Arthroplasty of the lunate using bone marrow mesenchymal stromal cells

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Abstract Mesenchymal stromal cells have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage and ligaments. The potential for the regeneration of bone with cartilage coverage has still not been achieved. We evaluated the ability of bone marrow mesenchymal stromal cells to regenerate osteochondral defects in the cavity of the lunate in an animal model. Autologous mesenchymal stromal cells were harvested from the iliac crest of New Zealand white rabbits and expanded in vitro. Total lunate excision was performed in 24 animals and the isolated cells were loaded onto scaffolds. Cell-free scaffolds were implanted in the lunate space of the right wrists of all animals, and the left lunate spaces were filled with predifferentiated, cell-loaded scaffolds. Radiographic and histological analyses were performed after two, six and 12 weeks. In addition, the animals were injected with a fluorescent agent every five days, starting at day 30. After two and six weeks there was no radiographic evidence of ossification, whereas after 12 weeks all animals showed radiographic evidence of ossification. Histological sections showed increasing evidence of cartilage-like cell formation at the edges and new bone tissue in the centre of the newly formed tissue in all

groups. The histological examinations showed that bone tissue was located around the newly incorporated vascularisation. This study demonstrated that newly formed vascularisation is necessary for the regeneration of bone tissue with cell-loaded scaffolds.

Introduction

Bone marrow mesenchymal stromal cells (BMSCs) are a potential cell source for tissue engineering. They are multipotent progenitor cells, which have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligaments, muscle, fat and dermis [1–4]. The cells can be isolated from a variety of tissues [1, 5, 6] using different separation techniques and differentiated into the appropriate phenotype under defined culture conditions with the action of specific growth factors or cytokines [7]. Their advantages are unlimited supply and easy accessibility by bone marrow aspiration, the possibility of in vitro expansion of the cells and an extensively studied high osteogenic and chondrogenic potential. Cell-based strategies may include the injection of cells with osteogenic potential into the repair site and implantation of three-dimensional scaffolds that are seeded with osteogenic cells in the defect. Bone and cartilage tissue-engineering strategies involving the use of BMSCs have been explored in preclinical animal models [6, 8]. Some of these studies have suggested that the repair of full-thickness osteochondral defects may be enhanced by the implantation of scaffolds with BMSCs [9].

One of the most frequently used animal models is the rabbit. The carpus of the rabbit is a complex unit of eight

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bones arranged into a proximal and a distal row. The wrist owes its stability and range of motion to the interaction of the capsuloligamentous structures and the contact between the cartilage layers of each carpal bone [10]. Wrist instability can be caused by many diseases, resulting from arthritic changes and trauma with secondary changes. Loss of the normal ligamentous integrity and osseous deformities cause pain and loss of function for the patients [11]. The post-traumatic osteomalacia of the lunate (Kienböck's disease) was first described by Kienböck in 1910 [12]. Since then, numerous experimental and clinical studies on its aetiology and pathophysiology have been performed and surgical treatments have also been attempted. Various procedures have been described for treatment of the different stages of Kienböck's disease. Intercarpal arthrodesis [3, 13], proximal row carpectomy [14–16] and implantation of vascularised grafts [17] are all approaches in clinical use. Beside these clinical therapeutic options, there are additional scientific approaches for the regeneration of bone defects. The scientific challenge is to regenerate both the bone defect and also the cartilage tissue on the surface. Huang et al. achieved regeneration of the lunate in a rabbit model with a cell-loaded scaffold; however, the treatment of a bone defect with chondrogenic coverage has still not been achieved [30].

In our study, we wanted to visualise the new vascularisation with the injection of a blue-gelatine solution and show that the regenerative tissue is associated with newly formed blood vessels.

Materials and methods

Cell harvest and isolation

Rabbit bone marrow was harvested from the posterior iliac crest of New Zealand white rabbits (four to six months old). An 18-gauge needle was used to penetrate the cortex of the iliac crest, and 7–8 ml of bone marrow was aspirated into a syringe containing 3,000 units of heparin. Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) with 10% foetal bovine serum (FBS, Gibco, Karlsruhe, Germany) was added to the aspirate and the cells were plated and grown for 14 days at 37°C and 5% CO₂. The medium was changed after one week and then every three to four days.

Scaffolds

The scaffolds were a composite sponge of 70% esterified hyaluronan and 30% gelatine. The hyaluronan component was obtained from commercially available Laserskin (Fidia Advanced Biopolymers, Abano Terme, Italy). The

gelatine component was hydrolyzed bovine collagen (Sigma, Steinheim, Germany). The composite scaffold had pores of two different sizes, with ranges of 50–150 µm and 250–500 µm in diameter [5].

In vitro assay

After reaching 80% confluence, cells were harvested by treatment of 0.1% trypsin containing 1 mM EDTA (Gibco, Karlsruhe, Germany). Cells were washed, counted and resuspended in 40 µl of serum-free defined medium, at a final concentration of 10⁵ cells/µl. The cell suspension was loaded onto gamma-sterilised composite scaffolds (4×5×2 mm) and preincubated for two hours at 37°C. Cell-loaded composites were then cultured in a serum-free defined chondrogenic medium containing transforming growth factor-beta 1 (TGF-β1) (R&D Systems, Wiesbaden, Germany) (10 ng/ml), dexamethasone (10⁻⁷ M) (Gibco, Karlsruhe, Germany), ascorbate 2-phosphate (37.5 µg/ml) (Gibco, Karlsruhe, Germany), insulin transferrin selenite (ITS) (6.25 µg/ml) (Sigma Aldrich, Steinheim, Germany), pyruvate (0.1 mM) (Sigma Aldrich, Steinheim, Germany), and bovine serum albumin (1.25 µg/ml) (Gibco, Karlsruhe, Germany) with medium changes every three days. After 21 days, the preparations were harvested for histological and immunohistochemical analysis.

Histological and immunohistochemical analysis

Cell-loaded scaffolds were fixed in 10% neutral buffered formalin, rinsed with phosphate buffered saline, dehydrated, embedded in Tissue-Tek (Sakura, Zoeterwoude, Netherlands) and frozen in liquid nitrogen. Sections were taken of 10-µm thickness, air dried, fixed in acetone and washed with 0.5 M Tris-buffer. Samples were then stained in a 0.03% toluidine-blue solution for one minute.

For immunohistochemical analyses the specimens were rinsed three times with Tris-buffer and pre-digested with pepsin (0.1%) (Fluka, Buchs, Switzerland) for 15 minutes. Blocking of non-specific antibody binding sites was done by incubating the slides in 5% bovine serum albumin (BSA) (Sigma Aldrich, Steinheim, Germany) in phosphate-buffered saline (PBS) for one hour. Type-II collagen immunohistochemistry was performed using monoclonal mouse anti-type-II collagen antibody (Dianova, Hamburg, Germany) in phosphate-buffered saline solution containing 1% bovine serum albumin (BSA) over night at room temperature. The specimens were rinsed three times with Tris-buffer. Immunostaining was visualised by treatment with a secondary antibody (Biotin-SP-conjugated Affini-Pure Goat-anti-Mouse IgG; Dianova, Hamburg, Germany) for 60 minutes, followed by diaminobenzidine staining (DAB kit; Sigma Aldrich, Steinheim, Germany). Negative

controls were prepared following the same protocol without addition of the primary antibodies.

Lunate excision

Approval was given by the Institutional Animal Care and Use Committee of the University of Regensburg. Anaesthesia was given using 2% xylazinehydrochlorid (2 mg/kg body weight) (Rompun®, BayerVital GmbH & Co KG, Leverkusen, Germany) and 10% ketaminehydrochlorid (15 mg/kg body weight) (Ketavet®, Pharmacia & Upjohn GmbH, Erlangen, Germany) intramuscularly. The forelimbs of the 24 New Zealand white rabbits were shaved, prepared, sterilised and draped. A 2-cm dorsal longitudinal skin incision was made directly centred over the lunate in the line with the third metacarpal bone of the wrist. Extensor tendons were carefully retracted and an incision was made through the dorsal fat pad. The joint capsule was opened and the scapholunate and lunatocuneiform joint were identified. The entire lunate was carefully excised. Animals were divided into three groups (Table 1). In all groups, the lunate space of the left forelimb was filled with $4 \times 5 \times 2$ mm composite scaffolds loaded with BMSCs, which had been preincubated in a chondrogenic medium (experimental group). The lunate spaces of the right forelimbs were filled with the same sized scaffolds without cells (control group). Following placement of the implant, the dorsal joint capsule and fat pad were reapproximated to secure the scaffold in the lunate space. The extensor retinaculum and the skin were closed, and the wound was then covered with a soft compressive dressing with no additional immobilisation. The rabbits were allowed to bear weight as tolerated. At two, six and 12 weeks, tissues were harvested for histological and intravital labelling analyses.

Radiographic analysis

Standard posteroanterior radiographs of the wrists were made immediately after surgery and at the time of tissue harvest. The radiographs were evaluated independently by two blind reviewers (MN, PA) for presence of osseous tissue in the lunate defects.

Table 1 Number of animals in each group

Groups according to harvest time after implantation	Left wrist (scaffold with cells)	Right wrist (scaffold without cells)
Group 1 (two weeks)	8	8
Group 2 (six weeks)	8	8
Group 3 (12 weeks)	8	8

Intravital fluorochrome labelling

After implantation the animals were injected with a fluorescent agent every five days, starting at day 30, in order to label the process of bone formation. Injections were administered subcutaneously:

- On the 30th and 35th days: xylenol orange (90 mg/kg body weight, Merck, Darmstadt, Germany)
- On the 40th and 45th days: calcein green (10 mg/kg body weight, Merck, Darmstadt, Germany)
- On the 50th and 55th days: alizarin red komplexon (30 mg/kg body weight, Merck, Darmstadt, Germany)

By fluorescence microscopy, the polychromically labelled sequence was captured and the corresponding time of the highest rate of bone formation was determined.

Vascularisation

The vascularisation was marked by pressure perfusion of the brachial artery immediately after sacrificing the animals. The brachial arteries were cannulated with a common intravenous line and flushed with a buffer saline (0.1 M). A 4% phosphate-buffered formaline (pH 7.0) was injected, and the artery flushed again with a buffer saline (0.1 M) at body temperature. The arm was put into water (body temperature), and the Berliner blue solution (body temperature) (Chroma, Stuttgart, Germany) combined with gelatine (2%) was injected. To fix the blue gelatine solution, the arm was cooled in ice water.

Histological analysis of the forelimb

The wrists of the rabbits were harvested and fixed in 4% phosphate-buffered formalin (pH 7.0) and then embedded in polymethylmethacrylate (Technovit 7200 VLC®, Kulzer&Co, Friedrichsdorf, Germany). Coronal sections were then cut into 250 µm-thick slices using a water-cooled saw (Exakt, Norderstedt, Germany). The sections were ground and polished to a thickness of 150 µm. Toluidine-blue staining was performed by incubating the sections in 0.03% toluidine-blue solution for 15 minutes. Histological sections were reviewed by two blind reviewers (RK, PA).

Results

Cultivation of bone marrow mesenchymal stromal cells and cell-scaffold constructions

After proliferation the cells were loaded onto the gamma-sterilised scaffolds. All cell-loaded scaffolds cultured in chondrogenic medium retained their original size and

shape, with minimal swelling or distortion of the three-dimensional architecture. After 21 days in vitro, the surface of the cell-scaffolds exhibited cartilage-like morphology. Evidence of chondrogenic differentiation all over the scaffolds was confirmed by the presence of extensive toluidine-blue metachromasia and a positive type-II-collagen immunohistochemical staining (Figs. 1 and 2). No cross-reactivity between type-II antibody and the scaffolds was detected.

Radiographic analysis

The control group, in which the lunate excision had been performed with implantation of cell-free scaffolds, showed no radiographic signs of new bone formation at any time. The experimental group, in which the lunate excision had been treated with implantation of cell-scaffolds, demonstrated no bone formation at two and six weeks; however, after 12 weeks new bone formation was observed in all eight animals (Fig. 3).

Histological analysis

At the end of the in vivo tests, no signs of infection or chronic inflammation of the wrists could be detected. The lunate spaces of the control group of all animals, which were treated with cell-free scaffolds, demonstrated disorganised fibrous tissue. No evidence of vascularisation or

Fig. 1 Histological sections of bone marrow mesenchymal stromal cell-loaded scaffolds after incubation in chondrogenic medium for three weeks. The scaffold stained intensely for toluidine-blue metachromasia, indicating the abundance of proteoglycan deposition ($\times 2$, bar=1 mm)

Fig. 2 Chondrogenic differentiation was further confirmed with a positive type-II collagen immunohistochemical staining ($\times 20$, bar=200 μ m)

bone or cartilage formation was observed histologically in any of these animals (Fig. 4a, b). After two weeks, the wrists of the experimental group in which the cell-scaffolds were implanted, showed small remnants of the original

Fig. 3 Radiographs of group-3 animals at 12 weeks. **a** The right wrist after lunate excision and implantation of a cell-free scaffold. No evidence of bone formation was seen. **b** The left wrist after lunate excision and implantation of a cell-loaded scaffold. New bone formation was seen in the repair tissue within the lunate space in all animals (white arrow)

Fig. 4 Histological sections of the control group after 12 weeks. Staining was performed with toluidine blue. **a** An overview of the lunate space, which is filled with disorganised fibrous tissue. No

evidence of organised tissue, blood vessels, cartilage or bone formation can be seen. Magnification $\times 4$. **b** Magnification $\times 20$ of the area highlighted in **a**

hyaluronan-gelatin-composite scaffolds between newly formed scar tissue (Fig. 5). Detached blood vessels were seen in the lunate space (arrow, Fig. 5). No evidence of new bone formation was seen in the animals after two weeks.

The lunate replacement with the cell-scaffolds showed good defect filling after six weeks. Fibroblastic cells were seen on the contact surface to the surrounding carpal bones (Fig. 6). Compared to the two-week results, increasing vascularisation and ossification was seen in the centre of the defects (arrow, Fig. 6). In animals in which the cell-scaffolds were implanted for 12 weeks, the lunate space was filled with repair tissue (Fig. 7a). At higher magnifi-

cation, the cell formation on the surface resembled differentiated chondrocytes with surrounded extracellular matrix deposition. On the right side of Fig. 7b we saw hyaline cartilage tissue of the normal bones. On the left side we observed regenerated cartilage-like tissue on the surface (Fig. 7b). Evidence of bone formation was observed with islands of ossicles within the lunate space. Vascularisation was seen in the centre of the lunate space, without penetrating the surrounding cartilage-like cell layers (arrow, Fig. 7a). Compared with the six-week animals, the 12-week rabbits had more newly formed cartilage tissue with more extracellular matrix and also more bone tissue within the lunate space. The grade of vascularisation was also advanced.

Fig. 5 Histological sections of the experimental group after two weeks. Toluidine-blue staining showed small remnants of the original hyaluronan-gelatin-composite scaffolds between newly formed scar tissue. Detached blood vessels were seen in the tissue of the lunate space (arrow). Visibility of the blood vessels was obtained as previously described. No evidence of bone or cartilage-like tissue was seen (toluidine-blue staining; magnification $\times 4$)

Fig. 6 Toluidine-blue staining shows the lunate space of the experimental group after six weeks. Detached bone tissue formations and increasing vascularisation are seen in the centre of the lunate space (arrow) (toluidine-blue staining; magnification $\times 4$)

Fig. 7 **a** Histological sections of toluidine-blue staining of the experimental group after 12 weeks (magnification $\times 4$). The lunate space is filled with repair tissue. In the centre new bone formation could be detected (arrow). **b** Magnification of the area highlighted in

a. Cell formation on the surface. Extensive extracellular matrix could be detected. Hyaline cartilage tissue can be seen on the *right side* and regenerated cartilage-like tissue on the *left side* (toluidine-blue staining, magnification $\times 20$)

Intravital fluorochrome labelling

Confocal laser scanning microscopy analyses were performed after 12 weeks of implantation for both groups (experimental and control group). In the right wrists (control group), the lunate space was filled with cell-free scaffolds, and no evidence of bone formation was seen (Fig. 8a, b). In the left wrists (experimental group), in which the lunate space was filled with the cell-loaded scaffolds, new bone tissue was detected (Fig. 9a, b). As seen in the fluorochrome labelling, the highest rate of bone tissue production occurred 40–45 days after implantation, demonstrated by the small green bands in the middle of the defect. This bone formation was identified concentrically around new blood vessels.

Discussion

In this study, we have investigated the repair of lunate defects caused by complete excision, using composite scaffolds and bone marrow derived mesenchymal stromal cells. Most scientific studies only consider the regeneration of single tissue defects using BMSCs; however, we considered complete bone and cartilage regeneration with blood vessels and developed a defect model in a small animal (New Zealand white rabbit) where the lunate was completely excised. The regenerated tissue could be analysed histologically, radiologically and with intravital fluorochrome labelling. The vascularisation was detected by injection of a blue gelatine solution. Two different tissue engineering strategies were used in this study: the implan-

Fig. 8 **a** A section of the lunate space of the control group after 12 weeks (toluidine-blue staining, magnification $\times 4$). **b** Results of the confocal laser scanning microscopy of the same section of the control group (magnification $\times 4$). After implantation, the animals were

injected with three different fluorescent agents as further explained. No evidence of new bone formation was detected in the confocal laser scanning microscopy of the control group

Fig. 9 **a** A section of the lunate space of the experimental group (toluidine-blue staining, magnification $\times 4$). **b** Results of the confocal laser scanning microscopy of the same section showed newly formed bone tissue in the middle of the defect (*white arrow*)

tation of a cell-free scaffold and a cell-loaded and predifferentiated scaffold. The scaffold used in our study was a composite of esterified hyaluronan and hydrolyzed collagen. This material was chosen because the composite and its components possess a number of properties suitable for tissue repair. Among a variety of different hyaluronan analogs, the complete benzylesterified hyaluronan (commercially available as Laserskin®) has been shown to possess excellent biocompatibility and biodegradability [18]. Its moderate degradation rate over a period of two months gives sufficient scaffold stability for tissue formation. Previous studies also show a good ability to support cell loading and differentiation *in vitro* and *in vivo* with a hyaluronan-based scaffold combined with hydrolyzed collagen [19–22]. Our study also confirmed that bone marrow derived cells, loaded onto the composite scaffold and precultured in chondrogenic medium, underwent chondrogenesis with an extensive extracellular matrix production of cartilage-like tissue as determined by histological examination. The scaffold retained its dimensions allowing for the creation of appropriately sized implants.

The complete excision of the lunate of the rabbit without subsequent treatment resulted in a disorganised fibrous tissue, as demonstrated previously [23]. Based on these outcomes, we used a cell-free and a cell-loaded scaffold in our study. We used predifferentiated cell-loaded scaffolds, which showed cartilage-like tissue in the histological analyses before the implantation. Several authors have reported success after using preformed cartilage-like constructions to promote healing of bone defects [24, 25]. Vacanti et al. showed bone defects in a rat model which had been filled with periosteal progenitor cells and contained repair tissue which appeared to be cartilage-like at an early stage [25]. This tissue then progressed to produce bone through an endochondral ossification cascade. For the predifferentiation we chose a chondrogenic medium instead

of an osteogenic medium to try to promote the onset of endochondral ossification.

The results of the implanted cell-free scaffold showed disorganised fibrous tissue, without evidence of bone or cartilage tissue or vascularisation in all groups. The wrist treated with the cell-loaded scaffolds showed small remnants of the original hyaluronan-gelatine-composite scaffold between newly formed scar tissue at two weeks. An expanded cell formation was seen on the surface area but no evidence of new bone formation or vascularisation. However, after six weeks the defect was improved. Radiologically there was no evidence of bone formation, but in the histological analyses we saw a loose vascularisation in the centre of the defect, surrounded by a collection of cells on the surface. Finally, after 12 weeks, radiological evidence showed small bone formation in the centre of the defect. Bone formation was also affirmed by intravital fluorochrome labelling. The highest rate of new bone tissue was seen after 40–45 days, documented by the green band. The bone formation seemed to be concentrically around the detected new blood vessels, possibly attributed to better nutrition in this area. Another important ability of mesenchymal stem cells is the secretion of a number of cytokines and growth factors that improve angiogenesis [26]. This could explain the absence of vascularisation in the group with the cell-free scaffold. In summary, the 12-week animals had more bone and cartilage formation than the six-week animals. The central portions of the lunate space were replaced by osseous tissue, and the periphery of the osteochondral repair tissue remained cartilaginous. The cells on the periphery had not undergone endochondral ossification and showed a good regenerated cartilage-like tissue, which probably is sufficient for functional repair.

In our study we demonstrated that tissue engineering is a promising approach for the treatment of Kienböck's disease. But for further conclusions, long-term studies are

required to improve comprehension of the advanced differentiation of the cells. Another obvious limitation is the use of small animal models to study tissue engineering. It is not understood if the described strategy would be effective in a large animal model or even in humans. The use of large constructions has additional demands on diffusion of nutrients and metabolic products, and cell loading also becomes much more difficult.

The rabbit wrist is a weight-bearing joint. To allow the rabbit to bear weight after the operation may have enhanced the formation of osteochondrogenic repair tissue within the lunate space. In previous studies we demonstrated that mechanical loading showed an up-regulation of chondrogenesis and osteogenesis in in vitro tissue engineered constructs [27]. Several other studies also showed the positive effect of biomechanical conditioning on cartilaginous and osseous tissue formation [28–30].

In this study, we have demonstrated that it is possible to use mesenchymal stromal cells for the reconstruction of bone tissue with a cartilage-like layer, using tissue engineering. These strategies should be further explored for situations in which normal healing of bone defects is impaired or complete bones are missing. For further information on the potential use of mesenchymal stromal cells and the strategies in tissue engineering, long-term analyses in large animal models are required before translating these strategies into human treatment.

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