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Regeneration of ovine articular cartilage defects by cell-free polymer-based implants

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Abstract

The aim of our study was the evaluation of a cell-free cartilage implant that allows the recruitment of mesenchymal stem and progenitor cells by chemo-attractants and subsequent guidance of the progenitors to form cartilage repair tissue after microfracture. Chemotactic activity of human serum on human mesenchymal progenitors was tested in 96-well chemotaxis assays and chondrogenic differentiation was assessed by gene expression profiling after stimulating progenitors with hyaluronan in high-density cultures. Autologous serum and hyaluronan were combined with polyglycolic acid (PGA) scaffolds and were implanted into full-thickness articular cartilage defects of the sheep pre-treated with microfracture. Defects treated with microfracture served as controls. Human serum was a potent chemo-attractant and efficiently recruited mesenchymal progenitors. Chondrogenic differentiation of progenitors upon stimulation with hyaluronan was shown by the induction of typical chondrogenic marker genes like type II collagen and aggrecan. Three months after implantation of the cell-free implant, histological analysis documented the formation of a cartilaginous repair tissue. Controls treated with microfracture showed no formation of repair tissue. The cell-free cartilage implant consisting of autologous serum, hyaluronan and PGA utilizes the migration and differentiation potential of mesenchymal progenitors for cartilage regeneration and is well suited for the treatment of cartilage defects after microfracture.

Keywords: Cartilage regeneration; Microfracture; Cell-free implant; Stem cells; Chemotaxis

1. Introduction

Chondral or osteochondral lesions of the articular cartilage were found in about 60% of the patients in consecutive knee arthroscopies. Up to 20% of the patients showed focal chondral or osteochondral defects [1,2]. Since articular cartilage has a low intrinsic regenerative capacity and cartilage lesions may potentially lead to severe osteoarthritis, a variety of reparative techniques evolved that aim covering of the cartilage defect, formation of cartilaginous repair tissue and resurfacing of the articular cartilage. Among others, bone marrow-stimulating techniques like drilling [3], abrasion [4] or microfracturing [5] are frequently used surgical options for the treatment of focal cartilage defects.

The microfracture technique is a minimally invasive procedure that induces a healing response by establishing access to the subchondral bone marrow in regions of articular damage. Arthroscopically, debridement of the defect is performed by removing all free or marginally attached cartilage in and around the defect. Damage to the subchondral bone should be avoided and a well-attached healthy cartilage rim is prepared that circumscribes the defect. Adjacent to the cartilage rim and in the center of the

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defect, multiple perforations are introduced in the subchondral bone. These microfractures allow the influx of blood, blood-derived cells and bone marrow-derived mesenchymal stem and progenitor cells (MSCs) into the defect, forming a blood clot. Mesenchymal progenitor cells have a multipotential differentiation capacity that allows development along the chondrogenic lineage [6] and may be induced by growth factors from the subchondral bone to develop a cartilaginous repair tissue [7–9]. The clinical results after microfracture treatment of full-thickness defects, especially in patients aged 40–45 or younger, have shown good to excellent results with improvement in function and reduction of pain [10–12]. On the cellular level, the repair tissue induced by microfractures has been shown to be of a hyaline to fibrous appearance with limited short-term durability. Therefore, methods for the advancement of the microfracture technique are demanded that may enhance the content of key matrix components and improve the cartilaginous repair tissue [13].

In recent years, a variety of biocompatible biomaterials have been shown to support and enable the regeneration of cartilage defects by cell-based tissue engineering approaches. The use of autologous chondrocytes (autologous chondrocyte implantation, ACI) has been shown extensively to be well suited for the repair of cartilage defects [14–16]. Advanced cartilage tissue engineering grafts combine the regenerative potential of autologous cells with biocompatible graft-stabilizing scaffolds for the treatment of focal cartilage defects. Clinically applied scaffolds are based on e.g. hyaluronan [17], collagen [18] or synthetic polymers [19].

Especially alpha-hydroxypolyesters are frequently used synthetic polymers in cartilage tissue engineering. In particular, polylactic (PLA) and polyglycolic (PGA) acids as well as their copolymer poly-lactic-*co*-glycolic acid (PLGA) have been studied in detail. For instance, perichondral cells have been shown to form a cartilaginous extracellular matrix with chondrocyte-like cells when embedded in PLA and implanted in rabbit osteochondral defects [20]. The implantation of autologous chondrocytes, pluronic and PGA in porcine articular defects gave rise to well-integrated hyaline cartilage with good biochemical and biomechanical properties [21]. PLGA has been shown to support the development of human articular chondrocytes towards hyaline cartilage in vitro [22] and showed formation of hyaline-like cartilage with firm bonding of the repair tissue to the adjacent cartilage and to the subchondral bone in an equine defect model [23]. These bioresorbable polymer-based scaffolds allow the homogenous three-dimensional distribution of cells within the biomaterial, may guide the formation of the repair tissue, ensure initial mechanical stability and manageability during surgery and, in case of performing ACI, avoid the use of additional cover materials like periosteum, resorbable sheets or foils [24].

Taking advantage of the mechanical and tissue-guiding properties of bioresorbable polymers and the differentiation potential of mesenchymal progenitor cells that are activated by the microfracture procedure, this study evaluates the suitability of a cell-free cartilage implant consisting of PGA, hyaluronan and autologous serum for articular cartilage regeneration. Here we show that human serum acts as a chemo-attractant and stimulates the migration of human MSCs. Hyaluronan supports the differentiation of human MSCs along the chondrogenic lineage as shown by gene expression analysis. Ovine full-thickness articular defects covered with cell-free implants and pre-treated with microfracture showed formation of a cartilaginous repair tissue, partially filling the defect. In defects treated with microfracture only, no repair tissue was evident. Therefore, covering of defects pre-treated with microfracture by a cell-free cartilage implant based on a PGA scaffold and hyaluronan is considered to be a promising approach to regenerate articular defects.

2. Materials and methods

2.1. Isolation and cell culture of human mesenchymal progenitor cells

As described previously, human adult MSCs were isolated from bone marrow aspirates ($n = 8$, age 48–69) [25,26] and from subchondral bone marrow ($n = 3$, age 40–62) [27]. The bone marrow aspirates were derived from donors who were examined to exclude hematopoietic neoplasm and were histologically diagnosed as normal. The study was approved by the Ethical Committee of the Charité Universitätsmedizin Berlin. MSCs from subchondral bone marrow were isolated from excised bone obtained from the lateral tibia head during high tibial closed wedge osteotomy. Cells were grown in complete Dulbecco's modified eagle (DME) medium (Biochrom, Germany) containing 2 ng/ml basic fibroblast growth factor (bFGF, Tebu, Germany) and 10% fetal bovine serum (FBS, Perbio, Belgium) or 5% human serum (German Red Cross, Germany). The medium was exchanged every 2–3 days. MSCs were routinely tested for the presence of the typical MSC-related cell surface antigens SH2 and SH3 as well as for the absence of CD34 and CD45.

2.2. Preparation of cell-free cartilage implants and MSC recruitment assay

Resorbable felt ($15 \times 20 \times 1.1\,mm^3$) of pure PGA (Alpha Research Deutschland GmbH, Germany) was soaked in 330 µl hyaluronic acid (HA, 10 mg/ml Ostenil®, TRB Chemedica AG, Germany). Implants were freeze-dried for 16 h using a lyophilisator (Leybold-Heraeus, Germany) and stored in a desiccator at room temperature. To evaluate the capacity of human serum to recruit MSCs, freeze-dried felts of PGA and HA were incubated with 1 ml of human serum from whole blood (German Red Cross, Germany) for 10 min. After dripping off the surplus of serum, the felt was centrifuged in a reaction tube for 10 min at 720*g*. After recovery of the fluid, the mixture of HA and serum was diluted in a 1:1 ratio with DME medium containing 0.5% bovine serum albumin (BSA) and used for the cell migration assay. Cell-free implants re-hydrated with 1 ml physiological saline served as controls.

For the cell migration assay, the ChemoTX® Assay System (Neuroprobe, USA) with a polycarbonate membrane (pore size: 8 µm, pore density: 1×10^3 pores/mm^2) was used according to the manufacturer's recommendations and as described previously [27]. All tests were performed in triplicate. Individual mixtures of HA and serum obtained from cell-free implants ($n = 5$) were used as chemo-attractant. Mixtures obtained from individual cell-free implants ($n = 3$) that were re-hydrated

with physiological saline, DME medium containing 0.5% BSA (negative control, $n = 3$) and DME medium containing 10% FBS (positive control, $n = 3$) served as controls. All migration assays were performed in triplicate. After migration of MSCs (passage 3), cells were fixed with acetone/methanol 1:1 (v/v), rapidly stained with Hemacolor and counted in three representative visual fields. The number of migrated MSCs was extrapolated to the total area of the well (25 mm^2).

For statistical evaluation, the *t*-test was applied and differences in pair-wise group comparisons were considered significant at $p < 0.05$.

2.3. Chondrogenic differentiation of human mesenchymal progenitor cells

For differentiation studies, MSCs (passage 3) obtained from individual donors ($n = 3$) were pooled in equal amounts and 250,000 MSCs were centrifuged to form a high-density culture [28]. Differentiation was induced for up to 2 weeks with DME medium supplemented with ITS + 1 (insulin–transferrin–sodium–selenite, Sigma, Germany), 0.1 μM dexamethasone (Sigma, Germany), 1 mM sodium pyruvate (Sigma, Germany), 0.17 mM ascorbic acid-2-phosphate (Sigma, Germany), 0.35 mM proline (Sigma, Germany) and 10% (v/v) HA (10 mg/ml Ostenil®). DME medium supplemented with ITS + 1, dexamethasone, sodium pyruvate, ascorbic acid and proline served as control. The medium was exchanged every second day.

2.4. Polymerase chain reaction (PCR)

Per point in time, 25 high-density MSC pellets were homogenized in 1 ml TriReagent (Sigma, Germany) using an UltraTurrax. Bromochloropropane was added, incubated at room temperature for 10 min and centrifuged at 12,000*g* for 15 min. The supernatants were used for the isolation of total RNA using the Qiagen Rneasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Subsequently, total RNA (3 μg) was reverse transcribed with the iScript cDNA Synthesis Kit according to the manufacturer's recommendations (BIO-RAD, Germany). The relative expression level of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, up 5′–3′: GGCGATGCTGGCGCTGAGTAC, down 5′–3′: TGGTCCACACCCATGACGA) was used to normalize marker gene expression. Real-time PCR using the i-Cycler PCR System (BIO-RAD, Germany) was performed with 1 μl of cDNA sample using the SYBR Green PCR Core Kit (Applied Biosystems, USA). Relative quantitation of the expression of type IIα1 collagen (up 5′–3′: CCGGGCAGAGGGCAATAGCAGGTT, down 5′–3′: CAATGATGGGGAGGCGTGAG), cartilage link protein (up 5′–3′: GCGTCCGCTACCCCATCTCTA, down 5′–3′: GCGCTCTAAGGGCACATTCAGTT), cartilage oligomeric matrix protein (up 5′–3′: GGGTGGCCGCCTGGGGGTCTT, down 5′–3′: CTTGCCGCAGCTGATGGGTCTC) and aggrecan (up 5′–3′: CCAGTGCACAGAGGGGTTTG, down 5′–3′: TCCGAGGGTGCCGTGAG) was performed and is given as fold change compared to non-stimulated controls.

2.5. Implantation of cell-free implants in cartilage defects of the sheep and histology

The study was designed in accordance with the German law for animal protection and has been approved by the Review Board for the Care of Animal Subjects at the Regierungspräsidium Tübingen, Germany. Eight adult Merino sheep (female, age: 3 years) were used in this study. For anesthesia, sheep were fasted for 24 h and pre-medicated with an intramuscular injection of 0.05 mg/kg atropine, 0.1 mg/kg xylazine and 10.0 mg/kg ketamine. Anesthesia was induced through a cephalic-vein cannula with 2–3 mg/kg propofol, and after endotracheal intubation, maintained by inhalation of 1.5–2.0% isoflurane delivered in oxygen and air (FiO_2 0.4). Respiratory rate and tidal volume were adjusted to maintain end-expiratory carbon dioxide tension ($P_{et}CO_2$) between 35 and 45 mmHg. To ensure analgesia 4 mg/kg carprofen and 4 μg/kg fentanyl were given preemptively by intravenous bolus injections followed by a continuous infusion of 4 μg/kg fentanyl throughout the surgical procedure. All surgical procedures were performed under aseptic conditions. The left stifle joint was opened by an antero-medial approach and the medial condyle of the femur was exposed. Degenerative joint disease and skeletal abnormalities were excluded by visual inspection and full-thickness cartilage defects of $11 \times 8\ mm^2$ were created in the weight bearing cartilage using a scalpel and a sharp spoon. Nine microfracture perforations were introduced in the subchondral bone using a chondropick®. The depth of the perforations was 2 mm and bleeding was observed. Freeze-dried cell-free cartilage implants ($11 \times 8 \times 1.1\ mm^3$), as described above, were soaked in autologous sheep serum for 10 min, used for covering of the defect ($n = 4$) and securely fixed trans-osseously as described previously [29]. Cartilage defects with microfracture perforations but without covering with cell-free implants served as controls ($n = 4$). Wound closure was performed in layers and protected by a plaster. Post-operatively, maintenance of analgesia was achieved by daily application of 4 mg/kg carprofen for at least 2 days. Sheep were allowed to move freely in boxes for 10 days and out at feed, thereafter. At 14 days ($n = 2$) and 3 months ($n = 6$), sheep were anesthetized and killed by administration of 100 mg/kg thiopentone and 2 mmol/kg potassium chloride intravenously.

Joints were fixed in formalin, decalcified and embedded in paraffin. Sections (6 μm) were stained with safranin O/fast Green and sections through the center of the defect ($n = 3$ per group) were evaluated histologically.

3. Results

3.1. Human serum is a chemo-attractant and stimulates the migration of human MSC

For analyzing the activity of human serum to recruit human mesenchymal progenitors, the ChemoTX® Assay System was used. MSCs are separated from the chemo-attractant by a porous polycarbonate membrane and migrate through the membrane when a chemotactic stimulus is given (Fig. 1). Using human serum from whole blood as a chemo-attractant, MSCs were recruited and migrated through the membrane and attached to the lower surface of the membrane (Fig. 1A). In contrast, only few MSCs migrated when serum was absent (Fig. 1B). The average number of MSCs recruited by different chemo-attractants is given in Fig. 1C. The positive control (FBS) recruited about 4.849 MSCs/25 mm^2. In contrast, 181 MSCs migrated spontaneously, when medium was used as a negative control. The number of migrating MSCs was significantly ($p < 0.05$) enhanced by using HA as a chemo-attractant that recruited about 633 MSCs in the absence of serum. The mixture of HA and human serum significantly ($p < 0.0001$) elevated the number of recruited MSCs and guided 2.524 MSCs to migrate through the membrane.

3.2. HA induces the expression of cartilage matrix molecules in human MSCs

Bone marrow-derived MSCs were stimulated for up to 2 weeks in high-density micro-mass culture with HA. MSCs developed a compact pellet of vital cells that

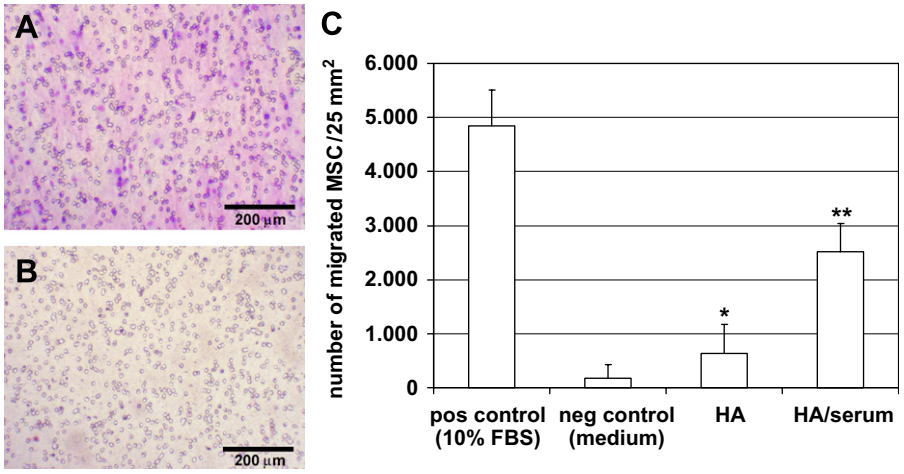

Fig. 1. Chemotactic recruitment of human mesenchymal progenitors by human serum. Upon stimulation with human serum, human mesenchymal stem and progenitor cells (MSCs) migrated through the pores of the polycarbonate membrane, attached to the under-side of the membrane and were stained with hemacolor (A). MSCs were not evident in negative controls stimulated with medium (B). Quantitation of migrated progenitors (C) showed that hyaluronic acid (HA) significantly ($*p<0.05$) stimulated the migration of the progenitors compared to negative controls. Using the combination of HA and human serum, the amount of migrating mesenchymal progenitors was significantly ($**p<0.0001$) elevated compared to negative controls and to progenitors stimulated with HA. The bars show the mean number of migrated MSCs and the standard deviation is given.

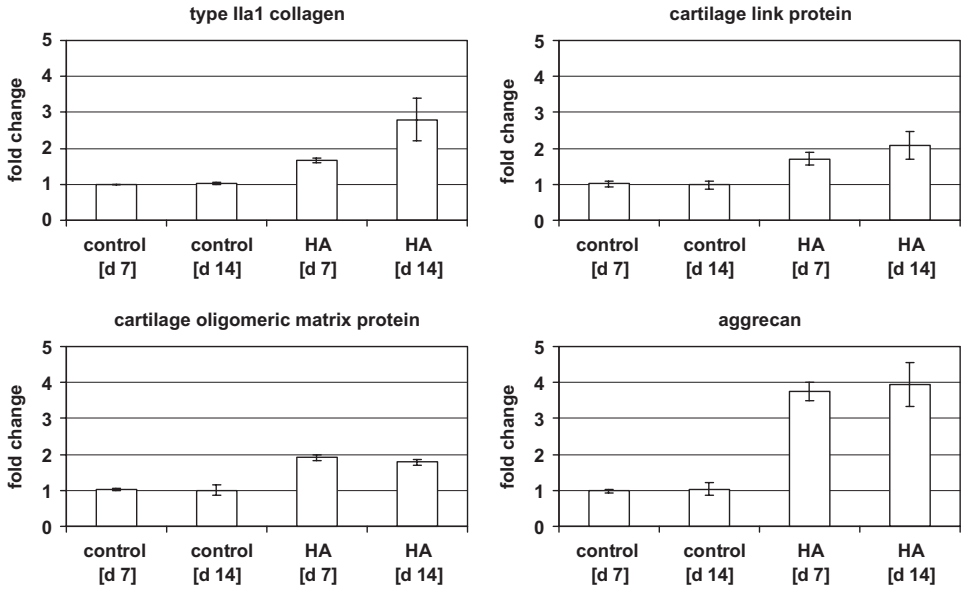

Fig. 2. Semi-quantitative real-time gene expression analysis of typical chondrogenic marker genes in human mesenchymal progenitors upon stimulation with hyaluronan. The expression of the chondrogenic marker genes *type II collagen*, *cartilage link protein*, *cartilage oligomeric matrix protein* and *aggrecan* increased when human mesenchymal progenitors were cultured in high-density in the presence of hyaluronan for up to 14 days. The expression of marker genes is given as fold change compared to non-stimulated progenitors. The mean of each triplicate well is plotted and the error bars represent standard deviation.

showed, after histological staining, virtually no formation of a cartilaginous extracellular matrix (data not shown). In contrast, gene expression analysis of typical cartilage marker genes (Fig. 2) showed that prolonged culture of MSCs in the presence of HA for 14 days induced the expression of *type IIα1 collagen* (2.8 fold), *cartilage link protein* (2.1 fold), *cartilage oligomeric matrix protein* (1.8 fold) and *aggrecan* (3.9 fold) compared to untreated controls.

3.3. Microfracture treatment and implantation of the cell-free polymer-based implant in sheep articular defects

After showing that serum may have the capacity to guide MSCs into the implant and that HA may promote the chondrogenic differentiation of MSCs, the capacity of HA and serum to regenerate articular cartilage was analyzed in a sheep articular cartilage defect model (Fig. 3). The bioresorbable felt-like textile scaffold of pure PGA was

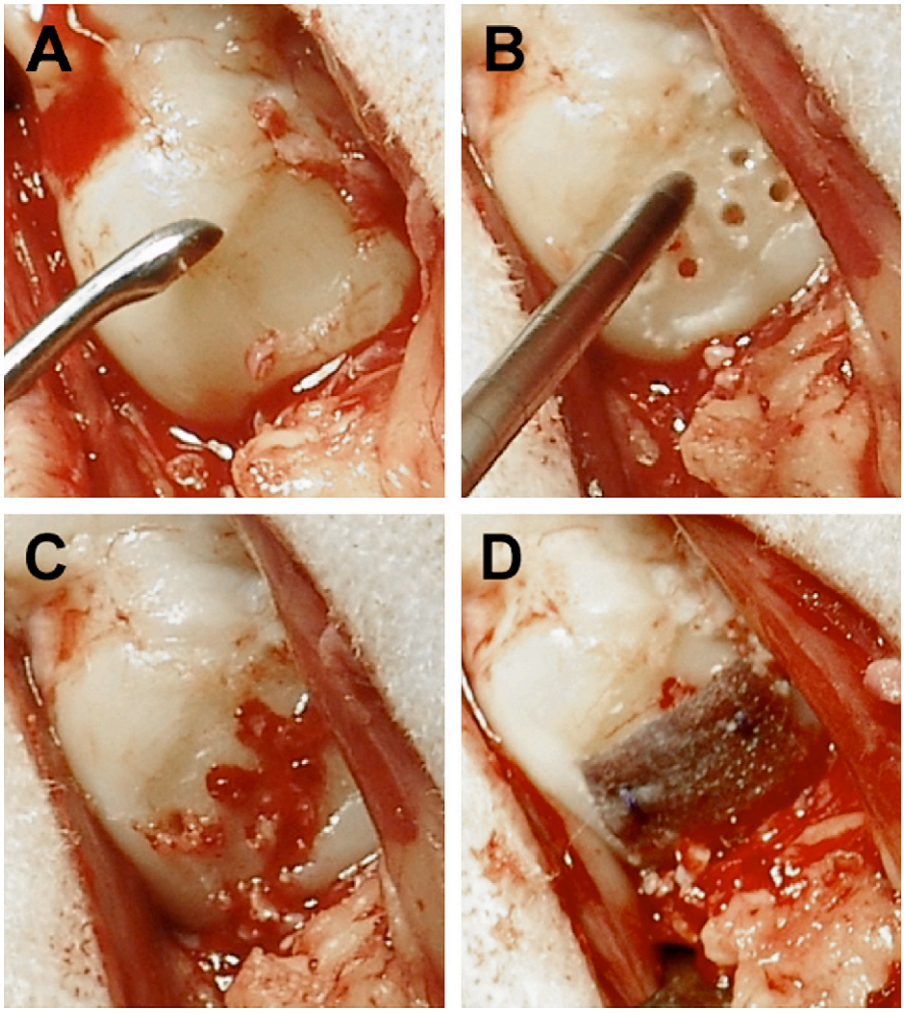

Fig. 3. Implantation of the cell-free implant in full-thickness defects after pre-treatment with microfracture. Full-thickness cartilage defects ($11 \times 8\,mm^2$) were created by debridement of the articular cartilage down to the subchondral bone (A). Microfracture perforations were introduced in the subchondral bone using a chondropick® (B) and bleeding of the subchondral tissue was observed (C). The cell-free implant consisting of the polyglycolic acid scaffold, hyaluronan and autologous serum was used to cover the defect and fixed by trans-osseous suturing (D).

soaked with HA, freeze-dried and was re-hydrated directly prior to the implantation by adding autologous serum. For implantation, a full-thickness articular cartilage defect was created (Fig. 3A). To provide access to progenitors residing in the subchondral bone marrow, microfracture perforations (Fig. 3B) were introduced into the subchondral bone until bleeding was observed (Fig. 3C). Thereafter, the cell-free implant was used to cover the defect and securely anchored by trans-osseous fixation with resorbable sutures (Fig. 3D).

At day 14, histological analysis of the defect area was performed (Fig. 4). The cartilage defect treated with microfracture perforations but without covering by the cell-free implant showed complete debridement of the articular cartilage down to the subchondral bone and no development of repair tissue (Fig. 4A). The cell-free implant covering the defect (Fig. 4B) showed a superficial layer of a cell-rich fibrous tissue towards the joint space (white arrows, Fig. 4C) and a layer firmly bonding the implant to the subchondral bone (black asterisk, Fig. 4D). In the central region of the implant (Fig. 4E), the fibers of the polymer (black double arrow, Fig. 4F), Safranin O-stained extracellular matrix and cells (Fig. 4F, white arrows) were evident.

3.4. Histological evaluation of repair tissue formation in sheep articular defects

After 3 months, defects treated with microfracture, only, showed macroscopically no development of a repair tissue (Fig. 5A). The articular cartilage defects showed debridement down to the subchondral bone with some protrusion of tissue from the cartilage rim (cartilage flow). In the center of the defect, remnants of the microfracture perforations with bleeding of the subchondral bone tissue were evident (Fig. 5A, black arrows). Defects treated with microfracture and covering with the cell-free implant showed formation of a repair tissue that is characterized by a hyaline appearance (Fig. 5B). The repair tissue partially filled the defect and exhibited small cavities with

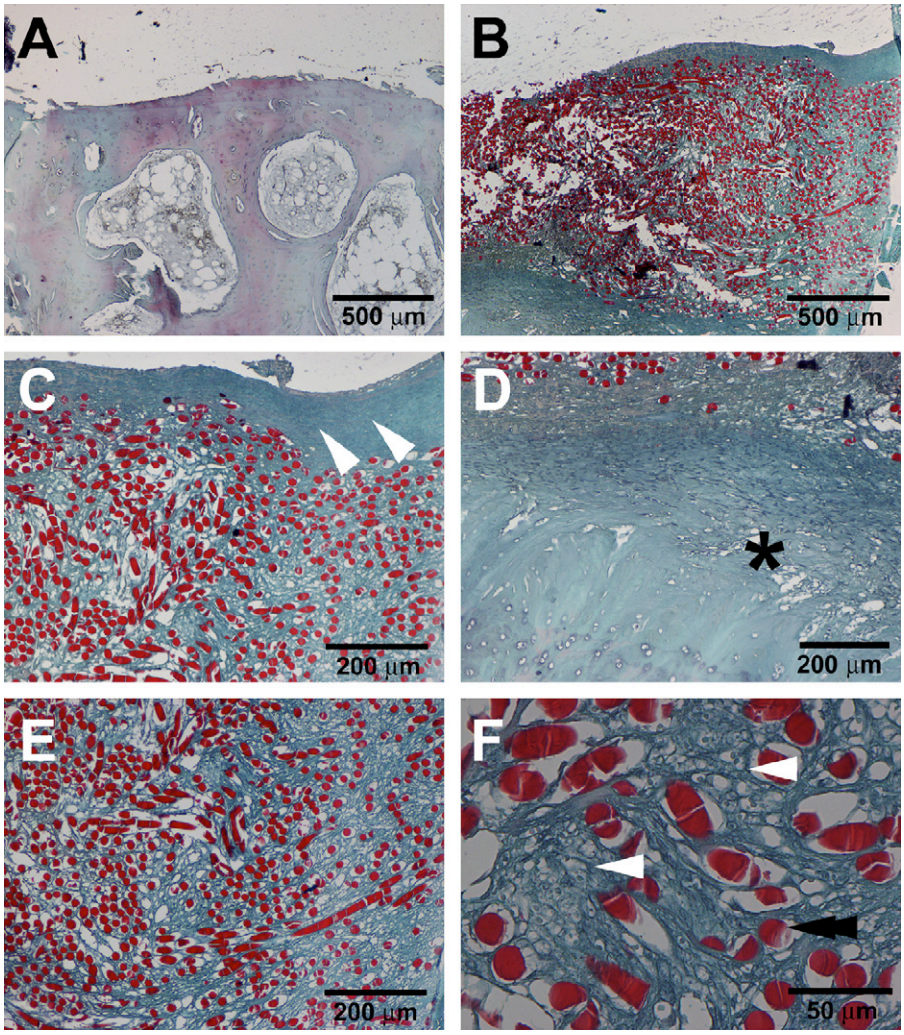

Fig. 4. Histological analysis of cartilage defects, 14 days after treatment with microfracture and implantation of the cell-free implant. At day 14, safranin O staining showed that the articular cartilage was completely removed down to the subchondral bone and no development of repair tissue in the defect treated with microfracture was apparent (A). The cell-free implant covered the defect (B), showed a layer of cell-rich fibrous tissue in the superficial zone of the implant (C, white arrows) and firm bonding to the subchondral bone (D, black asterisk). In the center of the implant (E), fibers of the polymer (F, black double arrow), extracellular matrix and cells were evident (F, white arrows).

no or marginal repair tissue formation (Fig. 5B, black arrow). In all samples treated with the cell-free implant, a distinct area was evident with marginal development of repair tissue (Fig. 5B, black asterisk), which may occur due to anatomical properties of the sheep stifle joint and/or localization of the patella and ligaments scraping the implant.

Histological analysis of defects treated with microfracture as well as the repair tissue formed after microfracture treatment and covering with the cell-free implant was performed by staining with Safranin O and is shown for each individual defect (Fig. 6). After 3 months, the defects treated with microfracture without covering with the cell-free implant showed no formation of repair tissue, histologically (Fig. 6A–C). The articular cartilage was removed completely down to the subchondral bone and few incidental remnants of the cartilage were evident (Fig. 6B, black arrow). The microfracture perforations in the subchondral bone, were filled with a cell-rich repair tissue, showed ongoing osseous tissue formation and achieved the level of the subchondral calcified layer of the end-plate, but not of the articular cartilage (Fig. 6C, black arrow). As shown in high-power magnification micrographs (Fig. 6D–F), the mature cortical bone of the end-plate (Fig. 6D, black asterisk) is sharply defined from the calcified zone of the subchondral bone plate (Fig. 6D, black arrows). Articular cartilage or repair tissue covering the subchondral bone was not evident.

Histological analysis of the repair tissue of defects after microfracture treatment and implantation of the cell-free implant showed the formation of a cartilaginous tissue with intense staining of proteoglycans (Fig. 6G, H). The repair tissue developed in one of the defects showed a cell-rich but unstructured tissue with an emerging extracellular matrix containing proteoglycans (Fig. 6I). Development of a proteoglycan-containing tissue is shown by the bright and

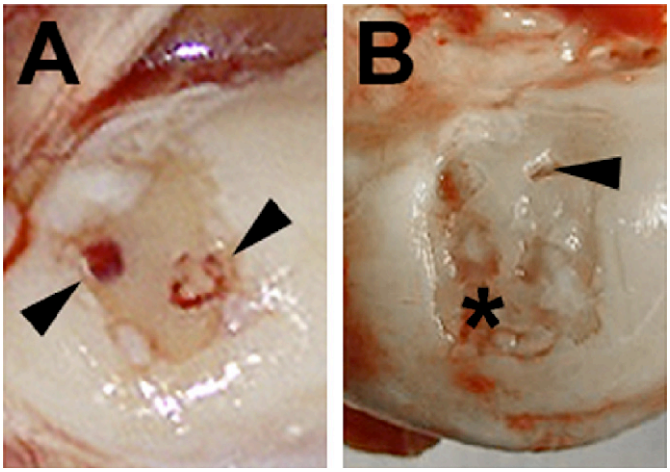

Fig. 5. Macroscopic view of cartilage defects, 3 months after treatment with microfracture and implantation of the cell-free implant. Three months after treatment with microfracture only (A), the defects showed no formation of a repair tissue. The subchondral bone was still visible and some cartilage flow was evident. Remnants of the microfracture perforations showed bleeding of the subchondral bone tissue (A, black arrows). Defects covered with the cell-free implant after pre-treatment with microfracture (B) exhibited a cartilaginous repair tissue of a hyaline appearance with some cavities (B, black arrow). All defects covered with the implant showed a spatially confined area with marginal repair tissue formation, which may due to anatomical characteristics of the sheep stifle joint (B, black asterisk).

faint pink staining of the tissue located towards the joint space (Fig. 6L, black arrows). Subjacent to the cell-rich repair tissue, a just developing calcified zone (Fig. 6L, black asterisks) can be seen contiguous with the underlying cortical bone. Specimens with cartilaginous repair tissue showed progressive remodeling of the subchondral bone tissue (Fig. 6G, H, black arrows) adjacent to the cortical bone (Fig. 6G, asterisk). The repair tissue was void of polymer fibers and showed viable cells (Fig. 6J, K). The cells were round-shaped with some cells of fibroblastic appearance and mainly showed a columnar distribution with some clustering. There were no signs of abnormal calcification, infiltration of immunological cells, apoptosis of cells nor necrosis within the repair tissue.

4. Discussion

In the present study, we demonstrated that the cell-free cartilage implant made of a textile PGA scaffold and HA allows the in situ recruitment of MSCs by serum as a chemo-attractant and subsequent guidance of the progenitors towards formation of cartilage repair tissue. Using a 96-well chemotaxis assay system, we showed that human serum is a potent chemo-attractant and stimulated the migration of human MSCs from bone marrow. Culture of human MSCs in the presence of HA in high-density micromasses stimulated the progenitors to differentiate along the chondrogenic lineage as shown by the induction of typical chondrogenic marker genes. In the ovine cartilage defect model, histological analysis documented that covering of defects pre-treated with microfracture by the cell-free implant led to the development of a cartilaginous repair tissue that was rich in cells, showed a typical proteoglycan-rich extracellular matrix and partially filled the defect. In contrast, control defects treated with microfracture, only, formed no repair tissue.

Perforation of the subchondral bone is followed by the migration or flow of mesenchymal progenitors from the bone marrow and subsequent formation of a fibrous to cartilaginous repair tissue [5]. The mechanisms and factors that guide the migration and differentiation of mesenchymal cells in microfracture-mediated repair have still to be elucidated. Early events in the reparative sequence include the formation of a hematoma with fibrin deposition and binding of platelets. It is suggested that these platelets release vasoactive factors, growth factors and cytokines that may influence cell migration, differentiation and tissue development [7]. In addition, the bone and its marrow contains growth factors and cytokines that may be important key molecules for vascularization, cell migration and repair of osteochondral defects [30,31]. Recently, it has been shown that chemokines [32,33], bone morphogenetic proteins (BMP) [34] and human synovial fluid [27] stimulate mesenchymal stem cells to migrate in vitro. As shown here, human serum that contains a variety of chemokines and growth factors is also a potent elicitor of stem cell migration and may support the influx of stem cells into the defect during microfracture. This is consistent with a report of Kramer and colleagues that shows that multipotential mesenchymal stem cells were evident in a porcine collagen I/III matrix that was soaked with blood during the microfracture procedure for 5 min and subsequently fixed with fibrin glue and serum [35]. Therefore, the autologous serum used in the cell-free implant may, in cooperation with synovial fluid and factors from the blood, enhance or promote the migratory activity of mesenchymal stem cells activated by microfracture and may attract to as well as enrich MSCs within the implant.

MSCs have the capacity to develop into a variety of mesenchymal tissues including bone and cartilage [6]. Especially their chondrogenic developmental capacity may value MSCs as an attractive cell source for regenerative medicine [36]. Initiation of chondrogenesis in MSCs was achieved by culturing MSCs three-dimensionally in the presence of transforming growth factors and selected BMPs [28,37,38]. Interestingly, as also shown here, the chondrogenic differentiation sequence of MSCs may not only be initiated by distinct growth and differentiation factors but structural components of mesenchymal tissues like HA. HA is frequently used for the treatment of pain associated with knee osteoarthritis, lubricates the joint, is a shock absorber, has a protective effect on articular cartilage and may exert its effect by enhancement of chondrocyte HA and proteoglycan synthesis as well as by suppressing potentially pro-inflammatory mediators [39]. As shown here, in human MSCs, HA induced the

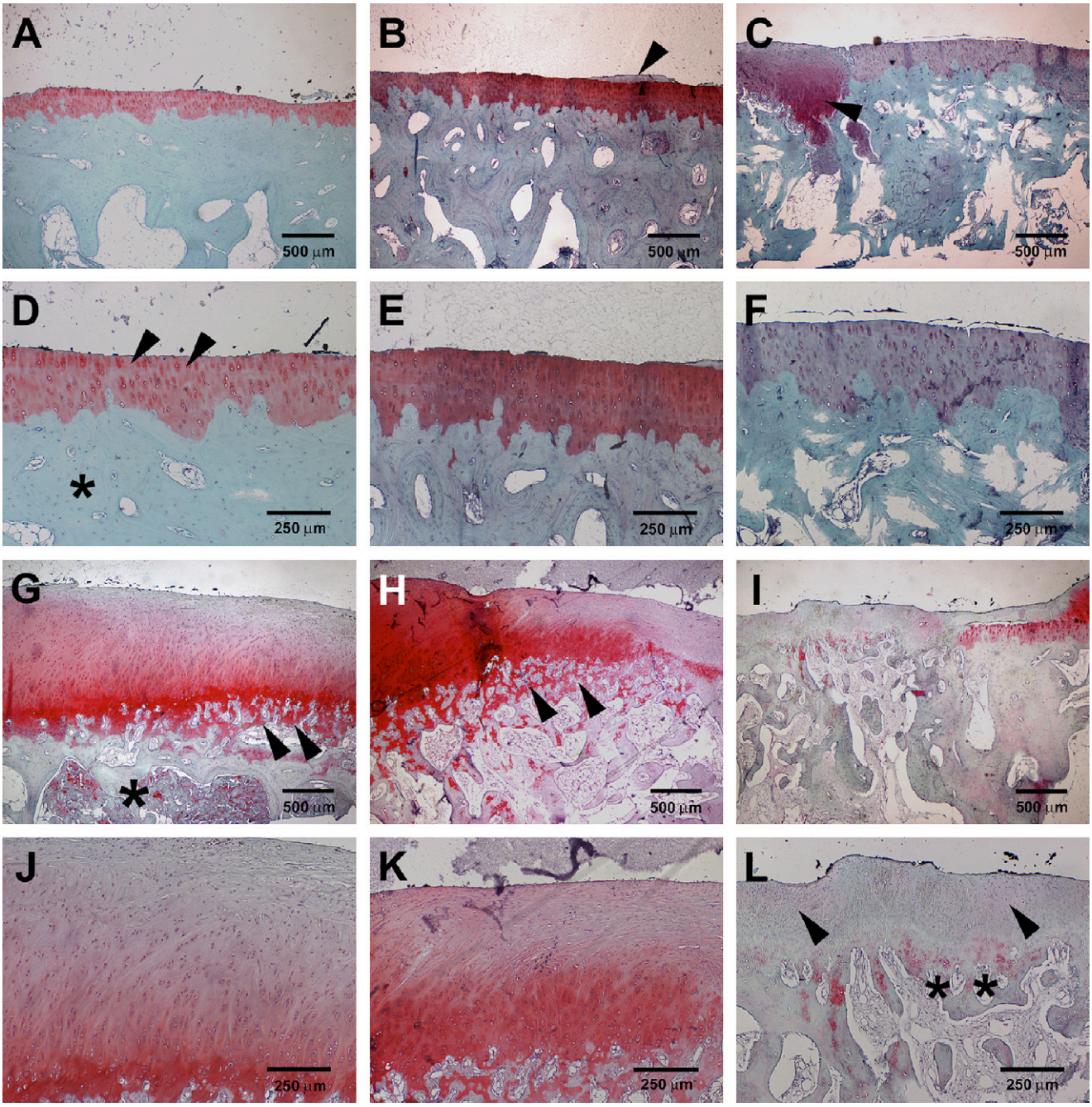

Fig. 6. Histological analysis of cartilage defects, 3 months after treatment with microfracture and implantation of the cell-free implant. Safranin O staining of the individual specimens of defects, that were treated with microfracture but without covering with the cell-free implant, showed no formation of repair tissue (A–F). The articular cartilage was completely removed and occasionally some remains of the cartilage were evident (B, black arrow). Microfracture perforations were visible and were filled with repair tissue that achieved the level of the subchondral calcified layer (C, black arrow). Defects treated with microfracture explicitly showed the calcified zone of the subchondral plate (D, black arrows) and mature cortical bone (D, black asterisk). Tissue covering the defect was not evident. All defects that were covered with the cell-free implant after pre-treatment with microfracture developed repair tissue (G–L). Histologically, the repair tissue showed cartilaginous characteristics (G, H) or was unstructured with faint staining of proteoglycans (L, black arrows) and a calcified zone just evolving (L, black asterisks). Adjacent to the cortical bone (G, black asterisk) the subchondral bone tissue showed progressive remodeling (G, H, black arrows). The cartilaginous repair tissue was rich in round-shaped cells with columnar distribution and showed intense staining of proteoglycans (J, K).

expression of typical chondrogenic marker genes including type II collagen and aggrecan. The chondrogenesis-inducing properties of HA were also shown in the equine and rabbit model. In the equine model, HA as well as autologous synovial fluid stimulated chondrogenesis of MSCs in different concentrations [40] and transplantation of autologous MSCs embedded in HA gel sponges showed good repair of rabbit osteochondral defects [41]. This suggests that HA may induce or at least support the chondrogenic development of mesenchymal progenitors.

In the cell-free implant, human serum and HA with their migration and differentiation stimulating properties were combined with the initially mechanically stable PGA scaffold and used for covering of ovine full-thickness cartilage defects pre-treated with microfracture. Three months after implantation of the cell-free implant in large defects, the defects showed the formation of a cell-rich repair tissue of cartilaginous appearance and partial filling of the defect. The effectiveness of covering defects pre-treated with microfracture was also shown in the canine model. Articular defects of the trochlear grooves of adult dogs were treated with microfracture, with covering by a type II collagen membrane after pre-treatment with microfracture, and with autologous chondrocytes embedded in the collagen matrix. The best filling of the defect was achieved by covering the defect after microfracture [42]. In contrast, using the ovine model, covering of defects with a porcine collagen matrix after pre-treatment with microfracture did not enhance the healing response compared to microfracture without covering after 4 and 12 months. Instead, regeneration of hyaline-like cartilage was achieved after introducing microfracture perforations and implanting the collagen matrix augmented with chondrocytes [43,44]. Recently, the use of cell-free collagen matrices combined with fibrin glue and autologous serum in terms of autologous matrix-induced chondrogenesis (AMIC) is suggested to be a promising treatment option to cover defects after microfracture [45]. In osteochondral defects, Gotterbarm and colleagues used a cell-free two-layered implant consisting of tri-calcium phosphate for bone development and a superficial type I/III collagen layer for cartilage regeneration. Treatment of porcine defects with the osteochondral implant improved defect filling and subchondral bone repair. The addition of a mixture of growth factors improved the mechanical and histological properties of the repair tissue in the early (12 weeks) but not in the late phase (52 weeks) of cartilage regeneration [46].

All these cell-free approaches have in common that the use of cell-free cartilage or osteochondral implants may have the advantage that only one surgical intervention is necessary and donor site morbidity is avoided, in contrast to cell-based grafts. Since cell-free implants are storable and have a considerably longer shelf life than cell-based grafts, the cell-free implants can be used on demand for the treatment of focal cartilage defects. In addition, from the surgical point of view, the textile structure of textile polymer-based scaffolds and especially PGA scaffolds ensures secure fixation of the implant in the defect by cartilage suturing, trans-osseous suturing or by resorbable pins [47–49]. The enhanced biomechanical stability of the scaffolds allows for early loading of the treated defect, as demonstrated in this study. This contrasts with longer periods of limited weight bearing recommended after microfracture treatment. From the cellular point of view, covering of the defect after pre-treatment with microfracture may enhance the healing response by supporting cell migration and cell differentiation. In addition, the textile structure of the implant may allow enrichment of the amount of cells at the defect site and may prevent acute negative events caused by bleeding into the joint cavity. In a dog model, a single bleeding resulted in a disturbed cartilage matrix turnover and mild synovitis in the short term. However, in the long term, the joints recovered and normal cartilage metabolism as well as absence of synovial inflammation were evident [50,51].

5. Conclusions

In summary, we have shown that the components of the cell-free implant, HA and serum, induce and/or support the migration and chondrogenic differentiation of human mesenchymal progenitor cells derived from bone marrow. Covering of full-thickness ovine cartilage defects by the cell-free implant after pre-treatment with microfracture showed the formation of cartilaginous repair tissue. In contrast, in this model, large articular defects treated with microfracture, only, showed no healing and formed no repair tissue. Therefore, the implantation of polymer-based cell-free implants of HA embedded in a textile PGA scaffold soaked with autologous serum into cartilage defects is suggested to be a promising approach to cover and regenerate articular cartilage defects after microfracture, clinically.

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