Abstract

**Purpose** The purpose of this study was to investigate the influence of continuous perfusion and mechanical stimulation on bone marrow stromal cells seeded on a collagen meniscus implant.

**Methods** Bone marrow aspirates from 6 donors were amplified in vitro. \(10^6\) human BMSC were distributed on a collagen meniscus implant. Scaffolds were cultured under static conditions (control) or placed into a bioreactor system where continuous perfusion (10 ml/min) or perfusion and mechanical stimulation (8 h of 10% cyclic compression at 0.5 Hz) were administered daily. After 24 h, 7 and 14 days, cell proliferation, synthesis of procollagen I and III peptide (PIP, PIIIP), histology, and the equilibrium modulus of the constructs were analyzed.

**Results** Proliferation demonstrated a significant increase over time in all groups \((p < 0.001)\). PIP synthesis was found to increase from 0.1 ± 0.0 U/ml/g protein after 24 h to 2.0 ± 0.5 (perfusion), 3.8 ± 0.3 (mechanical stimulation), and 1.8 ± 0.2 U/ml/g protein (controls) after 1 week and remained significantly elevated under the influence of perfusion and mechanical stimulation \((p < 0.01)\) after 2 weeks. Mechanical stimulation increased the equilibrium modulus more than static culture and perfusion after 2 weeks \((24.7 \pm 7.6; 12.3 \pm 3.7; 15.4 \pm 2.6 \text{ kPa}; p < 0.02)\).

**Conclusion** Biomechanical stimulation and perfusion have impact on collagen scaffolds seeded with BMSCs. Cell proliferation can be enhanced using continuous perfusion and differentiation is fostered by mechanical stimulation.

**Level of evidence** Non-applicable experimental study.

**Keywords** Tissue engineering · Meniscus · Bone marrow stromal cell · Collagen implant · Mechanical stimulation

Introduction

The menisci play a key role in load transmission, nutrient transfer, stabilization, and thus, articular cartilage protection within the knee joint. Partial or entire loss of the meniscus has been attributed with articular cartilage degeneration, joint instability, loss of sports activity level, and pain [15].

Numerous scaffolds have been suggested for use in order to engineer a bioartificial meniscus [38]; however, only the collagen meniscus scaffold [32] and a polyurethane scaffold [13] have been investigated following in human implantation. The 5-year follow-up examination of the collagen meniscus implant (CMI) demonstrated that a
new biomechanically competent meniscus-like tissue forms and that patients benefit from implantation by higher activity levels [18, 32, 44].

Besides scaffolds, cells are necessary for the tissue engineering of the meniscus. In previous studies, fibrochondrocytes, articular chondrocytes, and mesenchymal stem cells have been used [21, 29, 42]. Due to the limited availability of the fresh fibrochondrocytes, advantages for the use of mesenchymal stem cells are seen [3, 10]. A study with the human mesenchymal stem cells [1] reported adherence to a hyaluronan/gelatin composite scaffold and cell differentiation into fibrochondrocytes. Improved mechanical properties of cell-seeded gels have been reported as a function of gel cross-linking and time [8].

For tissue engineering of cartilage, growth factors and mechanical stimulation are key factors that enhance hyaline cartilage formation [12]. The same principles apply to meniscal tissue [9, 11, 37]. Positive effects of mechanical stimulation of tissue engineered grafts of cartilage and bone are reported frequently in the literature [2, 19, 23]. Until the present point, there is only limited knowledge of the effects of mechanical stimulation for tissue engineering of the meniscus [21, 34, 40]. Most of the studies demonstrated a positive effect on the meniscal cells due to the mechanical stimulation. But the effect seems to be dependent on the amount of the applied load or compression. In the aforementioned studies, dynamic compression of 0.08–0.16 MPa with 5 Hz over 24 h and of 0.1 MPa over 24 h implemented as static compression was applied. These strain levels correspond with a low physiological load calculated in a finite element model [26, 36]. An experimental study with a compressive load of 5 bar could not demonstrate a positive effect by mechanical stimulation on the meniscal cells; strain levels of more than 5 bar were found to be toxic for the cells [28].

However, ongoing studies without bioreactors also demonstrated that the mechanical stimulation has a positive effect on the meniscal regeneration. Animal studies in rats have shown that mobilization increases the synthesis of collagen and proteoglycan, while immobilization inhibits both aggrecan and accumulation of collagen [14, 41].

The hypothesis of this study is that there is a positive effect of mechanical compression and perfusion regarding cell proliferation, procollagen synthesis, and the biomechanical properties of a tissue engineered meniscus construct in a bioreactor system.

**Materials and methods**

**Bone marrow aspiration**

Bone marrow aspirates were obtained during routine surgical exposure of the iliac crest in 6 patients (age: 27–55 years) after informed consent. The procedure was approved by the local ethical committee. The samples were aspirated in a syringe that was filled with 1,000 IU of heparin and were processed within 24 h. A density gradient (Biocoll, Biochrom AG, Berlin, Germany) was used in order to select cells of an equivalent molecular weight. For the 2-dimensional cell culture, a fibroblast differentiation media (DMEM/HAM’s F-12 (1:1) with t-Glutamine, 10% FCS (Biochrom AG, Berlin, Germany), 5 µg/ml ascorbic acid, 3 ng/l FGF-2 ([20], Biochrom AG, Berlin, Germany) was used. After the first passage, cells were pooled, and 7 cell cultures from the same cell pool were used for the experiments (n = 7). At this point, cell phenotype was analyzed using the following protocol: $10^6$ viable (trypan blue negative) bone marrow mononuclear cells were stained with the following non-diluted antibodies (all obtained from BD, Franklin Lakes, USA): CD45-APC, CD235-APC, CD271-PE, and CD73-PE [39]. The fraction of bone marrow mesenchymal stem cells (PE+, APC−) that were selected using a fluorometric cell sorter (BD FACSAriaTM, BD, Franklin Lakes, USA) was 89 ± 6%.

Cells of the 2nd or 3rd passage were counted, and $10^6$ cells were resuspended in the above-mentioned media. The cells were then seeded on the collagen scaffold (Table 1).

**Collagen meniscus implant**

Collagen meniscus implants (CMI, Menacell™, Regen® Biologics Inc., Hackensack, NJ, USA) designed for animal studies were used for this project. For the CMI, potential toxic effects have been described, and washing of the media before seeding has been recommended [28]. Therefore, the scaffolds were washed in PBS for 1 week before further processing.

Two of the constructs were fit on a permeable disk (20 mm diameter). $10^6$ cells resuspended in 300 µl of serum were seeded in portions of 30 µl into the matrix using a 22G-syringe and the before mentioned culture media. The syringe was inserted into the central portion of the scaffold and 10 µl of media was released. Then, the syringe was pulled back gently and another 10 µl of fluid was injected. The procedure was repeated 3 times. The cells were allowed to adhere during a 24-h period of incubation.

**Bioreactor**

Two CMI’s were put in a modified perfusion bioreactor introduced by Bader [5], designed by the Institutional Research Laboratory. The bioreactor was continuously perfused with a rate of 10 ml/min. Cyclic compression was achieved by means of a magnetic field engine (Fig. 1). An electronic length gauge could monitor the compression.
In a pilot study, the strain rate could be increased up to 20% achieving compression forces of 64 kPa. The stimulation frequency can be modulated between 0.2 and 10 Hz. For the experiments, a 10% cyclic compression (0.4 mm) and 0.5 Hz repetition rate were applied for 8 h per day followed by 16 h of rest. Samples were harvested after 24 h (1 CMI), such as 1 and 2 weeks (one additional scaffold) after beginning of the culture (Table 1).

Radioimmunoassay

Synthesis of collagen I and III was investigated by quantification of the C-terminal propeptides of collagen I and III. A PINP and PIIINP [125I] radioimmunoassay kit was purchased from Orion Diagnostica, Espoo, Finland, and performed in accordance with the manufacturer’s recommendations. For the determination of the total amount of protein, a micro-Lowry assay for microplates was used. The reagents were purchased in a kit (No. 690-A: Sigma Chemical, St. Louis MO). Aliquots were frozen and stored at −70°C until assayed.

A standard curve was prepared (data not shown, linear fit, six points, n = 3, \( R^2 = 0.98 \)). After standards and samples were diluted and transferred to the microplate, 200 ul of biuret reagent was added to each well and mixed thoroughly with repeated pipeting. Biuret reagent was prepared by mixing 0.5 ml of 1% cupric sulfate with 0.5 ml of 2% sodium potassium tartrate, followed by the addition of 50 ml of 2% sodium carbonate in 0.1 N NaOH. The mixture was then allowed to incubate at room temperature for 10 min. After incubation, 200 ul of biuret reagent was added to each well and mixed thoroughly with repeated pipeting. The absorbance of each well was measured at 540 nm using a spectrophotometer.

Fig. 1 Schematic showing the bioreactor system used for the mechanical stimulation of the collagen meniscus implants:

- Two CMI’s were put in the bioreactor chamber, where a custom-made plunger implemented a cyclic compression of 10% on the scaffold. An electromagnetic field engine induced compression. There was a continuous perfusion of the constructs with 10 ml/min. Length, force and pressure changes were continuously monitored.

Table 1 Protocol of bone marrow aspiration, cell expansion, passaging, and seeding of the CMI

<table>
<thead>
<tr>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow aspiration patients 1-6</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
</tr>
<tr>
<td>2-dimensional cell culture</td>
</tr>
<tr>
<td>Passage 1</td>
</tr>
<tr>
<td>Pooling of cells from 6 different individuals</td>
</tr>
<tr>
<td>7 separate cell cultures: Cell expansion</td>
</tr>
<tr>
<td>Passages 2 and 3</td>
</tr>
<tr>
<td>Seeding of CMI’s (n=7)</td>
</tr>
</tbody>
</table>
temperature for 10–15 min prior to the addition of 20 μl
per well of 1.0 N Folin and Ciocalteu’s reagent. Samples
were mixed immediately with repeated pipeting with each
addition. Color was allowed to develop for 30 min at room
temperature and the absorbance measured at 650 nm and
blanked on the water-only control. Although in these
experiments the plates were read immediately, the reaction
was found to be stable for up to an hour. All absorbance
determinations were made using an ELISA microplate
reader (BP 800, Biohit, Finland) with the reader controlled
by an external PC running KC3 data reduction software
(BioTek Instruments, Winooski, VT). Regression analysis
and statistics of the curve were performed using KC3.

MTS assay

The MTS assay was used to investigate the cell metabolism
of BMSCs seeded on the scaffold. Cells were incubated
with 0.5 mg/ml MTS for 2 h at 37°C; the solution was then
removed and formazan salts dissolved with dimethyl
sulphoxide, and the absorbance of the resulting solution
was determined at 490 nm using a plate reader (BP 800,
Biohit, Finland). Cell number was determined from the
 calibration data, (not shown linear fit, six points, n = 3,
R² = 0.97) obtained using cells from the same culture [35].

H&E stain

Samples were frozen immediately after harvesting at a
freeze rate of −15/°C/min until achieving −80°C. A freeze
section was performed. After air drying, H&E staining
solution was added for 10 min. After rinsing with distilled
water, a cover glass was put on top of the sample. The
specimens were examined with a fluorescent light micro-
scope in the dark chamber. H&E stain was investigated
using a light microscope (Olympus CX41, Olympus
Deutschland GmbH, Hamburg).

Biomechanical testing

As described previously [7, 17], the equilibrium modulus
was determined under confined compression via stress
relaxation tests. Constructs were trimmed in size using a
mosaicplasty harvester 6 mm inner diameter (OATS;
Arthrex; Naples, FL, USA). The diameter was confirmed
using a contact-less laser micrometer. The construct was
placed in a cylindrical chamber with a diameter of 6 mm.
The plunger had a 5.5 mm diameter, and a 4.5-mm hole was
centered below the support chamber. A computer-activated
micro-stepper motor controlled the displacement of the
plunger. Steps (10–50 μm) were imposed on the scaffolds
and resultant loads were fit to a poroelastic model
(EnduraTech; Electroforce (ELF) 3200 System, Minne-
tonka, MN).

Statistical analysis

Results were expressed as mean values ± standard devia-
tion. If normality and equal variance tests were passed, a
one-way repeated measures analysis of variance (RM-
ANOVA) was used to identify time-dependent differences
within and between the groups. Pairwise multiple com-
parison procedures were conducted with a post-hoc Tukey
 test (SPSS 11.5; SPSS, Chicago, IL, USA). A p-level of
0.05 was considered to be statistically significant.

Results

Cell morphology

During the observed time period, there was more extra-
cellular matrix present in samples cultivated with perfusion
and both perfusion and mechanical stimulation. Cells under
mechanical loading demonstrated less cell to scaffold
adhesions compared with static cultures and cultures under
continuous perfusion (Fig. 2).

Metabolic cell activity

After 24 h, metabolic cell activity was determined with
0.27 ± 0.06 (absorbance at 490 nm). There was an
increase up to 0.31 ± 0.08 (mechanical stimulation),
0.33 ± 0.09 (perfusion), and 0.27 ± 0.06 (static control)

| Table 2  | Study protocol of the CMIs seeded with BMSC including the static control group, the perfusion group, and the group with biome-
| Stimulation: compression/frequency | Static controls | Perfusion | Perfusion/Mechanical Stimulation 10%/1 Hz |
| Duration | Continual | Perfusion/continual | Mechanical stimulation: 8 h/day |
| Number | 7 | 7 | 7 |
| Samples | 24 h after seeding, 1 and 2 weeks following the beginning of stimulation |
| Analysis | H&E stain, viability assay, MTS test, PIP, PIIP, and protein |

 Springer
after the first week and up to 0.35 ± 0.07 (mechanical stimulation), 0.37 ± 0.08 (perfusion), and 0.36 ± 0.07 (static control) after the second week.

Metabolic cell activity demonstrated time-dependent increase \((p < 0.001)\). The multiple comparison procedure revealed a highly significant increase in between 24 h and 2 weeks \((p < 0.001, \text{Fig. 3})\). There was no significant difference between the three culture conditions \((\beta = 0.95)\).

Procollagen I and III propeptide

Procollagen I and III propeptide (PIP) synthesis was found to increase from 0.1 ± 0.0 U/ml/g protein after 24 h to 2.0 ± 0.5 (perfusion), 3.8 ± 0.3 (mechanical stimulation), and 1.8 ± 0.2 U/ml/g protein (static control, lower than perfusion and mechanical stimulation, \(p < 0.05\)). These differences were also evident after 2 weeks (2.7 ± 0.3, 4.0 ± 0.6, and 1.8 ± 0.2 U/ml/g protein, \(p < 0.01\); Fig. 4). There was a significant increase in PIP over time \((p < 0.001)\). All three groups synthesized significantly different amounts of PIP \((p < 0.001)\).

Procollagen II propeptide (PIIIIP) synthesis was found to increase from 0.1 ± 0.0 U/ml/g protein after 24 h to 2.9 ± 0.7 (perfusion), 3.1 ± 0.9 (mechanical stimulation), and 1.6 ± 0.3 U/ml/g protein (static control, lower than perfusion and mechanical stimulation, \(p < 0.01\)). These differences were also evident after 2 weeks (2.8 ± 0.5, 3.4 ± 0.9, and 1.5 ± 0.3 U/ml/g protein, \(p < 0.01\); Fig. 5). There was a significant increase in PIIIIP over time \((p < 0.001)\). All three groups synthesized significantly different amounts of PIIIIP \((p < 0.001)\).
Under static culture conditions, the equilibrium modulus of the constructs decreased from 10.5 ± 2.3 after 24 h to 6.7 ± 1.1 kPa and then increased to 12.3 ± 3.7 kPa; \( p < 0.02 \). Samples subjected to continuous perfusion demonstrated increased mechanical properties (10.6 ± 3.4 after 1 week and 15.4 ± 2.6 kPa after 2 weeks). The equilibrium modulus after 2 weeks was significantly increased compared with 24 h (\( p < 0.04 \)) and 1 week (\( p = 0.05 \)). Mechanical stimulation increased the equilibrium modulus after 1 week (14.1 ± 3.0 kPa; \( p < 0.01 \)) and 2 weeks (24.7 ± 7.6 kPa; \( p < 0.02 \)). The data is presented in Fig. 6.

There was a significant effect of time (\( F = 26.4; p < 0.01 \)), culture condition (\( F = 18.1; p < 0.01 \)), and the combination of time and culture condition (\( F = 5.9; p < 0.01 \)) on the equilibrium modulus. Mechanical stimulation enhanced the equilibrium modulus compared with static culture after 1 week (\( p < 0.01 \)). After 2 weeks, mechanical stimulation increased the equilibrium modulus compared with both static controls and samples that were cultured under continuous perfusion (\( **p < 0.01 \)).

**Discussion**

The most important finding of the present study was that perfusion and mechanical stimulation have a positive impact on proliferation and differentiation of bone marrow stromal cells cultured on a collagen meniscus implant.

Different scaffolds and cell sources have been described for tissue engineering of the meniscus: The use of fibrochondrocytes is limited by number [30]. The use of
autologous chondrocytes is associated with an additional trauma, and the use of allogeneic chondrocytes is associated with the possible risk of disease transmission and immunologic reaction [31, 43]. Mesenchymal stem cells have the advantage of being autologous and are available from the iliac crest. Pooling of mesenchymal stem cells from several donors is a common practice in tissue engineering and generates higher and more stable immunosuppression, particularly suppression of lymphocyte proliferation. The problem of rejection is expected to be of importance when not pooling stem cells and using only cells from one individual [33].

Up to now, there are only a few studies using MSC for meniscal tissue engineering. There is evidence for these cells that the production of extracellular matrix is higher in comparison with fibrochondrocytes [6].

There was a significant influence of time, culture condition, such as perfusion and mechanical stimulation, as well as the combination of time and culture condition on construct equilibrium modulus. In spite of the tissue compatibility of the CMI in the clinical studies, problems with cell seeding in experimental studies are described [28]. An extensive washing of the CMI with culture medium at least for 6 days before cell seeding is recommended and was used in this study.

Various forms of mechanical stress such as static load, dynamic compression load, dynamic tensile forces, and rotary cell culture systems have been used [4, 16, 22, 27, 34, 40]. Static compression leads up to relative inhibition of the matrix production and collagen synthesis [4, 22, 40], whereas under the influence of dynamic compression forces in some studies, an increase in the protein and proteoglycan synthesis was seen. Marsano [27] found in his study with a scaffold and chondrocytes in a rotary cell culture system locally different compositions of the generated tissue. In the aforementioned studies, dynamic compression of 0.08–0.16 MPa with 5 Hz over 24 h and of 0.1 MPa over 24 h lead up to an increase in cellular proliferation. In contrast to this study, static compression was used. High compressive loads of 500 MPa could not demonstrate a positive effect by mechanical stimulation on the meniscal cells; strain levels of more than 500 MPa were found to be deleterious for the cells [28]. We used a strain rate of 10% and a stimulation time of 8 h per day because of our previous experience in 2-dimensional cell culture [24, 45]. The amount of pressure delivered on the loaded scaffolds was monitored and limited to 16 MPa.

Regarding the PIP and PIIIP synthesis, the group with mechanical stimulation and perfusion showed a significant increase of released PIP and PIIIP after 1 and 2 weeks. This corresponds with the observation of other authors using osteoblastic cells [25] or fibroblasts [45] regarding procollagen I peptide or collagen I in a two-dimensional cell stretching apparatus. We have used procollagen I and III instead of collagen I or III because the scaffold material itself contains collagen I and has shown positive reactions to collagen I and III antibodies after immunostaining in a pilot study.

The findings from the present study support the recommendation of initial static culture after thorough rinsing of the CMI when seeding the scaffold with BMSC [28]. Thereafter, mechanical loading can enhance BMSC differentiation and construct equilibrium modulus.

Limitations of this study are the limited observation time period of 2 weeks and the application of only cyclic compression, but not rotary cell culture systems as other authors.

Further in vivo studies are needed in order to clarify the benefit of cell seeding of meniscus scaffolds and to elucidate the benefit from mechanical loading. Longer time periods and a modulation of the perfusion and compression rates should be analyzed in future studies.

As a perspective for the clinical future, collagen meniscus implants seeded with human bone marrow stromal cells prior to implantation could be a promising approach for patients with meniscal defects, leading to earlier and better ingrowth than CMI without BMSCs.

Conclusion

In this experimental study, we could show that biomechanical stimulation and perfusion can promote proliferation and differentiation of bone marrow stromal cells seeded on a collagen meniscal scaffold.

Acknowledgments We wish to thank J. Viering and H. Schumann for the construction of the bioreactor system used in this study. We acknowledge the writing assistance of Gavin Olender, MSc. We are grateful for the financial support of the “Deutsche Arthrosehilfe” and the German Society of Orthopaedic Traumatologic Sports Medicine (GOTS). We gratefully acknowledge the support of W. Rodkey and Regen Biologics Inc. for this study.

References

human vascular aortic tissue based on a xenogenic starter matrix. Transplantation 70(1):7–14


