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Original Article

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Sonic hedgehog protein promotes proliferation and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells in vitro

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Abstract

Background. Sonic hedgehog (Shh) protein is known to be an important signaling protein in early embryonic development. Also, Shh is involved in the induction of early cartilaginous differentiation of mesenchymal cells in the limb and in the spine.

Methods. The impact of Shh on adult stem cells, human bone marrow-derived mesenchymal stem cells (MSCs), was tested. The MSCs were treated either with recombinant Sonic hedgehog protein (r-Shh) or with transforming growth factor-beta 1 (TGF-β_1) as a positive control in vitro for 3 weeks. The effects on cartilaginous differentiation and proliferation were assayed.

Results. MSCs when treated with either Shh or TGF-β_1 showed expression of cartilage markers aggrecan, Sox9, CEP-68, and collagen type II and X within 3 weeks. Only r-Shh-treated cells showed a very strong cell proliferation and much higher BrdU incorporation in cell assay systems.

Conclusions. These are the first data that indicate an important role of Shh for the induction of cartilage production by MSCs in vitro.

Introduction

Articular cartilage is composed of a dense extracellular matrix and a sparse population of chondrocytes. When mature cartilage is damaged, the repair tissue is fibrocartilaginous and lacks the mechanical strength of the native hyaline tissue. Adult stem cells derived from the bone marrow may be used to generate new tissues in vitro. Bone marrow-derived mesenchymal stem cells (MSCs) are homogeneous and can differentiate reliably and reproducibly in many tissues such as cartilage, bone, or fat.[1] To date, attempts to generate biochemically and biomechanically normal hyaline cartilage in vitro have failed, and research now focuses on the identification and characterization of the involved molecules. In normal cartilage development in vivo, Sonic hedgehog protein (Shh) plays a critical role in the induction of early chondrogenic differentiation. Shh is one of the vertebrate homologues of the Drosophila hedgehog signaling protein that patterns the elements eye, wing, and leg in the *Drosophila* embryo. In vertebrates, Shh is expressed in the embryo by the floor plate and the notochord as well as in the limb bud.

Murtaugh et al. have shown that presomitic chicken mesoderm differentiates into cartilage when cultured in vitro in the presence of Shh. In this differentiation pathway, the expression of bone morphogenetic proteins and their receptors (BMPRs) seems to be a downstream target of Shh action.[2] This observation was supported by a role of the hedgehog mediator Gli in the direct activation of BMP promoters.[3]

Both subcutaneously implanted fibroblasts expressing Shh protein and Indian hedgehog protein (Ihh) in nude mice were shown to induce cartilage formation followed by endochondral ossification at the donor site.[4] Ihh regulates the longitudinal bone growth directly by stimulation of proliferating chondrocytes in the growth plate.[4,5]

The BMPs as well as human transforming growth factor-beta 1 TGF-β_1 and TGF-β, belong to the TGF-β superfamily of signaling molecules. The chondrogenic properties of TGF-β_3 on MSCs in vitro are well known.[1,6–8] TGF-β_1 and TGF-β_3 share similar effects on mammalian cells and differ mainly in their potency.[9]

Another important feature of hedgehog signaling is its proliferative influence on many cell types because it induces activation of GLI, a transcriptional factor that enhances cell proliferation. Mutations of the hedgehog pathway can even cause uncontrolled cell proliferation.[10] Constitutively, hedgehog signaling due to a mutated PTHrp receptor may lead to enchondromatosis (Ollier and Mafucci diseases), and transgenic

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mice expressing the hedgehog transcriptional regulator GLI2 develop enchondromatosis-like lesions.[11]

The matrix proteoglycan aggrecan, which is already expressed in early stages of differentiation, and the matrix protein collagen type II, which indicates mature hyaline cartilage, are commonly used as gene markers of chondrogenic differentiation. CEP-68 (chondrocyte expressed — protein, also named cartilage acidic protein 1) may be used to distinguish chondrocytes from osteoblasts and MSCs. CEP-68 is still a relatively new cartilage-specific marker that harbors an epidermal growth factor-like calcium-binding domain.[12]

The aim of this study was to identify a growth factor that induces chondrogenic differentiation in human MSCs and may help us to reach the goal to engineer true hyaline cartilage in vitro and in vivo.

Materials and methods

Cell culture

Mesenchymal stem cells (MSCs) were derived as previously described[1] by harvesting bone marrow of the proximal femur metaphysis during hip replacement surgery from three healthy adults (two males, 37 and 46 years old; one female, 19 years old). After review of the study protocol by the institutional review board (#302-04), written informed consent was obtained from each patient.

About 2.5 ml bone marrow was collected into vacutaine tubes. Tubes were immediately centrifuged with Ficollpaque plus for 30 min at 1500 g. The mononuclear cell layer that was formed by the centrifugation was collected and resuspended in essential medium (Gibco; MEM) containing 20% fetal bovine serum, antibiotics, and glutamine. Cells adhered and grew. Once the desired confluency was obtained (about 70%), 10^5 cells/well were plated in six-well plates.

After adhesion, MSCs were treated with medium (MEM) containing 20% fetal bovine serum alone or 20% serum with 1 ng/ml TGF-β_1 as a positive control or with 20% serum and 100 ng/ml recombinant Sonic hedgehog protein (R&D Systems, Minneapolis, MN, USA) referred to as r-Shh, each in duplicate, for 3 weeks.[13] This incubation time was used as previous cell culture studies had shown changes of cell number and shape[7] as well as an increase of chondrogenic markers[14] for up to 3 weeks after induction of differentiation. The cell medium was changed in all groups twice per week.

Shh has already been studied in differentiation experiments at a concentration of 100 ng/ml.[4] Using different concentrations in our assay system, this concentration was confirmed as excellent for proliferation and thus used again. It was shown in previous studies[8,9] that TGF-β_1 seems to have its highest efficacy for proliferation and differentiation at a concentration around 1 ng/ml.

Alcian blue staining

After 3-week-long incubation, staining was performed with Alcian blue, pH 2.5, for 30 min followed by 3% acetic acid for 5 min. Then, the wells were washed with water for 1 min.

Immunocytochemistry

The cells were fixed with formalin. A polyclonal rabbit antibody against human aggrecan (Acris, Hiddenhausen, Germany) was employed as primary antibody at a dilution of 1:100. After an extravidin-biotin reaction with the secondary antibody, diaminobenzidine (DAB) was used for immunocomplex visualization.

RNA isolation

RNA was prepared from untreated MSCs, from TGF-β-treated MSCs, and from Shh-treated MSCs, always on day 21. Total RNA was extracted from cell pellets with TRIZOL (Invitrogen, Karlsruhe, Germany). RNA derived from human chondrocytes was used as the positive control (kind gift of Sven Schmitt, Department of Orthopaedic Surgery, Offenbach, Germany). RNA integrity was checked with gel electrophoresis.

RT-PCR

Detection of human chondrocyte-specific transcripts was performed by reverse transcriptase-polymerase chain reaction (RT-PCR); 1 µg RNA of intact samples was reverse transcribed using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RT was carried out at 42°C for 50 min. PCR primers (Biospring, Frankfurt, Germany) were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-GAA-GGT-GAA-GGT-CGG-AGT C-3′, antisense, 5′-GGA-AGC-CCA-TCA-CCA-TCT-TC-3′; aggrecan (AGC) sense, 5′-GGG-TCA-ACA-GTG-CCT-ATC-AG-3′, antisense, 5′-GGG-TGT-AGC-GTG-TAG-AGA-TG-3′; collagen type II (COL2A1) sense, 5′-TGG-CCT-GAG-ACA-GCA-TGA C-3′; antisense, 5′-AGT-GTT-GGG-AGC-CAG-ATT-GT-3′; collagen type X (COL10A1) sense, 5′-GCA-ACT-AAG-GGC-CTC-AAT-GG-3′; antisense, 5′-GAG-CCA-CTA-GGA-ATC-CTG-AG-3′; Sox9 sense, 5′-AGA-CCT-TTG-GGC-TGC-CTT-AT-3′, antisense, 5′-TAG-CCT-CCC-TCA-CTC-CAA-GA-3′; and chondrocyte expressed protein-68 (CEP-68) as described.[12] After initial dena-

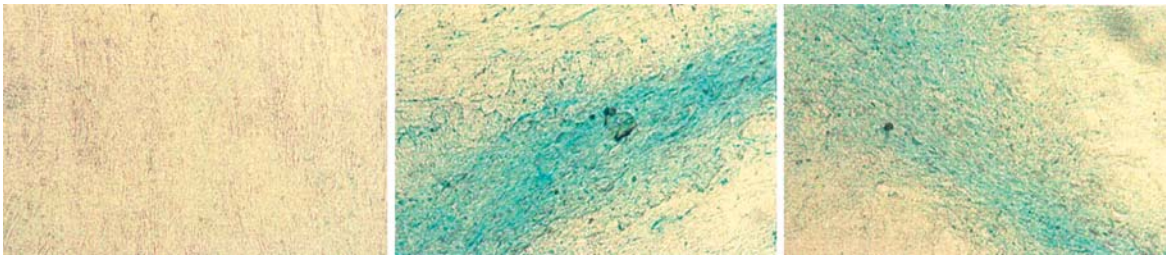

Fig. 1. Alcian blue stain after 3 weeks of culture. *Left*, treatment with medium plus 20% serum alone; *Middle*, treatment with 100 ng/ml Sonic hedgehog protein (Shh); *right*, treatment with 1 ng/ml transforming growth factor (TGF)-β_1

turation at 95°C for 10 min, thermal cycles were 35 cycles at 95°C for 30 s, at 56°C for 45 s, and at 72°C for 50 s. The reaction products were resolved with electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

Proliferation assay and cell counting

Cells were plated at a concentration of 0.7×10^4 cells/well into a 12-well plate and could adhere in a medium containing 20% serum. After 24 h, the medium was decanted and the cells were washed with phosphate-buffered saline (PBS) and starved in MEM with 0.5% serum for 36 h. Then, the medium was changed again and medium was added either alone with 0.5% serum or with either TGF-β_1 1 ng/ml, r-Shh 100 ng/ml, or medium only with 20% serum as a positive control. After 60 h, the cells were harvested using trypsin/ethylene diamine tetracetic acid (EDTA) and counted in a hemocytometer. All proliferation assays were performed in triplicate.

BrdU incorporation

Cells were grown in a 12-well plate and treated as described above. After 60 h bromodeoryuridine (BrdU) (Sigma, Germany) was added at 4 µg/ml. Cells were then incubated for 2 h and then fixed. Detection of BrdU was performed with a mouse anti-BrdU (Sigma) and a secondary antimouse-biotinylated antibody. DAB was used for visualization. Five areas per well were randomly selected and automatically counted with a computer-connected light microscope.

Results

Differentiation

The bone marrow-derived MSCs could be passaged several times without any detectable loss of proliferative capacity. MSCs were cultured for 21 days with r-Shh- or TGF-β-containing medium for the induction of cartilaginous differentiation.

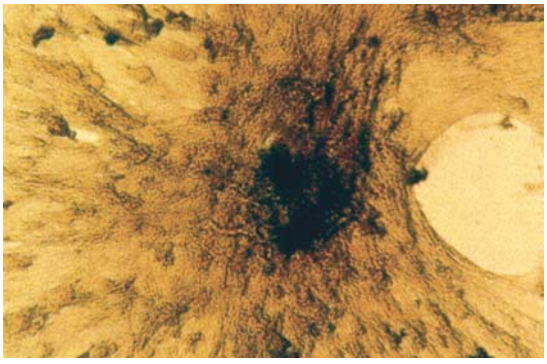

Fig. 2. Growing human stem cells stained with an anti-aggrecan antibody [diamino benzidine (DAB) stain]

MSCs treated with r-Shh or TGF-β stained slightly positive for Alcian blue, mainly in small formations of cell clusters. Control treated cells did not stain with Alcian blue (Fig. 1). With the antiaggrecan antibody, both r-Shh-treated cells and TGF-β-treated cells showed a positive reaction, mainly in nodule-like cell concentrations in the wells (Fig. 2). Control cells cultured with 20% serum presented only very weak staining in a few areas.

After 3 weeks of treatment with either r-Shh or TGF-β, a distinct mRNA expression of the cartilage-specific markers Sox9, collagen type II and X, and CEP-68 was present. Also, a weaker expression of aggrecan was observed whereas control treated cells had no expression of aggrecan or of collagen type II and X and only very little staining of CEP-68 and Sox9 (Fig. 3). These findings were consistent for all donors.

Proliferation

The putative proliferative effect of Shh on MSCs was investigated with a standard cell growth assay and BrdU incorporation. The results of the proliferation assay indicated a distinctively enhanced proliferation of the cells cultured with r-Shh in comparison to TGF-β_1-treated cells (Fig. 4). The addition of r-Shh to the medium containing 0.5% serum caused a twofold increase

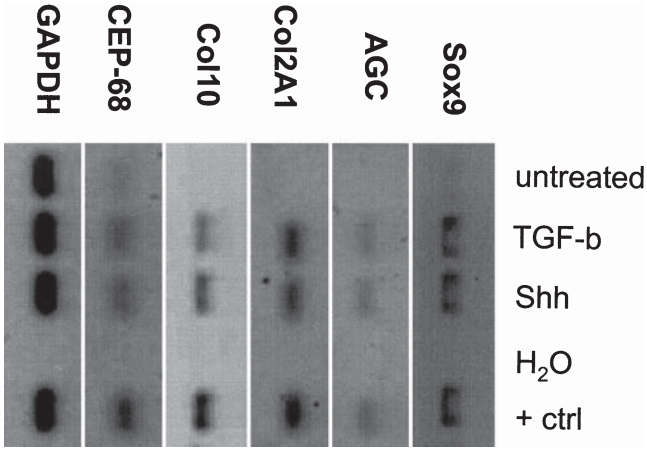

Fig. 3. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) (Woman, 19 years old) show particularly distinct results in the cartilage–specific collagen type II gene. The positive control was mRNA of normal human chondrocytes. No obvious differences could be detected in cultures from different donors. MSCs were treated with TGF-β or Shh as described in Methods. RNA was extracted from the MSC cultures and analyzed for the presence of transcripts of the indicated cartilage markers [*Sox9*; *AGC* (aggrecan); *Col* (Collagen)*2A1*, *Collo*; *CEP* (chondrocyte–expressed protein)-*68*; *GAPDH* (glyceraldehyte phosphate dehydrogenase)]. Expression was validated using cDNA from human cartilage as positive control and Aqua dest. and RNA from an untreated MSC sample as negative controls

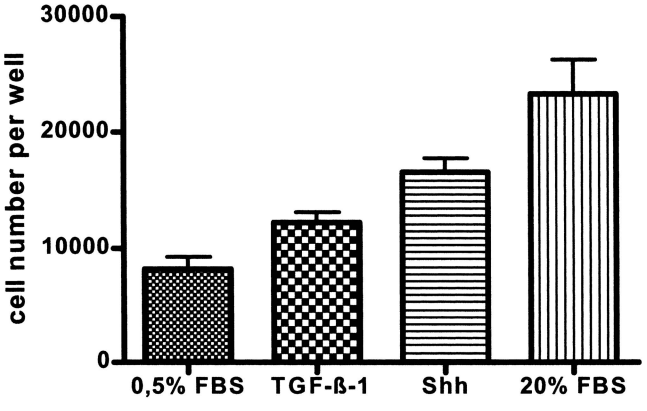

Fig. 4. Proliferative capacity of human cells (presented as mean ± 1 SD). The resulting cell numbers are the means of four independent experiments each performed in triplicate. Shown are the representative experiments of one donor (Woman, 19 years old). *FBS*, fetal calf selum

in cell number compared to culturing with 0.5% serum alone.

Similar results were obtained from BrdU incorporation analysis. Here, a more than tenfold increase in BrdU incorporation of r-Shh-treated cells was found compared with cells grown in 0.5% serum alone. r-Shh-

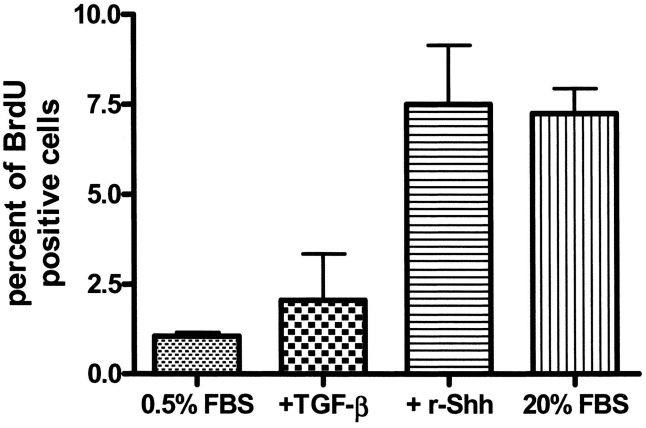

Fig. 5. Results of bromodeoxyuridine (*BrdU*) incorporation. The reported numbers are the mean ± 1 SD of counts of positive cells relating to overall cell count in five areas. Each assay was performed in triplicate. Shown is the representative experiment of one donor (Woman, 19 years old)

treated cells showed more than fivefold increase when compared to TGF-β-treated cells (Fig. 5).

The time-dependent and dose-dependent effect of r-Shh was examined. The optimum dose for proliferative activity was found between 50 and 100 ng/ml (Table 1). No significant differences were observed regarding the three different donors.

Discussion

Despite great advances in the field of tissue engineering, normal hyaline cartilage cannot be produced either in vitro or in vivo. Many attempts have resulted in fibrous — like cartilage tissue in which the matrix has low resistance to biomechanical stress.

Shh is known to play a critical role in normal cartilage differentiation and has proliferative influence on several cell types in vitro and in vivo. The aim of this study was to shed light on the functional role of Shh as a growth factor in human MSC proliferation and differentiation in vitro, as we assumed that Shh plays a major role in the induction of chondrogenic differentiation in human MSCs. Unfortunately, all in vitro and even in vivo systems have limitations; e.g., our model of differentiation is only two dimensional whereas complete cartilaginous differentiation of cells may be more efficient and complete in a three-dimensional environment. This restriction may explain why not all of the examined differentiation markers were strongly expressed.

To our knowledge, this is the first study that indicates an important role for Shh in MSC differentiation and proliferation. Shh induced chondrogenic differentiation of MSCs in all investigated markers in a way comparable with TGF-β, and Shh promoted an even more

Table 1. Effect of time and dose of recombinant sonic hedgehog protein (r-Shh) on proliferation of human bone marrow-derived mesenchymal stem cells (MSCs)

	72 h		96 h		120 h	
r-Shh (ng/ml)	Cell count ($\times 10^3$)	Proliferation (%)	Cell count ($\times 10^3$)	Proliferation (%)	Cell count ($\times 10^3$)	Proliferation (%)
0	21.7 ± 7.7	0	25.0 ± 2.7	0	22.1 ± 2.6	0
1	22.2 ± 5.0	2.3	26.3 ± 3.1	5.2	25.4 ± 2.6	14.9
50	27.9 ± 5.0	28.6	28.3 ± 1.2	13.2	30.4 ± 2.1	37.6
100	26.3 ± 5.1	21.2	29.6 ± 2.1	18.4	30.0 ± 3.1	35.7

Values are means ± SD (n = 3)
Cells were plated at 10^4 per well
Treatment began after 48 h
Proliferation is indicated as percent of difference to according control (0 ng/ml)

distinct proliferation than TGF-β. Similar to MSCs, embryonic mesoderm cells appear to be multipotent mesenchymal cells. Previous studies of Murtaugh et al. have already shown that exogenously added Shh is sufficient to initiate the entire chondrogenic program in explanted chick presomitic mesoderm. Also, they found that Shh treatment induced, after a lag period, endogenous BMP expression that completed the chondrogenic differentiation process.[2] Our results seem to indicate a similar role for Shh in MSCs.

In another study of the influence of Shh on mesenchymal cells, Enomoto-Iwamoto et al. made the observation that prechondrogenic RMD-1 and ATDC5 cell cultures exhibit a distinct positive Alcian blue staining when treated with recombinant Shh-N (rShh-N) for 1 week. However, the authors did not observe any expression of traits characteristic of chondrocytes in the more primitive cell line C3H/10T1/2 treated with either rShh-N or Indian hedgehog protein.[4] The fact that they did not achieve cell differentiation for C3H/10T1/2 cells may be explained by the lower initial cell density and by their shorter observation period.

Our observation that differentiation mainly occurs in nodules with high cell density is consistent with data from the literature. Cells need some density, i.e., confluency, for differentiation. To date, the best differentiation of mesenchymal cells has been achieved using the micropellet technique or three-dimensional gels.[6,15,16]

Shh has a positive influence on matrix formation, as shown by Kellner et al. They found that bovine articular chondrocytes produced up to 2.7 fold more extracellular matrix components than control constructs when grown in the presence of hedgehog proteins in a three-dimensional scaffold. Furthermore, the tissue obtained seemed more mature when grown in the presence of hedgehog proteins.[17] Articular chondrocytes are known to dedifferentiate when isolated and grown in plane cell culture. In an undifferentiated state, they may share a certain homology to MSCs. However, any procedure to harvest articular cartilage cells is more damaging than harvesting bone marrow cells. Therefore, if resulting in the same biomechanical quality under the influence of Shh, the use of MSCs with chondrogenic differentiation would be an advantage over the use of articular cartilage cells.

The chondrogenic effect of hedgehog proteins on MSCs seems to be mediated on one hand by their direct induction of SOX 9, which has been shown to bind to the promoters of the chondrocyte differentiation markers collagen IIa and aggrecan and activate their expression.[2,18] On the other hand, the hedgehog mediator Gli binds to BMP promoters.[3] Subsequent endogenous BMP expression is obligate for maintaining SOX 9 expression and complete chondrogenic differentiation.[2]

Comparing our results with previous studies, we believe that the proliferating and chondrogenic effect of Shh protein on bone marrow-derived MSCs is very promising and encourages further in vitro studies with MSCs in a three-dimensional environment.

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