Introduction

Bone morphogenetic proteins (BMPs) are a subfamily of the transforming growth factor-β super family and consist of about 20 members [1]. They are involved in embryonic development and tissue homeostasis and govern multiple functions, such as differentiation and cell proliferation. BMPs signal through serine/threonine kinase receptors (BMPRs), composed of type I and II subtypes [2, 3]. Three type I receptors (BMPR-Ia, BMPR-Ib and ActR-Ia) and three type II receptors (BMPR-II, ActR-II and ActR-IIb) have been identified [4–8]. Ligand binding leads to the assembly of a tetrameric complex of BMPRs, consisting of two type I and two type II receptors [9]. Signal transfer is obtained by Smad-1, -5 and -8, which are phosphorylated by the receptor complex. After phosphorylation, the Smad proteins associate with Smad-4. This complex is translocated in the nucleus and evokes alteration in the transcription of different genes [1].

BMP-14, also called growth and differentiation factor 5 (GDF-5), is expressed in developing bones during early cartilage condensation [10]. It has been suggested that GDF-5 is an enhancer but not an inducer for chondrogenesis [11]. GDF-5 mutations have been identified with brachydactyly, which causes shortened tubular bones in the hands and feet, and chondrodysplasia [12–14]. Conversely, overexpression of GDF-5 in chick limb buds increases the size of the affected elements due to an increased cell number [10]. Furthermore, GDF-5 is linked to a dedifferentiated state of the cells and to the formation of the embryonic joint interzone [15, 16].

The formation of the interzone and the process of cavitation require dramatic metabolic changes in protein expression [17].

Abstract

Human articular chondrocytes are expanded in monolayer culture in order to obtain sufficient cells for matrix-associated cartilage transplantation. During this proliferation process, the cells change their shape as well as their expression profile. These changes resemble those that occur during embryogenesis, when the limb anlagen form the interzone that later develops the joint cleft. We analysed the expression profile of genes that are reportedly important for these changes during embryogenesis within the dedifferentiation process of adult articular chondrocytes. We found GDF-5, BMPR-Ib and connexin 43 up-regulated, as well as a down-regulation of BMPR-Ia and noggin. Connexin 32 could not be detected in either native cartilage or in dedifferentiated cells. The newly synthesized proteins were detected by immunofluorescence. There is evidence from our results that dedifferentiated chondrocytes resemble the cells from the interzone in developing synovial joints.

Keywords: chondrocytes • dedifferentiation • GDF-5 • joint formation
Developmental biology describes this process as an active one, which is not only associated with the apoptosis of several cells within the cartilage zone, so that the limb anlagen form two structures articulating with each other [18].

Cells are in cytoplasmic contact with each other, with a complex pattern of gap junction protein expression (connexin 43 and connexin 32) [19]. At a molecular level, joint formation is associated with the appearance of BMP family members, such as GDF-5 and GDF-6 [15, 20] and with the down-regulation of type II collagen [21]. A similar process of dedifferentiation, associated with a down-regulation of collagen type II can be observed in monolayer cultures of human articular chondrocytes [22, 23]. The mechanisms that support this in vitro dedifferentiation process have not been well characterized, to date.

GDF-5 is described as one of the pivotal factors in joint development; thus we studied the role of GDF-5, its receptors (BMPR-Ia, BMPR-Ib and BMPR-II) and its antagonist (noggin) in the course of this in vitro dedifferentiation process. Furthermore we investigated the changes in the expression pattern of connexin 43 and 32 as important representatives of the intercellular communication system.

We suggest that some molecular expression patterns that have been described for the formation of the interzone can be found in the dedifferentiation process of human articular chondrocytes.

Material and methods

Isolation and monolayer culture of human articular chondrocytes

Human articular cartilage samples were collected from the hip of three patients (mean age 80 years) with no history of joint disease, who were scheduled to undergo joint replacement following femoral neck fracture (approved by the Ethics Board of the University of Vienna; code: 184/98). The isolation and cultivation of human articular chondrocytes was performed as previously described [22]. Chondrocytes from three different donors were pooled after cell isolation in order to obtain a sufficient number of chondrocytes for dedifferentiation experiments. From the same cartilage used for the cell culture a fraction was pooled and used as native cartilage control. Cells isolated from the ligamentum capitis femoris were used as a fibroblast control.

RNA extraction and purification

Total RNA was obtained from cells that were being cultivated in monolayer culture. Cells were harvested after cell isolation, at day 1, day 2 and then every second day until day 36, by adding 1 ml of TRI REAGENT® (Sigma-Aldrich, St. Louis, MO, USA). Lysis of the cells was performed directly on the culture dish. RNA isolation was performed according the standard protocol. For total RNA extraction from native cartilage, slices of human articular cartilage were frozen in fluid nitrogen and ground using a mortar and pestle. Further steps were performed with the RNeasy® plant mini kit (Qiagen, Germany). The procedure for isolating total RNA was executed as described in the RNeasy® Mini Handbook (Qiagen, Valencia, CA, USA).

cDNA synthesis

For cDNA synthesis, 0.2–1 μg of total RNA was diluted with nuclease-free water to a volume of 15 μl, and 4 μl iScript™ Reaction Mix, as well as 1 μl iScript™ Reverse Transcriptase, were added (Bio-Rad Laboratories, CA, USA). The reaction was incubated for 5 min. at 25°C, at 40°C for 30 min. and finally, heated at 85°C for 5 min.

Primers and probes for quantitative analyses

Primers and probes were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), which creates oligonucleotides with similar melting temperatures and minimal self complementarity. To avoid amplification of genomic DNA, the probes were placed at the junction of two exons. Gene specificity of the primers and probes and the absence of DNA polymorphism were confirmed by BLAST searches. Primers and probes were synthesized by biomers.net (Ulm, Hilden Germany). Primer concentrations were tested for each primer at concentrations of 50, 300 and 900 nM, and the combination that displayed the lowest threshold cycle value was chosen. Primer sequences are shown in Table 1.

Real-time PCR amplification and analysis

PCR amplification was performed and monitored using an ABI Prism® 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Hercules, CA, USA). The master mix was based on Brilliant™ QPCR Master Mix (Stratagene, Cycle Threshold, CA, USA). The thermal cycling conditions comprised the initial steps at 50°C for 2 min. and at 95°C for 10 min. Amplification of the cDNA products was performed with 40 PCR cycles that consisted of a denaturation step at 95°C for 15 sec. and an extension step at 60°C for 1 min. As an internal standard, 18S rRNA was chosen, using the pre-developed Taq Man™ assay (Applied Biosystems, Waltham, CA, USA). All cDNA samples (3 μl in 25 μl) were analysed in triplicate. The final numeric value was calculated as the ratio of the gene to 18S rRNA and was expressed in arbitrary units.

Immunofluorescence

Proteins, which were found up-regulated on the mRNA level in monolayer culture, were detected by immunofluorescence on the protein level.

Dedifferentiated chondrocytes from monolayer culture (4 weeks) were seeded onto 4-well chamber slides (about 40,000 cells per chamber). After a cultivation time of 3 days half of the slides were treated with 2 μM monensin (Sigma-Aldrich) for 24 hrs. Monensin, as an inhibitor of the intracellular protein transport, leads to an accumulation of newly synthesized pro-proteins in the endoplasmic reticulum and enhances the detection of cytokines that are expressed at low levels. The slides were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). After blocking with 2% bovine serum albumin, the slides were incubated for 12 hrs using the following primary polyclonal antibodies: GDF-5 (1:50, La Jolla, Santa Cruz, CA, USA); noggin (1:50, La Jolla); monoclonal antibodies: connexin 43 (1:100, Chemicon, Santa Cruz, CA, USA) and BMPR-Ib (1:50, R&D Systems, Inc., Minneapolis, MN, USA). Goat

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Table 1 Description of the designed primers and probes

| mRNA template | Primer sequence |
|---------------|----------------|
| GDF-5         | Left: 5′-ccacacacagcttattga-3′  
|               | Right: 5′-cagttctcctgatacgctgctg-3′  
|               | Probe: 5′-caagacgccacagcttattga-3′  
| BMPR-la       | Left: 5′-cagctatccctctgatacgctgctg-3′  
|               | Right: 5′-cctgtttcctgatacgctgctg-3′  
|               | Probe: 5′-aaataactcctgatacgctgctg-3′  
| BMPR-lb       | Left: 5′-gtcgctggctgatacgctgctg-3′  
|               | Right: 5′-ggtgcctgatacgctgctg-3′  
|               | Probe: 5′-caggaagccacagcttattga-3′  
| connexin 43   | Left: 5′-actggtgcctgatacgctgctg-3′  
|               | Right: 5′-agttctgtagctgatacgctgctg-3′  
|               | Probe: 5′-aaagatgtagctgatacgctgctg-3′  
| connexin 32   | Left: 5′-ctggacacagctgatacgctgctg-3′  
|               | Right: 5′-gtgacacagctgatacgctgctg-3′  
|               | Probe: 5′-cctggacacagctgatacgctgctg-3′  
| noggin        | Left: 5′-agatggagggctagttctgatacgctgctg-3′  
|               | Right: 5′-acacgccacatctgatacgctgctg-3′  
|               | Probe: 5′-aagagcagcctgatacgctgctg-3′  

Together with the increase in GDF-5 its specific receptor, BMP receptor 1b (BMPR-1b), was increased, but did not reach the level of ligament fibroblasts (Fig. 1b). Other BMPRs (BMPR-la and BMPR-lb), which are involved in BMP signal transduction, also altered their expression level, although to a lesser extent. We found that BMPR-la was slightly down-regulated during the entire cultivation period (Fig. 1c) and BMPR-lb was up-regulated around day 18 (Fig. 1d).

Along with the increase in GDF-5 there was a reduction of the inhibitor, noggin, which was diminished to one-tenth the expression level in native cartilage (Fig. 2a).

The mRNA level of connexin 43, a protein, which is responsible for the intercellular communication, was increased from day 12 to day 32, and corresponds quite well to the expression level of connexin 43, detected in human ligament fibroblasts (Fig. 2b).

Connexin 32, a protein that has been described as an important parameter for intercellular communication in the embryonic interzone, could not be detected in any sample (data not shown).

Immunofluorescence of intracellular and membrane proteins in chondrocyte monolayer culture

Protein expression of GDF-5 is shown in Fig. 3(a). The staining consistently extended over the whole cell and there was little difference from chondrocytes treated with monensin (Fig. 3b). Three-dimensional projection did not result in a clear reconstruction of the spatial organization of GDF-5. Incubation with monoclonal antibody against the GDF-5-specific receptor, BMPR-lb, resulted in an intensive staining of the cell membrane (Fig. 3c). Chondrocytes treated with monensin also showed many vesicles filled with BMPR-lb (Fig. 3d). The localization of these vesicles in the cytoplasm was verified by a three-dimensional projection of the cell. Figure 3(e) and (f) shows a double immunofluorescence staining of GDF-5 and BMPR-lb. As in the previous figures, GDF-5 is depicted in green, whereas BMPR-lb is demonstrated as red. Whether GDF-5 is bound to BMPR-lb could not be determined.

In order to prove whether cells form gap junctions, connexin 43 was stained with monoclonal antibodies. Connexin 43 staining appeared at the cell borders (Fig. 4a). Cells treated with monensin display connexin 43 staining in the perinuclear vesicles (Fig. 4b).

The expression of the BMP inhibitor, noggin, is demonstrated in Fig. 4(c). Noggin is mainly located in the perinuclear area. Monensin treatment caused the formation of vesicles containing noggin. These vesicles are located perinuclearly and throughout the cytoplasm (Fig. 4d).

Statistical analysis

All samples were assayed by real-time PCR in triplicates. The values were log transformed and reported as the mean ± S.D. of the real-time PCR analyses.

Results

Real-time PCR

The mRNA level of GDF-5 showed a 10-fold increase in expression after the first day, and remained unchanged during the entire cultivation period (Fig. 1a).
Fig. 1 Real-time PCR analysis of native cartilage (cart.), chondrocytes in monolayer culture (days 0–36) and ligament fibroblasts (lig.). Gene expression was normalized to 18S rRNA expression. Data were log transformed and expressed as mean ± S.D. of real-time PCR triplicate analysis. (a) GDF-5: There is a 10-fold up-regulation of GDF-5 expression in chondrocytes in monolayer culture compared to native cartilage. (b) BMPR-Ib: An up-regulation of BMPR-Ib expression can be found along with the up-regulation of GDF-5. (c) BMPR-Ia: There is a continuous down-regulation of BMPR-Ia from day 0 to day 36. Around day 24 a peak of BMPR-Ia expression is found. (d) BMPR-II: BMPR-II is expressed at a more or less stable level from day 0 to day 36. A peak of BMPR-II expression is found around day 24.

Fig. 2 Real-time PCR analysis of native cartilage (cart.), chondrocytes in monolayer culture (day 0–36) and ligament fibroblasts (lig.). Gene expression was normalized to 18S rRNA expression. Data were log transformed and expressed as mean ± S.D. of real-time PCR triplicate analysis. (a) Noggin: The expression level of noggin is diminished during the entire cultivation period in monolayer culture and shows a level similar to noggin expression in ligaments. (b) Connexin 43: After about 10 days connexin 43 expression is up-regulated to a level that is nearly equal to that of fibroblasts.
formation of the interzone can also be found in the dedifferentiation process of human articular chondrocytes.

Differentiation and proliferation are antagonistic processes. When chondrocytes are removed from their natural environment and transferred into monolayer culture, the arrest of the cell cycle is reversed and cells are stimulated to re-enter the propagation process.

Our studies indicate that one of the crucial players for the restart of propagation may be GDF-5. Within only 1 day in monolayer culture, there is a 10-fold increase in the synthesis of GDF-5. This up-regulation is maintained throughout the whole proliferation period, reaching its peak around day 22. This is also the period when the switch from type II collagen to type I collagen is completed [23].

This up-regulation of GDF-5 occurs together with the up-regulation of BMPR-Ib, indicating that both genes represent a functional unit and may augment the effect of GDF-5 on its target genes in this way.

The effect of GDF-5 is mediated by its binding to a tetrameric complex, consisting of BMPR-II and BMPR-I, and especially -Ib. We could show that, in cells that are transferred to monolayer culture, the level of BMPR-Ib is increased, while the expression of BMPR-Ia is down-regulated. One may speculate that this down-regulation happens in conjunction with the up-regulation of BMPR-Ib and is somehow an antagonistic process. The level of BMPR-II expression remains unchanged until day 18. After this time-point, there is an up-regulation of BMPR-II but also a slight increase of BMPR-Ia. These data suggest a synchronization of the expression profile of these three receptors.
A diminished amount of noggin also amplifies the effect of GDF-5. Noggin has been reported as an inhibitor of BMP-2, BMP-4, BMP-7 and GDF-5 by binding to these proteins and preventing interaction with their receptors [24]. If noggin is expressed at a lower level, a lesser amount of GDF-5 could be inhibited, and the proliferation process might be enhanced.

With the increase in cell number, there is also increased need for cell–cell communication. Thus, we tested to what extent cell–cell communication in dedifferentiated cells resembled communication during cavitation formation in the embryonic joint. Connexin 43 is a gap junctional protein, which is responsible for the increase in intercellular communication and synchronization during the formation of joint cavitation [25]. In the case of our dedifferentiation studies, we found that the expression of this gene is up-regulated around day 10, and again reaches its peak around day 18.

Studies suggest that expressions of GDF-5 and connexin 43 occur in conjunction with each other and display a similar spatiotemporal expression pattern in a variety of tissues. GDF-5 activity is one element of a mosaic of repressors and enhancers that are necessary to activate the connexin 43 promoter function. Conversely, the suppression of gap junctions inhibits the stimulatory effect of GDF-5 on chondrogenesis in micromass cultures. A functional gap junction may therefore be pivotal to ensure the action of GDF-5 [26, 27].

In the course of our studies, we also investigated connexin 32, another gap junctional protein that has been reported to be expressed together with connexin 43 during the formation of the joint embryonic interzone [19]. In this case, we were not able to detect any expression of this protein in the course of chondrocyte cultivation in monolayer culture.

Thus, considering our data on the expression of type I and type II collagen from previous studies [22, 23] together with GDF-5, noggin and connexin 43 from this experiment, we assume that the dedifferentiation process of chondrocytes in monolayer culture is established after a time period of about 20 days.

Around this period, it seems that the cells try to redifferentiate, as we found an up-regulation of BMPR-Ia, BMPR-II and noggin as well as a slight up-regulation of collagen type II in earlier studies [23]. We do not think that these findings are accidental, but suggest that this could be an ideal time-point for an external redifferentiation stimulus.

There is a striking morphological similarity between the cells of the interzone and dedifferentiated chondrocytes. Our molecular biological data highlight the similarity between those two events and indicate that dedifferentiation mechanisms may use, at least in part, signalling pathways and intercellular communication molecules that are similar to those used during embryonic development within the articular interzone.

The better these pathways are understood, and the more we know about events on a molecular biological basis in vitro, the better we can regulate proliferation and differentiation processes in vitro. A modulation of these signalling pathways may be an elegant way to influence the redifferentiation behaviour of cells destined for transplantation.

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