Osteoarthritis and Cartilage 23 (2015) 433–442

Osteoarthritis and Cartilage

Direct bone morphogenetic protein 2 and Indian hedgehog gene transfer for articular cartilage repair using bone marrow coagulates

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ARTICLE INFO

Article history:
Received 20 May 2014
Accepted 5 November 2014

Keywords:
Cartilage repair
Indian hedgehog
Bone morphogenetic protein 2
Cartilage
Bone marrow coagulates
Gene therapy

SUMMARY

Objective: Bone morphogenetic protein 2 (BMP-2, encoded by *BMP2*) and Indian hedgehog protein (IHH, encoded by *IHH*) are well known regulators of chondrogenesis and chondrogenic hypertrophy. Despite being a potent chondrogenic factor BMP-2 was observed to induce chondrocyte hypertrophy in osteoarthritis (OA), growth plate cartilage and adult mesenchymal stem cells (MSCs). *IHH* might induce chondrogenic differentiation through different intracellular signalling pathways without inducing subsequent chondrocyte hypertrophy. The primary objective of this study is to test the efficacy of direct *BMP2* and *IHH* gene delivery via bone marrow coagulates to influence histological repair cartilage quality *in vivo*.

Method: Vector-laden autologous bone marrow coagulates with 10^{11} adenoviral vector particles encoding *BMP2*, *IHH* or the Green fluorescent protein (*GFP*) were delivered to 3.2 mm osteochondral defects in the trochlea of rabbit knees. After 13 weeks the histological repair cartilage quality was assessed using the ICRS II scoring system and the type II collagen positive area.

Results: *IHH* treatment resulted in superior histological repair cartilage quality than *GFP* controls in all of the assessed parameters (with $P < 0.05$ in five of 14 assessed parameters). Results of *BMP2* treatment varied substantially, including severe intralesional bone formation in two of six joints after 13 weeks.

Conclusion: *IHH* gene transfer is effective to improve repair cartilage quality *in vivo*, whereas *BMP2* treatment, carried the risk intralesional bone formation. Therefore IHH protein can be considered as an attractive alternative candidate growth factor for further preclinical research and development towards improved treatments for articular cartilage defects.

Introduction

Available cell based treatments for articular cartilage repair fail to persistently restore hyaline articular cartilage *in vivo*[1]. One of the reasons for this failure, is an insufficient chondrogenic differentiation of transplanted or local multipotent cells[2]. To overcome this limitation, the delivery of relevant factors has been proposed as treatment option[2]. Several growth factors have been shown to stimulate chondrogenesis, including members of the transforming growth factor (TGF)-β superfamily[2,3]. One member of this family, bone morphogenetic protein 2 (BMP-2, encoded by *BMP2*), is emerging as a potent inducer of chondrogenesis *in vitro*[4–6] and *in vivo*[7–9]. BMP-2 is affecting gene expression via members of the SMAD protein family or mitogen-activated protein (MAP) kinase pathways after binding to BMP type I and type II receptors at the cell membrane[10,11]. However, along with other factors, it was

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http://dx.doi.org/10.1016/j.joca.2014.11.008
1063-4584/

observed to induce chondrocyte hypertrophy in osteoarthritis (OA), growth plate cartilage and adult mesenchymal stem cells (MSCs) *in vitro*[2,6,12]. While the induction of intralesional hypertrophy and bone formation in cartilage defects by BMP-2 was discussed previously, there is only limited data on this topic to date[2].

To achieve persistent hyaline cartilage regeneration, the stimulation of chondrogenic differentiation using different, non-hypertrophic, pathways is desirable[2]. The growth plate regulating Indian hedgehog protein (IHH, encoded by *IHH*) is an attractive candidate growth factor, which is observed to effectively induce chondrogenesis of MSCs *in vitro*, without driving chondrocyte hypertrophy[13,14]. IHH is affecting gene expression via Gli transcription factors[14]. Although BMP signalling might affect the *IHH* gene expression, no extensive activation of BMP signalling by IHH is described to date[15]. Despite of promising results *in vitro*, it is not known if IHH is capable of inducing chondrogenesis in cartilage defects *in vivo*.

The objective of this proof of concept study was to determine if IHH is capable to improve cartilage repair quality *in vivo* and thus resembles an attractive alternative growth factor candidate. Specifically, we hypothesized, that direct adenoviral gene transfer of *IHH* or *BMP2* into osteochondral defects, using autologous vector-laden bone marrow coagulates, results in higher histological repair cartilage quality and higher type II collagen deposition, than the transfer of *GFP* (encoding for the non-chondrogenic green fluorescent protein). Bone marrow coagulates in combination with adenoviral vectors were previously shown to be an efficient direct gene delivery system for the treatment of osteochondral defects[16,17].

Methods

Experimental animals, allocation, outcomes and comparisons

This controlled small-animal experiment was performed as approved by the Institutional Review Board of the Julius-Maximilians-University of Würzburg, Germany. Reporting is guided by the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines[18]. Ten female, skeletally mature New Zealand White Rabbits (with a mean weight of 3.11 kg ± 0.22 kg standard deviation; Charles River, Kißlegg, Germany) were randomly assigned to the respective groups: $n = 4$ animals in the *IHH* and $n = 3$ in the *BMP2* treatment groups, as well as $n = 3$ in the *GFP* control group. All animals received one osteochondral defect in each of their knees. In a one-stage procedure the defects were filled with an autologous bone marrow coagulate in combination with adenoviruses carrying complementary DNA (cDNA) of the respective transgene [Fig. 1(a)–(d)]. According to the allocation of the animal, the same gene was transferred in both knees, thus each animal contributed two observations. One additional animal was used to characterize the morphology of the treated lesion at the day of surgery [Fig. 1(e)].

Histological scoring, using the *overall assessment* parameter of the semiquantitative International Cartilage Repair Society II histological scoring system (ICRS II)[19] and type II collagen deposition (assessed by immunohistomorphometry) were designated as primary outcomes. Further, we assessed the remaining ICRS II parameters, the type X collagen deposition (as a marker of chondrocyte hypertrophy) and the type I collagen deposition (as a marker of fibrocartilage). The *BMP2* and *IHH* treated animals where compared to *GFP* controls at 13 weeks after surgery, which has previously been reported to be an appropriate time to evaluate long-term repair results in rabbits[20].

Adenoviral vectors

The first-generation, early region 1 and 3 (E1 and E3) deleted, serotype five adenoviral vectors carrying *BMP2*, *IHH* or *GFP* cDNA were previously generated and amplified as described before[6,21]. Suspensions of recombinant adenovirus were prepared by amplification in human embryonic kidney 293 cells followed by purification using three consecutive caesium chloride gradients[21]. Viral titres were estimated to be between 10^{12} and 10^{13} particles/ml by optical density at 260 nm and standard plaque assay[21].

Intervention, gene transfer system and animal care

Autologous bone marrow coagulates containing adenoviral vectors, were used as gene transfer system, as described and characterized before[16]. Briefly, animals received 10 mg xylazine (Bayer Vital, Leverkusen, Germany) and 200 mg ketamine

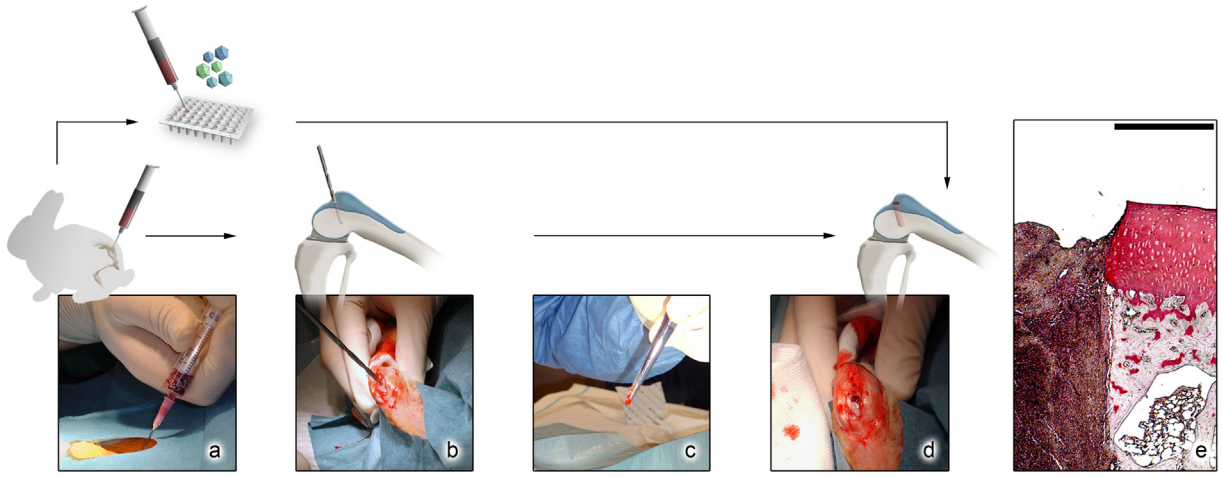

Fig. 1. One-stage gene transfer procedure and defect morphology at day 0. Aspiration of autologous bone marrow, transfer into wells of a 96-well plate and addition of 10^{11} adenoviral vector particles carrying the respective gene (either *IHH*, *BMP2* or *GFP*) into each well (a). Exposure of the femoral trochlea using a medial arthrotomy and generation of a 3.2 mm diameter osteochondral defect (b). The vector-laden bone marrow coagulate was obtained from the 96-well plate using forceps and implanted into the lesion without additional fixatives (c, d). Capsule, subcutaneous tissue and skin were closed in layers and the procedure was repeated for the contralateral knee (not shown). Type II collagen staining of the osteochondral defect after press-fit implantation of the autologous vector-laden bone marrow coagulate. Native adjacent cartilage and subchondral bone is depicted on the right side of the picture, while the vector-laden bone marrow coagulate is depicted on the left side. Note, that fixation and demineralization was necessary, which may have altered the coagulate surface. Bar representing 500 μm (e).