of the expression of chondrogenic factors, but rather with the reduction of the pro-inflammatory effects caused by IL-1β.

MSC differentiated under normoxic or hypoxic conditions expressed increased amounts of type I and II collagens, but also displayed increased expression of type X collagen mRNA. Type X collagen is a marker of chondrocyte hypertrophy, and its expression is associated with endochondral ossification[42,43]. Our results indicate that the chondrogenic differentiation of MSC under hypoxia does not prevent the expression of type X collagen. Induction of type X collagen is dependent on methylation patterns of its promoter[44] and

its expression can be suppressed *in vitro* by modification of the tissue culture vessels[45], but not by hypoxia.

Prior to any clinical application of MSC in ID regeneration, other issues must be addressed in addition to IL-1β and hypoxia. Among these are the cellular microenvironment and paracrine signals influencing chondrogenic differentiation *in situ*. Furthermore, expansion of MSC in co-culture with NP-cells or culture of MSC in suitable scaffolds improved chondrogenic differentiation of MSC[13,46–48]. However, in this study we focused on effects of hypoxia and inflammation on MSC differentiation. In summary, we conclude that hypoxia stabilizes the chondrogenic differentiation processes even in an inflammatory environment.

Conflict of interest

There is no conflict to be declared.

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