Enhanced migration of human bone marrow stromal cells in modified collagen hydrogels

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Abstract

Purpose Collagen I hydrogels are widely used as scaffolds for regeneration of articular cartilage defects. We hypothesised that ingrowth might be improved by removing the superficial layer of a compressed hydrogel. The control group consisted of the original unmodified product.

Methods The migration of human bone marrow stromal cells (hBMSCs) into the hydrogel was evaluated by confocal microscopy. We quantified the DNA concentration of the hydrogel for each group and time point and evaluated the chondrogenic differentiation of cells.

Results After one week, the detectable amount of cells at the depth of 26–50 μm was significantly higher in the modified matrix (MM) than in the non-modified matrix (NM) ($p=0.011$). The maximum depth of penetration was 75 μm (NM) and 200 μm (MM). After three weeks, the maximum depth of penetration was 175 μm (NM) and 200 μm (MM). Likewise, at a depth of 0–25 μm the amount of detectable cells was significantly higher in the MM group ($p=0.003$). After 14 days, the concentration of DNA was significantly higher in the samples of the MM than in the control group ($p=0.000$). Staining of histological sections and labelling with collagen II antibodies showed that a

chondrogenic differentiation of cells in the scaffold can occur during in vitro cultivation.

Conclusions Removing the superficial layer is essential to ensuring proper ingrowth of cells within the compressed hydrogel. Compressed hydrogels contribute better to cartilage regeneration after surface modification.

Introduction

The potential of human cartilage to regenerate itself is limited. Modern tissue engineering offers the ability to transplant stem cells into the defect in order to use their potential for differentiation in human cartilage cells [1]. The cells, however, need a carrier substance for differentiation, which simulates a tissue-specific environment [2, 3] and at the same time facilitates the implantation of the cells into the defect [4].

Cell-free compressed collagen hydrogels have been used to treat cartilage defects with excellent results and are already in clinical use in many non-US countries in orthopaedic and trauma surgery [5, 6]. They are used in operative procedures for the treatment of osteochondral defects, particularly when treating the knee joint (Fig. 1).

Currently, knowledge regarding how the adjacent cells from the bone marrow can infiltrate the collagen gel is very limited. Previous studies have shown that cell migration into collagen gels are dependent on scaffold composition and that there is potential for improvement [7].

The structure of these scaffolds was previously examined histologically. Collagen hydrogels have structural similarities to the extracellular matrix [8]. If compressed in an axial direction, hydrogels show a parallel arrangement of the structures, which suggests that migration of the cells could be hampered if cells seek to migrate into the scaffold from the base of the defect [9, 10].

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Fig. 1 The hydrogel is used for operative procedures for the treatment of osteochondral defects especially in the knee joint

Our hypothesis is that in the current composition the superficial parallel arrangement of collagen hydrogels hampers cellular migration into the scaffold and that a modification of the scaffold composition could enhance cell migration.

Materials and methods

Isolation and cultivation of human bone marrow stromal cells

Human bone marrow stromal cells (hBMSCs) were obtained during routine trauma surgery after informed consent and approval of the Institutional Ethics Committee. A cell pool of seven patients was created (four female and three male patients; mean age 41.6 ± 12 years). There were no signs of general arthritis or malignant tumours within the study population.

Isolation and cultivation were performed according to a modified protocol as previously described [10]. For each cell passage, the complete medium was removed, the cell culture was washed twice with phosphate-buffered saline (PBS) and was overlaid with 8 ml trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25 %/0.02 %). The solution was then incubated for five minutes (37 °C, 5 % CO₂, 95 % humidity) to loosen the cells from the bottom. Thirty-two millilitres of freeze medium [500 ml DMEM, 50 ml foetal calf serum (FCS), 5 ml penicillin/streptomycin (10,000 IE/10,000 µg/ml)] was added and the suspension was transferred into a Falcon tube. After an incubation period of ten minutes at room temperature, the cells were centrifuged (1,200 rev/min, ten minutes, 4 °C). After decanting the supernatant, the cell pellet was added to cell culture medium and was resuspended. Thirty-five millilitres of fresh cell culture medium was added and the solution was incubated (37 °C, 5 % CO₂, 95 % humidity) for proliferation.

Preparation of type I collagen hydrogel

The collagen I hydrogel (CaReS-1S®, Arthro Kinetics, Heidelberg, Germany) is prefabricated and cylindrical with

a diameter of 34 mm and a height of 8 mm. The rectangular scaffolds were frozen at −18 °C so that they could be cut into a size suitable for a tissue culture within a 24-well plate. Then they were contoured under sterile conditions using a cylindrical punch that was 18 mm in diameter and 8 mm in height.

In the modified matrix (MM) group, the hydrogel was modified prior to colonisation. One millimetre of the surface of the hydrogel was removed using a custom-made cutting guide (Institutional Research Laboratories, Head of Construction: J. Viering). The control group consisted of the unmodified scaffold (NM).

The hydrogel cylinders were transferred to a 24-well plate and were washed twice with one millilitre of cell culture medium. Subsequently, two millilitres of culture medium was added to the unseeded hydrogel and incubated for 48 hours.

Seeding of the hydrogel with hBMSCs

Third passage cells were used to seed the hydrogels. At 100 % cell confluency, the entire medium was removed. The cells were washed twice with 20 ml PBS and incubated for five minutes with 8 ml trypsin at 37 °C in order to detach the cells from the culture flask. Thirty-two millilitres of stop medium [500 ml DMEM, 50 ml FCS, 5 ml penicillin/streptomycin (10,000 IE/10,000 µg/ml)] was added. After an incubation period of ten minutes at room temperature, the cells were centrifuged (1,200 rev/min, 10 min, 4 °C). The supernatant was removed and the pellet was resuspended in cell culture medium.

The hydrogel cylinders were seeded with $5 \times 10^4/\text{cm}^2$ cells for the multiphoton/confocal microscopy and $5 \times 10^5/\text{cm}^2$ cells for the immunohistochemistry. The hydrogels were incubated (37 °C, 5 % CO₂, 95 % humidity) for seven, 14 and 21 days. The medium was changed every other day.

Confocal laser microscopy

The staining was performed according to the guidelines of the manufacturer using a LIVE/DEAD® Viability/Cytotoxicity Kit (L3224, Invitrogen, Molecular Probes, Life Technologies, Eugene, OR, USA). We used the Leica inverted-MP (Leica DM IRB, Leica Microsystems GmbH, Wetzlar, Germany). Viable cells stained with calcein AM were detected with an excitation wavelength of 785 nm and an emission of 480–550 nm. Dead cells stained with ethidium homodimer I were detected with an excitation of 543 nm and an emission of 560–680 nm.

Fields of view were taken at three separate areas of the scaffolds with a magnification of 200. The images were created with the program Leica Pro (Leica Microsystems GmbH, Wetzlar, Germany). The semi-quantitative assessment was performed using the program Leica LCS Lite

(Leica Microsystems GmbH, Wetzlar, Germany). To improve accuracy, the surface viable cells were not counted or evaluated due to the formation of a confluent monolayer of viable cells on the surface and the unspecific binding of calcein AM.

DNA quantification

DNA isolation was performed using a DNA Quantification Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The photometric quantification was performed using the Nano Drop ND-1,000 with the program V3.5.2 (NanoDrop Products, Wilmington, DE, USA); 1.5 µl of each sample was analysed in triplicate.

Chondrogenic differentiation of hBMSCs

To assess the differentiation of hBMSCs in the hydrogel, the cells were incubated for two weeks with differentiation medium [125 ml DMEM and Ham's F12 1:1, 12.5 ml FCS, 1.25 ml penicillin/streptomycin (10,000 IE/10,000 µg/ml), 250 µl ascorbic acid (25 mg/ml), 250 µl amphotericin B (250 µg/ml), 100 µl dexamethasone (50 µg/ml), 125 µl recombinant human transforming growth factor (rhTGF)-β₂ (5 µg/ml), 125 µl recombinant human insulin-like growth factor (rhIGF)-I (100 µg/ml), 100 µl proline (50 mg/ml)]. Subsequently, the cells were used for the colonisation of the modified hydrogels. Each cylinder was seeded with 5×10^5 cells/cm². After 21 days, they were fixed with formalin and cryosections were produced.

The cryosections were incubated for eight hours and initially stored at −18 °C. Next, they were fixed in acetone (−20 °C) and air-dried. The preparations on the slides were separated with a grease pencil (Dako Cytomation Pen, Dako, Glostrup, Denmark). Slides were buffered for three minutes [90 % aqua, 10 % Dako 10X wash buffer, 0.1 % bovine serum albumin (BSA)]. They were then blocked with peroxidase (0.3 % H₂O₂ in washing buffer) for 30 minutes. After rinsing once in washing buffer, the sections were incubated and doused with serum (10 % human serum in washing buffer). After 15 minutes, the antibody solution (Polyclonal Antibody to Collagen Type II, Biotin, Acris, San Diego, CA, USA) 1:200 dilution in staining buffer (wash buffer with 1 % BSA) was applied. The antibody incubation lasted one hour in a humid chamber. Sections were rinsed three times in buffer and doused for 30 minutes with the avidin-horseradish peroxidase (HRP) complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) (5 ml to one drop avidin staining buffer and one drop biotin), which was incubated in the dark prior to this. After the sections were rinsed three times in wash buffer, the DAB solution (Dako Liquid DAB + substrate-chromogen system, one drop DAB to one millilitre buffer, Dakocytomation, Carpinteria, CA, USA) was applied for

five minutes. Afterwards, the sections were rinsed with distilled water. To counterstain the cell nuclei, the sections were stained for two minutes in Harris haematoxylin (Roth, Karlsruhe, Germany) (1:3 in distilled water) and rinsed for ten minutes in tap water. After an ethanol series (2 min 96 % alcohol, 2 min 100 % alcohol), the sections were inlaid in xylene for 2×5 min. DPX was then added.

Images were digitised after examination under the light microscope with the program *analis getIT BX41* (Olympus, Hamburg, Germany). Human cartilage was used as a positive control. As a negative control, an unseeded section of the collagen I hydrogel and one treated with proliferation medium were used.

Toxicity testing of collagen I hydrogel biocompatibility

To exclude a toxic effect of the hydrogel or the preservation liquid, we used a cell suspension without hydrogel and added 1.5 ml of preserving liquid. Adherence after two, 24 and 48 hours was assessed. To exclude a direct toxicity of the hydrogel, a further control approach was modified. A piece of the hydrogel was placed onto the adherent cells. After an incubation period of two days the adherence was re-evaluated.

Statistical analysis

The data were processed with the statistical software package SPSS (version 20.0, SPSS Inc., Chicago, IL, USA). Interference statistical analyses were performed for two independent samples using the Mann–Whitney U test. The Friedman test was used to check several related samples. Two related samples were examined by a Wilcoxon test. Diagrams were created with the software package Excel (version 14.2.4, Microsoft® Excel®, Redmond, WA, USA).

Results

Confocal microscopy (migration, viability and proliferation of hBMSCs)

hBMSCs were fluorescently labelled using the LIVE/DEAD® Viability/Cytotoxicity Kit and analysed microscopically after seven, 14 and 21 days of culture. After one week, the detectable amount of cells at the depth of 26–50 µm was significantly higher in the MM than in the NM group. The maximum depth of penetration was 75 µm (NM) and 200 µm (MM). After two weeks, the detectable amount of cells at the depth of 0–25 µm was significantly higher in the MM than in the NM group. The maximum depth of penetration was 125 µm (NM) and 200 µm (MM). After three weeks, the maximum depth of penetration was 175 µm (NM) and 200 µm (MM). Even at a depth of 0–25 µm, the amount of detectable cells was

significantly higher in the MM group (Fig. 2). Over the time span of three weeks, the increase in cells at the different depths of penetration was significant in the MM (25–125 μm : $p=0.001$, 150–200 μm : $p=0.000$). In the NM, the increase was not significant over time. The total amount of viable penetrated cells was significantly higher in the MM group than in the NM group. The total amount of non-viable cells was extremely low overall. There was no significant difference between the two groups at the various time points (Fig. 3).

Quantification of DNA

We evaluated the amount of DNA which was contained in a matrix hydrogel at the different time points. After 14 days, the concentration of DNA was significantly higher in the samples of the MM than in the control group ($p=0.000$). However, there was no significant difference between the two groups after three weeks (Fig. 4). The increase in DNA over time in both groups was highly significant (MM: $p=0.001$, NM: $p=0.000$).

Chondrogenic differentiation of hBMSCs

We studied the ability of the matrix to allow hBMSCs to differentiate along the chondroblast lineage. It was found that there had been chondrogenic differentiation of the cells in the scaffold treated with differentiation medium. In comparison to the positive control of human cartilage, the differentiation was slightly attenuated. In comparison to the negative control only treated with proliferation medium and the unseeded scaffold, there was more chondrogenic differentiation of cells detectable.

Toxicity of the hydrogel

To exclude a toxic effect of the hydrogel or the preservation liquid, we used a cell suspension without hydrogel and added 1.5 ml of preserving liquid. Adherence after two, 24 and 48 hours was assessed. There was no change in the adherence at the different time points. To exclude a direct toxicity of the hydrogel, a

Fig. 2 After 1 week, the detectable amount of cells at the depth of 26–50 μm is significantly higher in the MM than in the NM group. The maximum depth of penetration is 75 μm (NM) and 200 μm (MM) (a). After 2 weeks, the detectable amount of cells at the depth of 0–25 μm is significantly higher in the MM than in the NM group. The maximum depth of penetration is 125 μm (NM) and 200 μm (MM) (b). After

3 weeks, the maximum depth of penetration is 175 μm (NM) and 200 μm (MM). Even at a depth of 0–25 μm , the amount of detectable cells is significantly higher in the MM group (c). The image gives an example of the distribution of cells in a matrix of the modified group. Each point on the scale of the box measures 10 μm (d)

Fig. 3 The total amount of viable penetrated cells is significantly higher in the MM group than in the NM group (a). The total number of non-viable cells is extremely low overall. There is no significant

difference between the two groups looking at the various time points (b). In confocal microscopy, living cells are green and dead cells are red using the LIVE/DEAD® Viability/Cytotoxicity Kit (c)

further control approach was modified. A piece of the hydrogel was placed onto the adherent cells. After an incubation period of two days, the adherence was re-evaluated. Again, no change in cell adherence appeared.

Fig. 4 After 14 days, the concentration of DNA is significantly higher in the samples of the MM than in the control group. However, there is no significant difference between the two groups after 3 weeks

Discussion

Migration into the matrix was significantly improved by modifying the surface of the hydrogel. Only a maximum penetration depth of 47 μm could be demonstrated in a liquid self-assembling collagen I gel [7]. The parallel arrangement of the superficial layer significantly complicates the migration into the matrix. However, this property can also have positive effects. The parallel alignment of the bottom of the matrix could protect them from the ingrowth of osteogenic progenitor cells and calcifications. Calcification of the matrix is desired in the treatment of bone defects [11–13]. However, it is a hindrance for the regeneration of cartilage.

We evaluated the migration of cells by confocal laser microscopy. This method provides a complete analysis of the matrix, since it offers the possibility to create a 3D image of the specimen [7, 14]. Therefore, it is by far superior to conventional microscopy with concomitant production of histological sections. However, this has its importance in the project by the evaluation of differentiation of hBMSCs to chondroblasts, which was analysed by collagen II antibodies in immunohistology.

Furthermore, we evaluated cell replication by using DNA analysis [15]. The amount of DNA after 14 days was significantly higher in the MM. After 21 days, we could no longer find a significant difference between the groups. Due to the faster migration into the matrix, there was initially a higher replication rate. However, the higher confluency of cells may, in turn, have an inhibitory effect on cell proliferation [16, 17]. There was a convergence of the group values for the amount of DNA. It must be considered that the entire DNA of the multilayer is included in the calculation, since the complete matrix is analysed.

Chondrogenic differentiation should be demonstrated with collagen II antibodies [18]. The chondrogenic differentiation of hBMSCs is currently an intense area of research [19, 20]. It was shown that it takes place in the presence of a scaffold [21] and that type I collagen hydrogel ensures chondrogenic differentiation of mesenchymal stem cells [22, 23]. In our study, it was clearly shown that a chondrogenic differentiation of cells in the matrix is possible by staining histological sections and collagen II antibody labelling.

A limitation of this study is that we used hBMSCs, which are potential chondrogenic progenitor cells, but in the minority in situ. Furthermore, the influences of local cytokines were neglected, because of their inter-individual differences to ensure reproducibility in vitro.

However, the possibility that the hydrogel itself has a toxic effect on the cells must be ruled out, as discussed in the literature [24, 25]. The toxicity of the hydrogel could not be detected in our experiment.

These results are of particular interest for further clinical applications, as it was demonstrated that a simple modification of the implant promotes better cellular ingrowth.

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