Soluble Signalling Factors Derived from Differentiated Cartilage Tissue Affect Chondrogenic Differentiation of Rat Adult Marrow Stromal Cells

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Key Words

Rat mesenchymal stem cells • Chondrogenic differentiation • Coculture • Signalling factors • Cartilage • MMP-13 • Collagen X • Sox9

Abstract

Background: Chondral defects show lack of proper regeneration whereas osteochondral lesions display limited regeneration capacity. Latter is probably due to immigration of chondroprogenitor cells from the subchondral bone. Known chondroprogenitor cells for cartilage tissues are multi-potent adult marrow stromal or mesenchymal stem cells (MSCs). *In vitro* chondrogenic differentiation of these precursor cells usually require cues from growth and signalling factors provided *in vivo* by surrounding tissues and cells. We hypothesise that signalling factors secreted by differentiated cartilage tissue can initiate and maintain chondrogenic differentiation status of MSCs. **Methods:** To study such paracrine communication between allogenic rat articular cartilage and rat MSCs embedded in alginate beads a novel coculture system without addition of external growth factors has been established. **Results:** Impact of cartilage on differentiating MSCs was observed at two different time

points. Firstly, sustained expression of Sox9 was observed at an early stage which indicated induction of chondrogenic differentiation. Secondly, late stage repression of collagen X indicated pre-hypertrophic arrest of differentiation. In the culture supernatant we have identified vascular endothelial growth factor alpha (VEGF-164 α), matrix metalloproteinase (MMP) -13 and tissue inhibitors of MMPs (TIMP-1 and TIMP-2) which could be traced back either to the cartilage explant or to the MSCs under the influence of cartilage. **Conclusion:** The identified factors might be involved in regulation of collagen X gene and protein expression and therefore, may have an impact on the control and regulation of MSCs differentiation.

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Introduction

A defect in articular cartilage caused by aging, chondral injury or a degenerative disease is seldom repaired, causes persistent pain and restricts mobility. Generally, the avascular nature of cartilage is held responsible for the lack of spontaneous and satisfactory rejuvenation; more specifically it might be due to the inability to attract a sufficient number of chondroprogenitor cells [1]. Therapies are usually restricted to surgical intervention includ-

ing the cell based therapeutic option of autologous chondrocyte transfer (ACT). The most important factor for this and for any other tissue - engineering method is the source and origin of the appropriate cells. For ACT autologous chondrocytes from healthy tissue of minor weight bearing areas are isolated by enzymatic digestion, expanded *in vitro* and then injected back at the injured cartilage site under a periosteal flap or a synthetic matrix [2, 3]. However, the generated tissue is mostly fibrous and rigid and the risk of permanent damage to the donor site is high. Furthermore, adult chondrocytes have a limited proliferation potential and they easily dedifferentiate *in vitro*. Thus, the need of specific chondroprogenitor cells for regenerative therapies becomes evident. The discovery of bone marrow derived MSCs as chondroprogenitor cells has opened new horizons for bone/cartilage reconstructive procedures. It is in the interest of cartilage regenerative medicine to understand and identify molecular events characterising their growth and differentiation towards stable articular chondrocytes [4].

Under specific culture conditions and proper stimulation MSCs can differentiate into multiple mesenchymal lineages including osteocytes and chondrocytes. The first stage of chondrogenic differentiation is conversion of undifferentiated MSCs to committed osteochondroprogenitor cells leading to cell condensation and growth arrest. The major proteins involved in condensation initiation are fibronectin and proteins of the transforming growth factor β superfamily (TGF β 1-3 and BMP1-7) [5]. Cells present at the centre of the condensation nodules first form pre-chondrocytes and then chondroblasts / chondrocytes which start to produce cartilaginous extracellular matrix (ECM) marked by upregulation of structural protein genes as *col2a1*, *col9a2*, *col11a2* and *aggrecan*. Collagens make up to 60% of the hyaline cartilage protein bulk; among them the most dominant (90-95%) form of cartilaginous collagen is collagen type II which forms a fibrillar alloy structure together with type XI and IX collagens and non-collagenous proteins [6]. Gene expression of collagen type X which according to the concept of endochondral ossification indicates the final step of chondrocyte maturation serves as a marker for hypertrophic and terminally differentiated chondrocytes [7].

The regulatory mechanisms involved in the chondrogenic lineage signalling cascade are not sufficiently understood, however, they are known to involve numerous growth factors, proteases and cytokines in a strictly controlled time dependent manner. Major players impera-

tive for proper skeletogenesis belong to proteases of the MMP-family and growth factors of the VEGF family. For example, activity of MMPs and their endogenous inhibitors (TIMPs) is not only fundamental to skeletal growth and repair, but also to pathology. One member of this large family of matrix degrading MMPs, MMP-13 is predominantly expressed in both, osteoarthritic cartilage and hypertrophic and calcifying cartilage of the mammalian growth plate [8, 9]. Together with other collagenases, and their endogenous inhibitors like TIMP-1, MMP-13 plays a significant role in collagen turnover by cleaving collagen II. Additionally, it also degrades collagen X [10, 11]. VEGF, expressed by hypertrophic chondrocytes, is considered as an essential coordinator of chondrocyte death, chondroclast function, ECM remodelling, angiogenesis and bone formation in the growth plate thereby holds responsible for proper endochondral ossification [12]. Little is known how these soluble factors affect differentiation pathway decisions and integration processes of chondroprogenitor MSCs.

Chondrogenesis is controlled by several transcription factors, important players are members of the Sox- and Cbfa- family. In particular, Sox9 has been characterized as a master transcription factor with essential direct or indirect regulatory effects along the entire chondrogenic differentiation pathway. It is expressed in all chondroprogenitor cells and in differentiated chondrocytes but not in hypertrophic cells [13-16]. Direct control of hypertrophy involves Cbfa1, also known as RUNX2, the major osteogenic transcription factor. Expression of Cbfa1 increases during maturation of chondrocytes and is necessary for inducing gene expression of collagen X [17, 18] and MMP-13 in terminally differentiated chondrocytes [19]. For normal skeletal development and maintenance a co-ordinated regulation of these chondrogenic differentiation associated genes is critical.

This *in vitro* study aimed to identify soluble signalling factors mediating a paracrine cross talk between differentiated cartilage tissue and undifferentiated MSCs. We have designed and set up a coculture system of rat MSCs and allogenic articular cartilage chips under serum-free chondrogenesis favourable 3D- conditions. This coculture system restricts the contact between the explant and MSCs to soluble diffusible molecules and additionally provides authentic growth factors originating from the cartilage explants. Coculturing of MSCs with differentiated cartilage sustained *Sox9* gene expression in MSCs and causes suppression of collagen X biosynthesis and secretion presumably mediated by cartilage secreted factors.

Table 1. Primers used for quantitative PCR

Gene / RefSeq#	Amplicon (bp)	Primer (5' - 3')
<i>β-Actin</i>	104	forward -GTAGCCATCCAGGCTGTGTT -3' reverse -CCCTCATAGATGGGCAC AGT-3'
NM_031144		
<i>Chfa1</i>	86	forward -GGCCGGAATGATGAGAACTA -3' reverse -AGATCGTTGAACCTGGCCACT-3'
XM_34016		
<i>Col1a1</i>	59	forward -TCCAGGGCTCCAACGAGA-3' reverse -CTGTAGGTGAATCGACTGTTGC -3'
RGD61817*		
<i>Col2a1</i>	60	forward -CCCCTGCAGTACATGCGG -3' reverse -CTCGACGTCATGCTGTCTCAAG-3'
NM_012929		
<i>Col10a1</i>	247	forward -CCC TAT TGG ACC ACC AGG TA -3' reverse -TCT CTG TCC GCT CTT TGT GA -3'
AJ131848*		
<i>Sox9</i>	140	forward -CTGAAGGGCTACGACTGGAC-3' reverse -TACTGGTCTGCCAGCTTCCT-3'
XM_343981		

*Locus ID

We have identified VEGF-164α, TIMP-1, -2 and MMP-13 in the coculture supernatant which in part originated from cartilage explants and thus could be important players in regulation and control of the chondrogenic phenotype of MSCs.

Materials and Methods

Cell isolation, coculture and differentiation

MSCs from rat bone marrow were isolated as described earlier [20]. Briefly, bone marrow removed by centrifugation from tibiae and femora of 6 weeks old male rats was seeded at a concentration of 2.5×10^5 cells/cm² and proliferated in α MEM medium containing 5% glutamate, 1% pen/strep and 10% FBS (Invitrogen, UK). Cells from ~75% confluent flasks were harvested and high density 3-D cultures were attained by preparing a cell-alginate amalgam in 1.2% alginate (Sigma, USA) at a concentration of 10^7 cells/ml. Alginate beads of 10^5 cells/bead were formed by dropping the suspension into 102mM CaCl₂ solution and 10 beads per insert (1μm pore size, BD Bioscience, USA) were placed in 12-well transwell tissue culture plates. One allogenic rat hip cartilage explant cut in 3 pieces was placed in the bottom well. Putative bone contamination of cartilage chips was checked by immunoblot analysis for collagen I prior to culture of explants (see immunoblotting). The culture was carried out for 3 weeks in 2.3 ml serum-free chondrogenic medium with medium change every 2-3 days. The chondrogenic medium contained ITS+ premix (6.25μg/ml insulin, 6.25ng/ml selenium acid, 6.25μg/ml transferrin, 1.25mg/ml BSA and 5.35μg/ml linoleic acid; BD Biosciences, USA), 110μg/ml pyruvate, 40μg/ml proline, 0.1μM dexamethasone, 50μg/ml ascorbate 2-PO₄ [21] in α MEM high glucose (Invitrogen, UK). Additionally, 10ng/ml TGFβ-3 (R&D Systems, USA) was added to the culture medium of monocultures and cocultures as indicated (Fig.1 A, B). Samples of cells, tissue and culture supernatant were taken once a week for gene and protein expression analysis. Alginate beads were depolymerised by 30min incubation

in 55mM sodium citrate and 0.15M sodium chloride buffer at 37°C on a shaker; cells were recovered after 3min centrifugation at 750xg. Osteogenesis was induced for 19 days in 6 well culture plates. 250,000 cells/well were cultured in osteogenic medium containing, 10% FCS, 1% pen/strep, 10nM dexamethasone, 50μg/ml ascorbate 2-PO₄ and 10mM β-Na glycerophosphate. At the end of the culture time cells were fixed with methanol for 10min and stained for 2min with 1% Alizarin Red. To induce adipogenic differentiation 200,000 cells /well were cultured in 6 well culture plates in adipogenic DMEM medium containing 10% FCS, 1μg/ml insulin, 1μM dexamethasone, 0.5mM IBMX (Sigma, USA). After 19 days cells were fixed with 4% paraformaldehyde for 10min, incubated for 5min in 60% isopropanol and subsequently Oil Red O (# O-0625, Sigma; USA) staining solution was added for 10min.

RNA isolation, reverse transcription and quantitative PCR analyses

All molecular biology techniques were carried out as described earlier [20]. Briefly, RNA was isolated by an affinity column chromatography method with Ambion's RNeasy4-PCR kit according to the manufacturer's protocol. Contaminating DNA was removed with *DNaseI* enzyme (*DNA-free*, Ambion, USA). Invitrogen's SuperScript II reverse transcriptase kit was used for conversion of 0.5-1μg of RNA into cDNA with 500μg/ml of oligo-dT primers and 200 units of SuperScriptII enzyme. Relative quantitative PCR was carried out in triplicate with SYBR Green Dye I master mix using an ABI 7000 Prism Sequence detection system (Applied Biosystems, USA). Amplification parameters were as follows, denaturation at 95°C for 15sec, annealing at 60°C for 1min in 40 cycles repeats. Relative quantification (RQ) values were calculated by using ABI Prism 7000 SDS software v1.1 employing the ΔΔCt method with β-actin as endogenous control and undifferentiated MSCs freshly suspended in alginate (day 0) as calibrator. Mean RQ values were plotted on a semi-logarithmic graph. Primers (table 1) were designed with either Primer3 or "Primer Express" software (Applied Biosystems, USA). All primers were manufactured at MWG – Biotech, Germany.

Immunofluorescence

Alginate beads were incubated for 30min in 100mM BaCl₂ for irreversible polymerization [22]. Then the beads were fixed with 3% paraformaldehyde (PFA), sequentially dehydrated with alcohol and then embedded in paraffin. 4-8µm sections were cut with a microtome (Leica, Germany) and 2 sections per slide were mounted. Slides were fixed again in 3% PFA and submerged in xylol for 5min to remove paraffin. Sections were sequentially rehydrated and incubated for 1h in blocking buffer containing PBS with 1% BSA. 1:50 dilution of collagen II polyclonal antibody (R1039, Chemicon, USA) was used for overnight incubation followed by incubation with Alexa488 conjugated secondary antibodies (goat anti rabbit, 5µg/ml; Molecular Probes, US). Slides were permanently mounted with DAKO fluorescent mounting medium (DAKO, USA) and evaluated with scanning laser microscopy using a C1 confocal microscope (Nikon, Germany). Photos were taken with a Nikon C4 camera and analyzed with the associated software.

Collagen isolation

Secreted collagens were isolated from the culture supernatant after pepsin digestion [23]. 1ml of medium was incubated for 2h on a rotary mixer with 10mg/ml pepsin followed by overnight salt precipitation in 4.5M NaCl. After centrifugation at 14,000xg for 30min at 4°C the protein pellet was precipitated overnight with absolute ethanol at -20°C. After a second centrifugation step the protein pellet was dissolved in 60µl of sample buffer containing 0.8M urea. Samples were separated under denaturing conditions on a 4.5%-15% gradient SDS-PAGE gel followed by Coomassie Brilliant Blue R250 staining (Serva, Germany).

Pulse-Chase

After 21 days in chondrogenic favourable 3D - environment alginate suspended cells cultured in the presence of external added TGFβ-3 were metabolically labelled for 1h in labelling DMEM-HG medium without methionine and cysteine (#21013-024, Invitrogen, UK), as described previously [24]. Briefly, cell culture inserts containing the alginate beads were removed from cocultures and pre-incubated in the labelling medium without radioactive isotopes for 1h. Afterwards beads were labelled in absence of cartilage with freshly added 2ml labelling medium plus 3.7MBq of [³⁵S]- methionine and cysteine (Tran ³⁵S- label from Qbiogene/ICN-USA) for 1h at 37°C (pulse). Labelling medium was then removed and after 2 washing steps with PBS the labelled cells were placed back in the culture vessel; in case of coculture together with cartilage. Newly secreted collagens were chased by continuing the culture in chondrogenic medium for 4h at 37°C. 1ml cell culture supernatant was retrieved after 0.5h, 1h, 2h and 4h of chase time. Secreted collagens were extracted, as explained above and samples (each ~155 cpm) were separated on a 4.5-15% gradient SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue R250 staining. The destained and fixed gels were incubated for 30min in Amplify™ solution (Amersham Biosciences, UK), dried and developed for up to 21 days at -80°C. The radiographic images (Hyperfilm™ MP- Amersham Biosciences, UK) were acquired with CURIX 60 film developer (Agfa, Germany).

Immunoblotting

Western blotting was carried out with MSCs, cartilage explants and alginate cell fraction lysates. 0.5 mg cartilage explants were pulverized in a freezer mill (C3-Analysentechnik, Germany) and the finely ground powder extract was treated similar as the cells. Briefly, ~10⁶ cells were lysed by suspending the cell pellet in 80µl of 250mM Tris buffer (pH 6.8) containing 6% EDTA, 4% SDS, 40mM DTT, 20% glycerine, 0.002% bromophenol blue and 1:10 protease inhibitors (Complete Mini, Roche, Germany). Cells were heated for 5min at 60°C immediately followed by incubation for 10min at 100°C. Cell debris was precipitated by centrifugation at 14000xg for 30min at 4°C and protein containing supernatant was TCA-precipitated and subjected to 4.5-15% SDS-polyacrylamide gel electrophoresis. Prestained protein standards within a range of 7.5 to 207kDa (BioRad, Germany) were used as molecular weight markers. Proteins were electrotransferred to a nitrocellulose membrane for 3h at 4°C. To reduce unspecific binding the membrane was blocked for 1h in blocking buffer (5% dry milk in PBS containing 0.1% Tween 20). Blots were incubated overnight with polyclonal antibodies against collagen I, (R1038 Acris, Germany), MMP-2 (AB 809, Chemicon, Germany, 1:100) and MMP-13 (RP1MMP13, Triple Point Biologics, USA, 1:1000). Detection was performed using Horseradish-peroxidase coupled secondary anti-rabbit antibodies (A2074, Sigma, USA; 1:50000). Signals were visualized with ECL reagent (Pierce, USA) and the membrane was exposed to blue light sensitive autoradiography film (Pierce, USA).

For dot blot analysis, 1ml of cell culture supernatant was precipitated with 20% Trichloroacetic acid (TCA) solution for 30min at 4°C. Extracted protein was pelleted by centrifugation at 14000xg for 5min and washed twice with chilled acetone. After the final washing step the pellet was resuspended in 40µl PBS. 1µl of the resuspended protein was spotted and air dried on a nitrocellulose membrane. After blocking for 1h the membrane was incubated overnight with an antibody against TGFβ-1/2/3 (sc-7892, Santa Cruz, USA, 1:200) and developed as above.

Antibody array analysis

RayBio™ rat cytokine antibody array I for conditioned medium (Tebu-bio, France) was used according to the manufacturer's instructions. Briefly, the membranes were blocked for 30min in 5% BSA in 0.01M Tris buffer with 0.15M NaCl (pH 7.6) followed by overnight incubation at 4°C in 1ml of cell culture supernatant. After washing and incubation with biotin coupled anti-cytokine antibodies for 2h membranes were again washed and afterwards incubated with HRP-conjugated streptavidin for 2h. Signals were detected with the solution provided with the kit, exposed ECL films (Amersham Bioscience, UK) were developed with CURIX 60 film developer.

Zymography

For gelatine and reverse gelatine zymography, aliquots of culture medium were mixed with equal volumes of two fold concentrated sample loading buffer (2mM EDTA, 2% SDS, 20% glycerol, 0.02% bromophenol blue, 20mM Tris/HCl, pH 8.0) and subjected to electrophoresis on 1% gelatine containing SDS-polyacrylamide gels. For gelatine zymography 4.5 - 15%

Fig. 1. The coculture model. Schematic representation of the coculture model (A). The coculture of alginate suspended rat MSCs and allogenic cartilage chips was set up in 12 - well transwell tissue culture plates and carried out in serum-free chondrogenic medium without external addition of growth factors. Exogenous growth factor TGF β -3 was added to monocultures and cocultures of MSCs suspended in alginate when analysing effects of cartilage explants on collagen X gene and protein expression (B).

gradient gels were used. Reverse zymography was carried out on 15% SDS-polyacrylamide gels in which 25% conditioned macrophage medium was included as MMP-source. Both types of gels were washed twice for 30min in 2.5% Triton-X 100, rinsed in distilled water, and developed for 16h at 37°C in 50mM Tris/HCl pH 8.5 containing 5mM CaCl₂. Finally, the gels were stained with Coomassie Brilliant Blue R250 (Serva, Germany) to visualize protease or protease inhibitor activity and were photographed.

Incubation of ¹⁴C-labelled collagen X with culture supernatant from MSCs

(¹⁴C)-proline labelled collagen X was produced and isolated from IGF-I stimulated cranial chicken sternum chondrocytes as described elsewhere [25]. Equal amounts (1000 cpm) were incubated with aliquots of different conditioned cell culture media or PBS for 7h at 37°C and analysed by 4.5-15 % gradient SDS-PAGE gel electrophoresis under reducing conditions followed by fluorography as described [23].

Statistical Analysis

One way ANOVA and student t test were employed for quantitative PCR experiments which were performed in triplicate.

Results

The coculture model

A novel coculture system of cartilage tissue explants and multi-potent adult MSCs suspended in 1.2% alginate was set up in tissue culture inserts placed into 12 - well

transwell culture plates under serum-free conditions. The inserts separated MSCs from cartilage chips by a 1 μ m porous membrane, thus restricting the contact between the explants and MSCs to soluble diffusible molecules (Fig. 1A). This coculture system not only limits cell-cell communication to the paracrine level it also provides authentic growth factors originating from the cartilage explants. Different experimental set ups used throughout this study are schematically represented in figure 1B.

Prior to the coculture, both the MSCs and the cartilage explants were characterized. These MSCs were CD45^{low}, D7Fib⁺ and CD49a⁺ as screened previously by immunofluorescence and magnetic sorting with selected surface markers [20]. Differentiation potential of MSCs was determined by culture under osteogenic, i.e. monolayer culture in osteogenic medium, and chondrogenic favourable conditions, i.e. embedding of MSCs in alginate beads. Positive Alizarin Red staining indicated successful osteogenic differentiation (Fig. 2Aa and 2Ab) while positive extracellular collagen II staining indicated successful chondrogenesis (Fig. 2Ac and 2Ad). Adipogenic differentiation potential of MSCs was determined by positive staining of lipid droplets with Oil Red O dye (Fig. 2Ae and 2Af). These results confirmed sustained differentiation potential of the multipotent MSCs. The cartilage explants were screened for contaminating bone tissue by western blot analysis of collagen I in culture supernatants. Lack of collagen I secretion from cartilage explants indicates absence of contaminating bone tissue

Fig. 2. Characterization of the bone marrow extracted MSCs and cartilage explants. Differentiation potential of the rat bone marrow isolated MSCs (A). After 19 days of culture in osteogenic differentiation medium accumulation of calcified ECM is evident by positive staining with Alizarin Red (a) compared with the MSCs cultured in proliferation medium (b). Chondrogenic differentiation potential of MSCs was established by positive collagen II staining after 21 days in 3 - dimensional alginate environment which favours chondrogenic differentiation (c) compared with 3D- alginate culture from day 0 (d). Bars represent 50µm. Adipogenic differentiation was demonstrated by Oil Red O staining of intracellular lipid droplets after 19 days in adipogenic medium (e) compared with cells cultured in proliferation medium (f). Bars represent 100µm. N = 3. Western blot analysis of cartilage explants shows lack of collagen I secretion indicative of cartilage purity; in comparison MSCs secrete collagen I (B). Dot blot analysis of endogenous TGFβ1-3 secreted into the culture supernatant by monocultured cartilage and monocultured MSCs. These cultures were not supplemented with exogenous TGFβ-3 (C). Ca = explant culture of rat hip cartilage. N = 3.

Fig. 3. Relative changes in gene expression of transcription factors *Sox9* and *Cbfa1*. Relative gene expression level of *Sox9* (A) and *Cbfa1* (B) was assessed during culture time by qRT-PCR employing the $\Delta\Delta CT$ method using SYBR I Green dye. Target gene expression was normalized to gene expression in MSCs on day 0, which was used as a calibrator and constitutes the baseline at 1. Expression level of each gene was compared between coculture (dotted bars) and monoculture (white bars). Grey bar indicates relative gene expression level of freshly isolated rat hip articular chondrocytes. N=5.

(Fig. 2B). Growth factor TGFβ can be detected in the culture supernatant of cartilage explants until day 14, from day 21 on TGFβ secretion is strongly reduced and becomes undetectable in culture supernatant. Compared with the cartilage explant cultures MSCs secrete only neglectable amounts of TGFβ (Fig. 2C).

Effect of cartilage on gene expression profile of Sox9 and Cbfa1

To account for the regulatory effect of Sox9 and Cbfa1 in chondrogenic differentiation of MSCs, expres-

Fig. 4. Relative gene expression and protein secretion of *Colla1* and *Col2a1*. Relative quantitative mRNA expression level of a marker for fibroblast-like cells, *Colla1* (A) was compared with a marker for chondrogenesis, *Col2a1* (B) by qRT-PCR employing the $\Delta\Delta CT$ method using SYBR I Green dye. Gene expression of day 0 MSCs was used as calibrator and constitutes the baseline at 1. Expression level of each gene was compared between coculture (dotted bars) and monoculture (white bars). Grey bar indicates relative gene expression level of freshly isolated rat hip articular chondrocytes. N=3. Analysis of collagen chains secreted into the culture supernatant. Collagens were precipitated from culture medium, pepsin digested, separated on a 4.5%-15% gradient SDS-PAGE under reduced conditions and stained with Coomassie Blue (C). N=6. Assessment of collagen secretion from mono- and cocultured MSCs by employing the pulse-chase method. After 21 days cocultured cells were separated from cartilage explants and like monocultured cells metabolically labelled with [35 S]- methionine / cysteine for 1h. During the chase period from 0.5h up to 4h secreted collagens were extracted from the culture supernatant. Samples were separated on a 4.5-15% gradient SDS-PAGE under reducing conditions (D). MC = monocultured MSCs, CC = cocultures. N=2.

sion of these transcription factors was investigated by qRT-PCR (Fig. 3). Application of 3D - chondrogenic culture conditions resulted in an early increase of gene expression level of transcription factor *Sox9* on the first day of culture (day 1). Cartilage exerted an impact on *Sox9* gene expression within 7 days as the cocultured MSCs showed sustained up regulation of *Sox9* mRNA expression when compared with the down regulated expression in the monoculture. From day 14 onwards *Sox9* gene expression in coculture was reduced to the initial level of undifferentiated MSCs (Fig. 3A). *Cbfa1* gene expression was induced about 3-5 fold at day 1 equally in all culture regimes, remained stable until day 14 in mono- and cocultures and became reduced to initial level in undifferentiated MSCs after 21 days. The presence of car-

tilage did not influence relative induction and maintenance of gene expression level of *Cbfa1* (Fig. 3B). Notably, induction of *Sox9* and *Cbfa1* gene expression did not require cues from growth factors as TGF β as the mRNA levels during the initial (day 1 for *Sox9*) and the middle (days 1-14 for *Cbfa1*) stages of the culture were upregulated also in monolayer culture.

Effect of cartilage on gene expression profile of Colla1, Col2a1 and Col10a1 and their biosynthesis

To identify the effect of cartilage explants on chondrogenesis of MSCs, expression and biosynthesis of different collagens were monitored. Altogether *Colla1* gene expression remained suppressed throughout the 3 weeks

Fig. 5. Relative gene expression, biosynthesis and secretion of collagen X. Relative mRNA expression level of *Col10a1* is assayed by qRT-PCR employing the $\Delta\Delta CT$ method using SYBR I Green dye (A). Gene expression of day 0 MSCs was used as calibrator which constitutes the baseline at 1. Expression level of each gene was compared between coculture (dotted bars) and monoculture (white bars). Gene expression of monocultured MSCs + TGF β -3 (black bar) and cocultured MSCs + TGF β -3 (mosaic bar) was determined in parallel. Grey bar indicates relative gene expression level of freshly isolated rat hip articular chondrocytes. N=4. Analysis of collagen X protein biosynthesis and secretion. Collagens were precipitated from the alginate beads and from the culture medium, pepsin digested, separated on a 4.5%-15% gradient SDS-PAGE under reduced conditions and stained with Coomassie Blue (B). N=4. Collagen X degradation assay: (14 C)-labelled collagen X, isolated from metabolically labelled chicken chondrocyte cultures, was incubated with supernatants from mono- and cocultured MSCs (C). MC = monocultured MSCs, CC = cocultures, S = culture supernatant, CF = cellular fraction, * = collagen X band in cellular fraction. N = 3.

of culture and it became less than the basal level during the culture period which was mainly independent of cartilage (Fig. 4A). On the contrary, mRNA expression of *Col2a1* was progressively elevated in both mono- and cocultured MSCs similarly; cartilage exhibited no significant influence on gene expression level of *Col2a1* (Fig. 4B). Secretion of $\alpha 1(\text{II})$ collagen chains was increased in cocultures when compared with the monocultured MSCs. Collagen chains originated from cartilage explants in cocultures may account in part for this observation, however, only to a minor extent. Notably, neither cartilage nor MSCs secreted detectable amounts of collagen I, indicated by the lack of the $\alpha 2(\text{I})$ chain (Fig. 4C). To deduce the origin of collagens detected in the coculture supernatant, both mono- and cocultured MSCs (day 21) were metabolically labelled with ^{35}S Met/Cys in a pulse/chase regimen after removal of the cartilage explants. After 1 hour of chase newly synthesized collagens were detected in the medium of MSCs which were previously cultured as cocultures with a secretion peak at 4 hours of chase. We assume that the majority of the collagen constitutes collagen II, because of the poor intensity of the lower $\alpha 2(\text{I})$ chain compared to the strong signal of the upper $\alpha 1(\text{II})$ band (Fig. 4D). Minuscule collagen secretion in monocultured MSCs corroborated the non-radioactive collagen preparation results of day 21 (Fig. 4C and D). These data suggest that factors derived from cartilage explants promote collagen II secretion of MSCs indicating a positive influence on biosynthesis and / or secretion of collagen II while not influencing gene expression rates.

Fig. 6. Antibody array based detection of secreted proteins at day 1, 7 and 14 of the culture. Culture supernatants were hybridized to nylon membrane based antibody arrays (RayBio™ rat cytokine antibody array I). Positive signals for TIMP-1 and VEGF-164 α showed differential secretion pattern depending on the time point and condition of the culture. Lane 1 = cartilage, lane 2 = cocultures, lane 3 = monocultured MSCs, lane 4 = negative control. N = 2.

Fig. 7. Reverse zymography for detection of TIMP-2. Aliquots of supernatants from monocultures and cocultures were subjected to SDS-PAGE under non-reducing conditions on a 4.5-15% gradient gel containing 1% gelatine and 25% conditioned macrophage medium. Ca = monocultured cartilage, MC = monocultured MSCs, CC = cocultures. N= 3.

Fig. 8. Gelatine zymography and western blot analysis of MMP-2 and of MMP-13. Aliquots of cell culture supernatants were subjected to SDS-PAGE under non-reducing conditions on a 4.5-15% gradient gel containing 1% gelatine. Gelatine zymographic analysis of the culture supernatant demonstrates secretion of an approximately 60 kDa sized proteolytic activity (pro-MMP-13) and a ca. 90 kDa sized band (presumably pro-MMP-9, black arrow) into the culture supernatant (A). N=3. Selected supernatant samples of cartilage monoculture were subjected to western blotting with antibodies against MMP-2 (a) and MMP-13 (b) allowing identification of the gelatine degrading MMP as MMP-13 (B). Ca = monocultured cartilage, MC = monocultured MSCs, CC = cocultures. N=3.

Compared with freshly isolated chondrocytes undifferentiated MSCs exhibited a very low baseline expression of *Col10a1* mRNA. In order to analyse the effect of cartilage factors on *Col10a1* gene expression we have set up mono- and cocultures in the presence of externally added TGFβ-3. In the absence of TGFβ-3 alterations in gene expression level between mono- and cocultures were below the detection limit, albeit too low to analyse a putative effect of cartilage explants on cocultured MSCs. Induction of chondrogenic differentiation did not alter *Col10a1* mRNA expression during the first two weeks of culture. Significant elevation of *Col10a1* mRNA was observed at the terminal stage of the culture (Day 28) in the presence of external TGFβ-3. While relative gene expression in the control cultures (monoculture + TGFβ-3) increased about 100 folds on day 28, gene expression in cocultured MSCs (+TGFβ-3) remained suppressed with a relative increase below 10 folds. This strong upregulation of collagen X gene expression in monocultured MSCs under the influence of

external added TGFβ-3 is therefore profoundly suppressed in the presence of cartilage (Fig. 5A). Concurrently, collagen X secretion and biosynthesis was strongly suppressed in the presence of cartilage compared to the monocultured MSCs which released high amounts of collagen X into the culture supernatant. Little collagen X was detected in the cellular fraction of monocultures, however, none was found in cell layers of cocultured MSCs (Fig. 5B). To exclude matrix metalloprotease (MMP) induced degradation of collagen X in the cell culture supernatant of cocultures ¹⁴C-labelled collagen X was incubated with the cell culture supernatants of mono- and cocultured MSCs from day 21 for 7 hours at 37°C. No proteolytic activity was observed in mono- or cocultured MSCs supernatants that generated degradation products of collagen X or reduced signal intensity of the bands. Therefore, we exclude MMP induced degradation (Fig. 5C) and attribute this strong impact of cartilage derived soluble factors on suppression of collagen X secretion from MSCs to the biosynthesis level.

Fig. 9. Soluble signalling factors derived from differentiated cartilage are involved in chondrogenic differentiation of rat MSCs. Cartilage explants display two significant effects on chondrogenic differentiation of rat MSCs: 1. Early upregulation of Sox9 indicates early chondrogenic induction and 2. Suppression of collagen X indicates a delay in hypertrophic differentiation. We have identified MMP-13, TIMP-1, TIMP-2 and VEGF which were secreted into the coculture supernatant. However, other signalling molecules not detected in our experimental set up surely have an impact on regulating and directing the differentiation process of MSCs, i.e. BMPs and Ihh / PthrP.

Profiling of secreted proteins

To identify soluble factors which may influence chondrogenic differentiation of MSCs cell culture supernatant was hybridized to an antibody array (RayBio™ rat cytokine antibody array I). Evaluation of the array containing antibodies to 19 secretory molecules resulted in identification of three molecules which exhibited differential secretion pattern dependent on the culture condition and time point of analysis. Vascular endothelial growth factor (VEGF-164α), tissue inhibitor of metalloproteases (TIMP-1) and monocyte attractant protein (MCP-1, data not shown) were observed to be secreted from MSCs and cartilage (Fig. 6). Strong VEGF secretion signals were observed at the onset of the culture both in cocultures and cartilage explants which decreases from day 14 on in the cartilage explant supernatant. Origin of secreted VEGF cannot be attributed solely to cartilage explants because at day 14 VEGF concentration appeared to be higher in the supernatant of cocultured MSCs while secretion is reduced in cartilage explant culture. However, the secretion is clearly positively influenced by cartilage in coculture because monocultured MSCs secrete minuscule amounts of VEGF throughout the whole culture time. Highest secretion of TIMP-1 was observed in the cartilage explants with slight decline during culture time, the same was observed in cocultured MSCs. Monocultured MSCs down regulated TIMP-1 secretion to almost negligible amounts on day 14. It appeared as if coculture supernatant did not contain more TIMP-1 compared with the cartilage explant culture, presumably mainly cartilage is responsible for TIMP-1 secretion in the coculture set up. Furthermore, based on the antibody array results secretion of the following pro-inflammatory cytokines and growth factors

could be excluded at any stage of the culture. These non-secreted factors included CINC-2, CNTF, Fractalkine, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX, Leptin, MIP-3α, β-NGF and TNF-α.

Analysis of secretion pattern of MMP-13 and TIMP-2 by zymography

Reverse zymography was used to detect TIMP-2 in the culture system. Monocultured MSCs did not secrete TIMP-2 at any time point; in contrast secretion of TIMP-2 by cartilage explants started at day 7 and remained detectable until the end of the culture period. Initially, less TIMP-2 secretion was detected in cocultures when compared with cartilage explant cultures however, at the end of the culture period the pattern was reversed (Fig. 7). Gelatine zymography results, applicable also for non-gelatinolytic MMPs [26], indicated secretion of MMP-13 and MMP-9. From the beginning of the culture cartilage and cocultures secreted increasing concentration of pro-MMP-13 without decline until day 21. In contrast, release of pro-enzyme from monocultured MSCs remained largely unchanged during the culture period. Notably, the active form of MMP-13 in coculture and cartilage culture became reduced in relation to the strongly increasing pro-form (Fig. 8A). In coculture and cartilage culture we have observed an additional gelatinolytic activity, which we assume to be pro-MMP-9 according to the molecular weight (black arrow in Fig. 8A). Western blot analysis of culture supernatants confirmed the identity of MMP-13 (Fig. 8Bb) in both monocultured cartilage explants and in MSCs (data not shown), while MMP-2 was not detected in the culture model at any stage (Fig. 8Ba).

Discussion

Paracrine factors released from cartilage exert various catabolic and anabolic effects on neighbouring cells and tissues. To identify factors with putative regulatory effects on the chondrogenic differentiation cascade, a coculture system between articular cartilage and pluripotent undifferentiated MSCs was established. The hypothesis suggested that differentiation of chondroprogenitor MSCs into chondrocytes is controlled and affected by soluble factors secreted by cartilage.

In the current culture system, where MSCs were kept in alginate stabilized 3D-environment known to induce chondrogenesis in MSCs [27, 28], *Sox9* gene expression was prematurely up regulated, which indicates early onset of chondrogenesis. Sustained gene expression of *Sox9* observed on day 7 under the influence of cartilage suggests involvement of chondrocyte released signalling factors in regulation of the chondrogenic inducer activity of *Sox9*. Several signalling pathways are involved in regulating *Sox9* and its downstream genes during cartilage differentiation. One of these pathways includes TGF β , which induces *Sox9* gene expression and transcriptional activity through Smad3 [29]. TGF β , released from cartilage explants until day 14, presumably accounts in part for the observed increase in *Sox9* gene expression. On the other hand, VEGF has been described to suppress *Sox9* mRNA levels during treatment of chick embryo limb buds [30]. In our system *Sox9* gene expression is reduced to about initial levels between day 7 and 14. Possibly, high level of VEGF in coculture supernatant induced this suppression of *Sox9* in MSCs, thereby counteracting the influence of TGF β . Despite suppression of *Sox9* mRNA to baseline level, gene expression of *Col2a1* remained up regulated. This observation indicates that an active *Sox9* gene above basal level of un-induced MSCs might not be mandatory in later stages of chondrogenic differentiation of MSCs for regulation of chondrogenesis associated genes as *Col2a1*, but is seemed to be imperative at initiation of culture. Notably, we observed auto induction of *Col2a1* gene expression and collagen II protein biosynthesis in rat MSCs which is different from human MSCs. Human MSCs require growth factors from the TGF β super family as TGF β 1-3 or BMP6 in order to induce chondrogenic differentiation. Rat MSCs appear to behave differently in that the 3-dimensional environment and the agarose scaffold provided by alginate beads is sufficient to induce and maintain chondrogenesis to a limited extend. The same has been observed in bovine MSCs which exhibited induc-

tion of chondrocyte marker genes as *Col2a1*, aggrecan and *Sox9* and the corresponding proteins upon culture in alginate hydrogels in absence of TGF β support [31]. This might hint to an intriguing difference between human and animal MSCs regarding structural and trophic requirements for chondrogenic induction.

The most profound effect of cartilage derived factors was observed on suppression of collagen X gene expression and protein secretion. MSCs did not secrete collagen X before day 21 corroborated by low gene expression until that time point. However, from 21 days on monocultured MSCs released high amounts of collagen X into the culture supernatant, while cocultures did not. Suppression of collagen X secretion in the presence of cartilage depicts delayed hypertrophy in our coculture model. We suggest that cartilage derived soluble factors have the ability to reduce collagen X secretion in MSCs. As we did not observe collagen X degradation products in culture supernatant we deduce that absence of collagen X secretion is due to suppression at the biosynthesis level. This suggestion is substantiated by significantly reduced gene expression in cocultured MSCs compared with the monocultured MSCs.

Paracrine factors derived from surrounding chondrocytes and endothelial cells alter differentiation status of chondrocytes. Chondrocytes of normal articular cartilage do not undergo terminal differentiation whereas growth plate chondrocytes do. Articular chondrocytes in osteoarthritic joints however, express markers of terminal differentiation like collagen X and Alkaline Phosphatase (AP) [32]. By coculturing articular and growth plate chondrocytes Jikko *et al.* could demonstrate that soluble paracrine factors alone were responsible for inhibition of terminal differentiation. Articular chondrocytes were able to suppress calcification and AP activity in cocultured growth plate chondrocytes, indicating that articular chondrocytes produce factors which inhibit terminal differentiation [33]. Also during endochondral ossification cartilage maturation and conversion of hypertrophic cartilage to bone is delayed by paracrine signals derived from periosteal / perichondrial tissue or from the cartilage proper itself. Formation of new blood vessels and their penetration into hypertrophic cartilage appeared to be essential for the elimination of this developmental barrier. Consistently with this notion, the terminal differentiation barrier in chondrocytes from the caudal part of 17-day old chick embryo sternum could be eliminated by coculturing them with endothelial cells *in-vitro*. The humoral activity secreted by endothelial cells consists of proteinases sufficient to break down the ter-

minimal differentiation barrier inherent to this chondrocyte population [34–36].

TIMP-1, TIMP-2, VEGF-164 α and MMP13 were the four prominent secreted molecules identified in the supernatant of cocultures and cartilage explants. Except VEGF-164 α which seemed to be mainly secreted by cocultured MSCs at least at the final culture stage, TIMP-1, -2 and MMP13 were additionally released by differentiated chondrocytes of the cartilage explants. We observed a profound increase in pro-MMP-13 secretion in coculture and cartilage monocultures during the culture period; however with declining activation status. This effect might be due to a concurrent secretion of TIMP-1 and -2, both known as efficient inhibitors of MMP-13 activity [37–39]. MMP-13 is known to degrade collagen X [40] however, lack of collagen X in supernatant of cocultured MSCs can not be due to proteolytic MMP-13 activities because collagen X degradation products or reduced signal intensities were never detected in the cultures. Additionally, in absence of cartilage MMP-13 activation rate was not higher whereas collagen X secretion was profoundly increased. In MMP-13 knockout mice Inada *et al.* have demonstrated a profound enlargement of the anatomic domain where collagen X is expressed by hypertrophic growth plate chondrocytes and an increase of collagen X deposition in this domain. In addition to proteolytic activity of MMP-13 this protease might exert a yet unknown regulatory impact on collagen X biosynthesis [41, 42]. Therefore, suppression of collagen X gene expression and accordingly biosynthesis in cocultures might be also due to a regulatory effect of MMP-13 and not to a proteolytic effect.

Multiple Cbfa1 consensus binding elements are located in the collagen X promoter region [43, 44]. Despite early induction (day 1) of *Cbfa1* gene expression in MSCs differentiation pathway collagen X expression is not significantly induced before day 28. Also, relative gene expression of *Cbfa1* was not significantly influenced by cocultured cartilage in our system during the whole culture period whereas gene expression of collagen X was. Therefore, we cannot attribute an influence of this transcription factor on mRNA expression or eventual transcription of collagen X.

Another key player in this system might be VEGF-164 α , released by cartilage explants and cocultured MSCs. VEGF as one of the most important mediators of angiogenesis is abundantly expressed during embryogenesis but in adults it is only found in restricted tissues and cell types, as in osteoarthritic chondrocytes, but not in

resting chondrocytes [45, 46]. Interestingly, we have detected VEGF-164 α in increasing amounts in cocultures and cartilage, however, main source seemed to be cocultured MSCs. VEGF is known to stimulate receptor phosphorylation of its VEGF-receptor 2 (flk-1 /KDR), thereby activating the mitogen activated protein kinases ERK/1/2 inducing long-lasting activation of the transcription factor AP-1 [47, 48]. As a consensus site for AP-1 has been identified within the promoter of collagen X, VEGF binding to its receptor (VEGFR1) might contribute to suppression of gene expression of collagen X, while at the same time it is known to induce secretion of MMP-13 [47].

All four members of the TIMP-family function as inhibitors of angiogenesis by blocking MMP activity. TIMP-1 and -2, both present in cocultures, presumably account for inhibiting generation of active MMP-13. Besides, TIMPs can exert MMP-independent functions, which might be important in our system. In addition to inhibition of MMPs TIMPs may also regulate cell growth and differentiation [49]. Cell surface binding of TIMP-1 and -4 has been reported, but the identification of specific cell surface receptors is missing [50]. Therefore, TIMPs secreted by differentiated chondrocytes and MSCs may act as inducers or repressors of specific factors necessary for chondrogenic differentiation of MSCs.

In conclusion, we have demonstrated that the coculture system including differentiated cartilage explants and undifferentiated MSCs is suitable for studying paracrine communication between both the components. Notably, *in vitro* supplementation of growth factors is not necessary for induction of chondrogenic marker genes and biosynthesis of the corresponding proteins. However, for long term maintenance of the gene expression level addition of growth factors like TGF β to the system is required as internal secretion by cartilage ceases from day 14 on. Cartilage derived soluble factors are sufficient to sustain *Sox9* gene expression and suppress collagen X expression/secretion while the 3-dimensional environment provided by alginate is capable of inducing chondrogenic marker expression. MMP-13 and VEGF may have a regulatory role in collagen X expression and secretion. Besides the identified secreted proteins, MMP-13, TIMP-1 and -2 and VEGF-164 α , other signalling molecules not detected by our experimental set up surely have an impact on regulating and directing the differentiation process of MSCs. It shall be kept in mind that in coculture on one hand cartilage derived factors influence the fate of MSCs; on the other hand MSCs released

molecules might have an impact on cartilage metabolism as well. These putative proteins may even abrogate the intrinsic blockade which prevents hypertrophy and eventual ossification in articular cartilage. For MSCs based cartilage repair, inhibition of hypertrophy and stabilization of the cartilaginous phenotype in explants as well as in the host is a prerequisite for success and long lasting vitality.

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