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Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel

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Background

Disruptions of the anterior cruciate ligament (ACL) of the knee joint are common and are currently treated using ligament or tendon grafts. In this study, we tested the hypothesis that it is possible to fabricate an ACL construct in vitro *using mesenchymal stem cells (MSC) in combination with an optimized collagen type I hydrogel, which is in clinical use for autologous chondrocyte transplantation (ACT).*

Methods

ACL constructs were molded using a collagen type I hydrogel containing 5×10^5 *MSC/mL and non-demineralized bone cylinders at each end of the constructs. The constructs were kept in a horizontal position for 10 days to allow the cells and the gel to remodel and attach to the bone cylinders. Thereafter, cyclic stretching with 1 Hz was performed for 14 days (continuously for 8 h/day) in a specially designed bioreactor.*

Results

Histochemical analysis for H&E, Masson-Goldner and Azan and immunohistochemical analysis for collagen types I and III, fibronectin and elastin showed elongated fibroblast-like cells embedded in a wavy orientated collagenous tissue, together with a ligament-like extracellular matrix in the cyclic stretched constructs. No orientation of collagen fibers and cells, and no formation of a ligament-like matrix, could be seen in the non-stretched control group cultured in a horizontal position without tension. RT-PCR analysis revealed an increased gene expression of collagen types I and III, fibronectin and elastin in the stretched constructs compared with the non-stretched controls.

Discussion

In conclusion, ACL-like constructs from a collagen type I hydrogel, optimized for the reconstruction of ligaments, and MSC have been fabricated. As shown by other investigators, who analyzed the influence of cyclic stretching on the differentiation of MSC, our results indicate a ligament-specific increased protein and gene expression and the formation of a ligament-like extracellular matrix. The fabricated constructs are still too weak for animal experiments or clinical application and current investigations are focusing on the development of a construct with an internal augmentation using biodegradable fibers.

Keywords

anterior cruciate ligament, collagen type I hydrogel, cyclic stretching, mesenchymal stem cells, tissue engineering.

Introduction

The anterior cruciate ligament (ACL) is regarded as critical for normal function of the knee joint. Its disruption causes functional impairment, meniscal lesions and the early onset of osteoarthritis [1]. Disruptions of the ACL are the most common ligamentous injuries of the knee, with more than 100 000 ACL reconstructions performed each year [2,3]. Currently, the most successful technique for the reconstruction of a torn ACL is the use of autologous grafts taken from other parts of the body, such as the central third of the patellar tendon (bone–patellar tendon–bone graft) and hamstring grafts usually

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using the semitendinosus or gracilis tendon [4]. But there are disadvantages, such as donor site morbidity, muscle weakening and the limited number of available grafts. These problems could be avoided by the use of fresh or cryopreserved allografts. However, there are then additional problems of allograft rejection, disease transmission, size miss-matching and insufficient blood supply.

Tissue engineering and cell-based technologies might overcome the limitations of autografts and allografts in the future. In particular, the use of multipotent mesenchymal stem cells (MSC) has opened up new therapeutic possibilities for the reconstruction of injured ligaments. Recent experiments coating tendon grafts with MSC have shown superior graft quality and osseointegration when replacing the ACL in a rabbit model [5]. MSC have also shown higher proliferation rates and synthesized more ligament-specific extracellular matrix proteins when seeded onto a biodegradable multifilament material designed for ACL repair, compared with ACL and skin fibroblasts [6]. As well as the cell type, the scaffold material used is of critical importance. The ideal ACL replacement scaffold should be biodegradable, porous, biocompatible, exhibit sufficient mechanical strength and able to promote the formation of a ligamentous tissue. Several groups have reported potential ACL scaffolds using collagen, silk, biodegradable polymers and composite materials [6–9]. Because the human ACL consists mainly of collagen type I, collagen type I matrices and especially hydrogels in combination with fibroblasts or MSC are high potential cell carrier materials for tissue-engineered ACL constructs.

In this study, we first aimed to fabricate an ACL construct *in vitro* in the shape of a bone–patellar tendon–bone graft with a non-demineralized bone cylinder at each end of the construct. For this purpose we used human MSC together with a collagen type I hydrogel, which is clinically approved for autologous chondrocyte transplantation (ACT). Second, we evaluated the influence of cyclic stretching on the protein and gene expression profile for typical ligament-specific markers compared with static conditions over a period of 14 days in a specially designed bioreactor.

Methods

Marrow-derived human mesenchymal stem cells (mhMSC)

The cell culture procedure was modified from Haynesworth *et al.* [10,11] and approved by the Institutional Review Board of the University of Würzburg (Würzburg, Germany). Briefly, trabecular bone plugs (5–10 mL) were harvested from the cutting plane of the femoral neck using a bone curet, and transferred to 50-mL conical tubes containing 20 mL DMEM/F-12 (PAA Laboratories, Linz, Austria). The tubes were vortexed to disperse marrow cells from the bone plugs and centrifuged (1000 r.p.m. for 5 min) to pellet-suspended cells and bone plugs. The supernatant was discarded and the pellets were reconstituted in 10 mL complete medium consisting of DMEM/F-12 supplemented with 10% FBS (Gibco BRL, Darmstadt, Germany), antibiotics (50 IU penicillin/mL and 50 μg streptomycin/mL; PAA Laboratories) and 50 μg/mL ascorbate 2-phosphate (Sigma, Deisenhofen, Germany). After vortexing, the released marrow cells were collected with 10-cc syringes fitted with 20-gauge needles and saved. The remaining cells in the bone plugs were extracted using an identical procedure for a total of five times, until the bone plugs appeared yellowish-white. The collected cells were pelleted (1000 r.p.m. for 5 min), resuspended in complete medium, counted with a hemocytometer, and plated at a density of 6×10^7 cells/150-cm^2 tissue culture flask (TPP, Trasadingen, Switzerland). Non-adherent cells were removed by aspiration with a Pasteur pipette after 2 days. Attached cells were washed with PBS and cultured in complete medium for 10–14 days to a subconfluent state. The medium was changed every 3–4 days.

Collagen type I gel and cell seeding

Collagen type I, dissected from rat tail, separated from non-soluble and non-collagenous ingredients, was dissolved in acetic acid and provided by Ars Arthro AG (Esslingen, Germany) at a concentration of 6 mg/mL, and was stored at −20°C. From this stock solution a collagen gel with a final concentration of 3 mg/mL containing 5×10^5 MSC/mL was produced. First, 1.25×10^6 MSC were resuspended in 1.25 mL of a special gel neutralization solution (special high-buffered cell culture medium provided by Ars Arthro AG to neutralize the acidic collagen solution). Second, 1.25 mL of the collagen type I stock solution (6 mg/mL) were added and gently mixed without air bubbles and kept on ice until use. For each construct 2.5 mL of collagen–MSC solution were prepared.

Fabrication of ACL constructs

Cylinders of non-demineralized bovine bone matrix (Lubboc®, Ost-Developpement, Clermont-Ferrand, France) with a diameter and length of 8 mm were penetrated in a transverse direction with an injection needle. A non-biodegradable suture wire (2.0 Seralon, Wiessner, Naila, Germany) was drawn through the hole of each cylinder and knotted. The top of a 2-mL plastic syringe was cut with a scalpel and one bone cylinder with an attached wire was put on the bottom of the syringe (Figure 1a). The prepared collagen–MSC solution (2.5 mL/construct) containing 5×10^5 MSC/mL was pipetted into the syringe (Figure 1b). A second bone cylinder was put into the syringe until it was totally penetrated with the gel solution (Figure 1c). The syringes containing the poured constructs were incubated at 37°C in a humidified 95% air–5% CO_2 atmosphere for 30 min. After polymerization, the cured constructs were pushed out of the syringe into a sterile Petri dish containing complete medium. Earlier studies had shown that the fabricated constructs were too fragile to be directly put into the bioreactor. Therefore, the constructs were cultured horizontally for 10 days to allow the cells to remodel the collagen gel matrix and attach to the bone plugs. During this time the constructs were pinned with injection needles in a specially designed Petri dish to avoid shrinking or rupture. The distance between the bone plugs of the pinned constructs was 2.5 cm. The medium was changed every 3–4 days.

Bioreactor and cyclic stretching

A specially designed bioreactor for cyclic stretching was fabricated based on a publication by Goulet *et al.* [12]. Briefly, a mobile anchor (top) and a fixed anchor (bottom) were installed vertically on a metal column in a glass cylinder (Figure 2a). The anchors were equipped with special devices for the fixation of the wires, which were earlier put through the bone cylinders of the ACL constructs (Figure 2b). An electric motor connected to a transformer was adjusted on the top of the bioreactor, which moved a rotating metal disk with a screw (Figure 2a, c). The screw could be turned up or down to control cyclic elongation, with amplitudes from 0 to 20 mm. A stretching frequency of 1 Hz and amplitude of 3 mm was performed for 14 days (continuously for 8 h/day). After the constructs were fixed, the bioreactor was filled with complete medium and kept at 37°C in a humidified 95% air–5% CO_2 atmosphere. The medium was changed every 3–4 days. Gas exchange was assured through a filter integrated into the metal top of the bioreactor. A total of six constructs was used for the stretching experiments. Six control constructs were pinned with injection needles in a specially designed Petri dish and cultured in a horizontal position without any applied strain.

Histochemical and immunohistochemical analysis

After 14 days, the bone cylinders were detached from the cyclic-stretched and control constructs and the

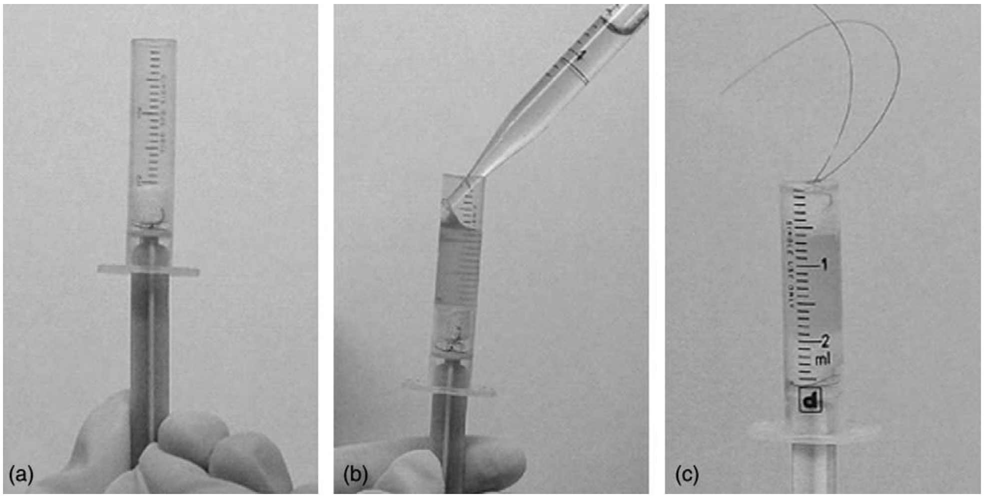

Figure 1. Fabrication method of ACL constructs from a MSC–collagen gel suspension and bone cylinders. (a) Syringe filled with a bone cylinder on the bottom. (b) Filling of the syringe with the collagen gel containing MSC. (c) Final molded ACL construct with one bone cylinder on the bottom and one at the top of the syringe. Note that for later fixation in the bioreactor both cylinders were provided with a non-biodegradable wire.

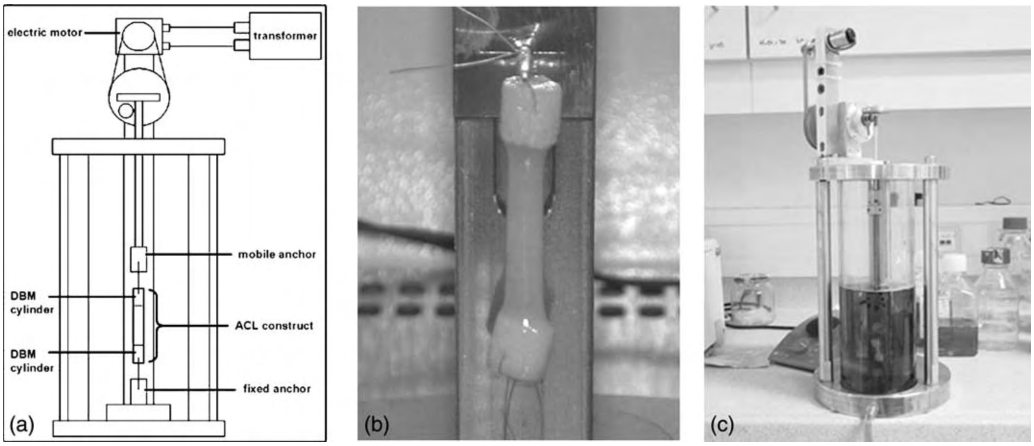

***Figure 2**. Bioreactor for cyclic stretching. (a) Schematic illustration of the bioreactor. (b) ACL construct attached to the mobile (top) and the fixed anchor with non-biodegradable wires. (c) Photograph of the bioreactor filled with medium and the ACL construct before starting the stretching experiment.*

collagen in the middle was divided into two parts. One part was used for histochemical and immunohistochemical analysis and the other part for RT-PCR analysis. The collagen gel was washed twice with PBS, embedded in Tissue Tek (Sakura, Horgen, Switzerland) and cryosections of 12 μm were performed. For histochemical analysis, sections were fixed for 10 min in acetone and stained with hematoxylin/eosin (H&E), Masson-Goldner or Azan. For immunohistochemical analysis of elastin, sections were pre-treated for 15 min with 6 m guanidin HCl/50 mm DTT in 20 mm Tris buffer. After rinsing with Tris buffer, incubation for 15 min with 100 mm iodoacetamide was performed. For all immunohistochemical analyses, sections were treated for 15 min with H_2O_2 in Tris buffer and incubated overnight at 4°C with the primary Ab diluted in 0.5% BSA. Primary Ab specific for human collagen type I (AB 745; Chemicon, Temecula, CA, USA), human collagen type III (AB 747; Chemicon), human elastin (MAB 1681; Calbiochem, San Diego, CA, USA) and human fibronectin (F0916; Sigma, Steinheim, Germany) were used. Sections were incubated with peroxidase-conjugated secondary and third Ab. Immunostaining was detected colorimetrically using DAB and slides were counterstained with hemalaun. As a control, an intact ACL of a 50-year-old man undergoing total knee arthroplasty (after informed consent and as approved by the Institutional Review Board of the University of Würzburg) was prepared and stained in an identical manner. For all immunohistochemical analyses, controls without the primary Ab were performed.

RNA isolation and RT-PCR analysis

The part of the collagen that was not used for histochemical analysis was minced with scissors. Total RNA was isolated using Trizol reagent (Gibco BRL), and an additional purification step using separation columns from the RNeasy kit (Quiagen, Hilden, Germany) was performed according to the manufacturer's protocol. The extracted RNA was converted to cDNA using Oligo dTs and M-MLV reverse transcriptase (Promega, Mannheim, Germany). Primers were selected to detect mRNA transcripts characteristic of ligament tissue (Table 1). The housekeeping gene *GAPDH* was included to monitor RNA loading. A 1-μL aliquot of the cDNA product was amplified using a Thermal Cycler (MJ Reasearch PTC-200 Peltier Thermal Multi Cycler; Biozym, Hessisch Oldendorf, Germany) in the presence of 2.5 U Taq polymerase (Amersham, Freiburg, Germany) at an initial denaturation at 94°C for 3 min, followed by different numbers of cycles (Table 1) of 1-min denaturation at 94°C, 1 min annealing with various temperatures (Table 1), 1 min extension at 72°C and a final incubation at 72°C for 5 min. The numbers of cycles and annealing temperatures were adjusted for each primer separately to obtain the highest possible sensitivity and specificity of the reaction. DNA from 15 μL of each PCR reaction was electrophoretically separated on a 1% agarose gel containing ethidium bromide and visualized using an imaging software (darkroom with camera support for table; LTF, Wasserburg, Germany). In order to control the sequence of the PCR products, bands were eluted using the MiniElute

Table 1. RT-PCR primer sequences and product size

Gene	RT-PCR primer sequences (5′ – 3′)	Cycle number	Annealing Temp (°C)	Product size (bp)
GAPDH	Fw: GTCAGTGGTGGACCTGACCT Rv: AGGGGTCTACATGGCAACTG	22	62	420
Col I	Fw: GGACACAATGGATTGCAAGG Rv: TAACCACTGCTCCACTCTGG	35	54	461
Col III	Fw: GCGGAGTAGCAGTAGGAG Rv: GTCATTACCCCGAGCACC	30	58	483
Elastin	Fw: GCAGTGCCTGGGGTCCTTGGAG Rv: GCTGCTTTAGCGGCTGCAGCTGG	30	58	211
Fibronectin	Fw: TGGAACTTCTACCAGTGCGAC Rv: TGTCTTCCCATCATCGTAACAC	30	58	451
Vimentin	Fw: GACCGCTTCGCCAACTACATCGAC Rv: GGTCATCGTGATGCTGAGAACTTCG	30	65	1060

Fw, forward; Rv, reverse.

Gel Extraction Kit (Quiagen). DNA was sequenced using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Darmstadt, Germany), analyzed with a 310 ABI automated sequencer (Applied Biosystems) and evaluated by the NCBI data bank.

Results

ACL constructs

Preliminary experiments had shown that a final concentration of 5×10^5 cells/mL of gel was a concentration with an acceptable gel contraction for the experimental set up. These experiments had also revealed that the cultivation of the constructs for 10 days in a fixed horizontal position was necessary to prevent the constructs from breaking if cyclic stretching was started too early. After culturing the constructs for 10 days in a horizontal position, they were stable enough to be transferred into the bioreactor and cyclic stretching applied. After 14 days of stretching, stretched constructs showed more gel contraction compared with control constructs cultured in a horizontal position without any tension.

Histochemical analysis

Cryosections of stretched constructs, non-stretched controls and a human ACL were stained with H&E, Masson-Goldner and Azan (Figure 3). The stretched constructs showed elongated fibroblast-like cells embedded in a wavy orientated collagenous matrix (Figure 3a, d, g). No orientation of cells and collagenous matrix was seen in the non-stretched control group (Figure 3b, e, h). Staining of a human ACL showed a wave-like structure of the collagenous extracellular matrix, as is typical for ligament tissue (Figure 3c, f, i). The cell density appeared to be slightly higher in the stretched compared with the non-stretched constructs, probably because of a pronounced contraction of the gel. The pictures of the stretched and control group represent parts of the constructs that were close to the bone plugs. In the middle part of the stretched constructs the cells showed less orientation and matrix synthesis.

Immunohistochemical analysis

Immunohistochemical staining of stretched constructs, non-stretched controls and a human ACL were performed for collagen types I and III, elastin and fibronectin (Figure 4). The stretched constructs showed a more intense staining for collagen types I and III and elastin (Figure 4a, d, g) compared with the non-stretched controls (Figure 4b, e, h). Staining of a human ACL showed intense staining of the extracellular matrix for collagen types I and III, elastin and fibronectin (Figure 4c, f, i, l). Pictures of the stretched constructs and the control group were taken from parts of the constructs that were close to the bone plugs. For each staining a no-primary Ab control was performed, which in all cases was negative for the stretched constructs and controls, as well as for the human ACL.

RT-PCR analysis

RT-PCR analysis revealed a higher gene expression of the ligament markers collagen types I and III, elastin and

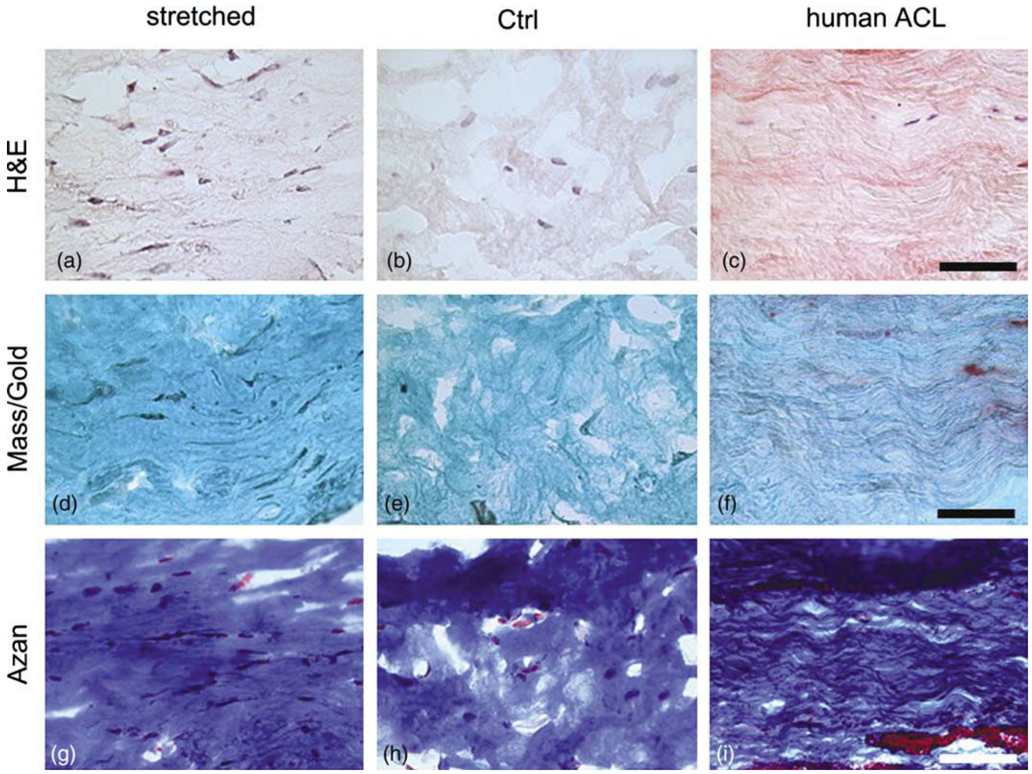

***Figure 3**. Histochemical analysis of stretched ACL constructs (stretched) after 14 days compared with non-stretched controls (Ctrl) and a human ACL (human ACL). (a–c) H&E staining, (d–f) Masson-Goldner staining and (g–i) Azan staining. Bars = 100 μm.*

fibronectin in stretched constructs compared with non-stretched controls (Figure 5). No difference was found for vimentin expression.

Discussion

In this study, ACL constructs have been fabricated *in vitro* from MSC and a collagen type I hydrogel, which is in clinical use for autologous chondrocyte transplantation and has been optimized for clinical application as a cell carrier material. A collagen type I hydrogel has been selected as a carrier material because the human ACL consists predominately of collagen type I [13].

Various research groups have shown that cells seeded in collagen gels can degrade and re-organize the surrounding extracellular matrix and adopt a specific orientation in a contracted collagen network as a function of culture condition and time [14,15]. For the fabrication of collagen gel-based ACL constructs, the concentration of collagen and the amount of cells used is of critical importance to achieve an optimum of primary stability and controlled gel contraction. Goulet *et al.* [16] have used a final collagen concentration of 1.5 mg/mL and a final concentration of 1×10^6 fibroblasts for their ACL constructs. We used a collagen concentration of 3.0 mg/mL for more stability and a cell concentration of 5×10^5 MSC for less gel contraction. Also, Goulet *et al.* [16] used ACL fibroblasts, which might have different effects on the stability and contraction of the collagen hydrogel than MSC.

The normal human ACL contains rows of fibroblasts within parallel bundles of extracellular matrix composed primarily of collagen type I fibers with a small proportion of collagen type III fibers, which separate the collagen type I bundles. Elastin, a fibrillar protein that affects the mechanical properties, is present only in very small amounts (less than 1% of dry weight). Cell cultures of human ACL fibroblasts express fibronectin, elastin, vimentin and collagen types I and III, as shown by immunohistochemisrty [16]. Besides staining with H&E, Masson-Goldner and Azan, these markers of ligamentous tissue and ACL fibroblasts have been used in our study for immunohistochemical and RT-PCR analysis.

Several investigators have shown that mechanical stimulation of collagen gels such as cyclic stretching can

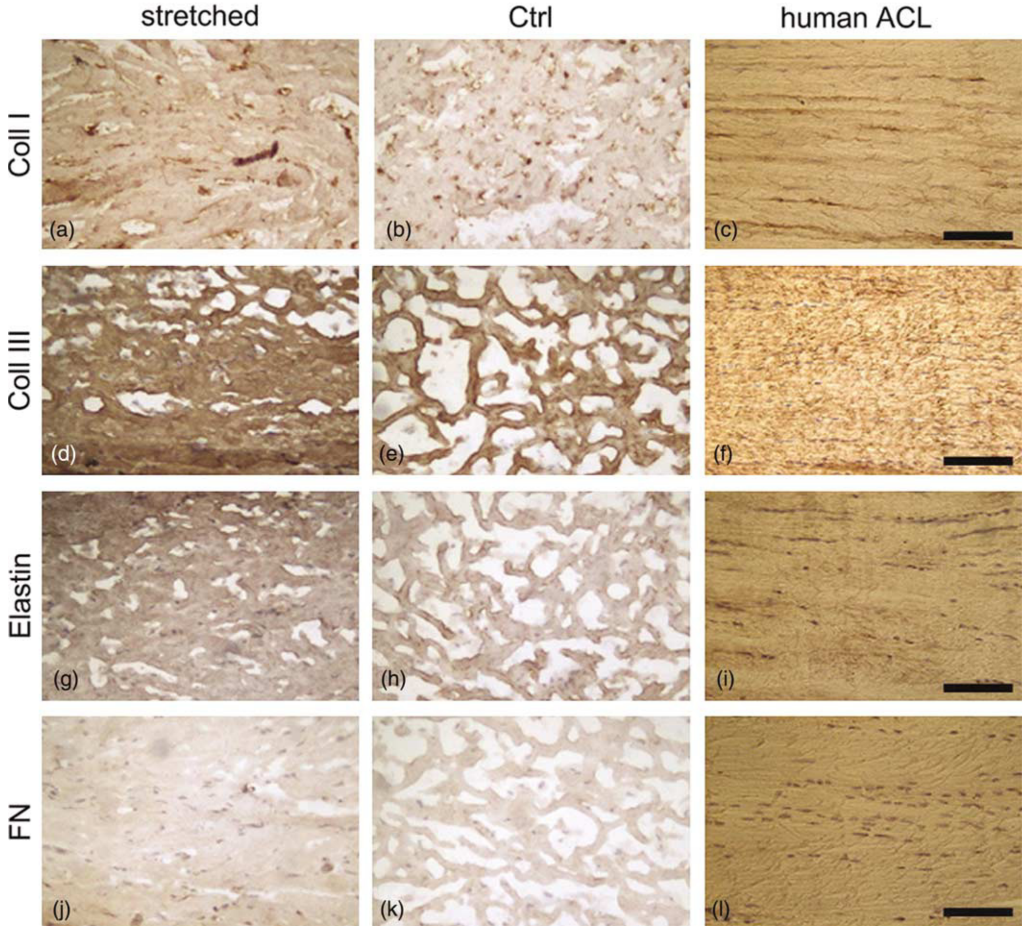

Figure 4. Immunohistochemical analysis of stretched ACL constructs (stretched) after 14 days compared with non-stretched controls (Ctrl) and a human ACL (human ACL). (a– c) Staining for collagen type I (Coll I), (d– f) staining for collagen type III (Coll III), (g– i) staining for elastin and (j– l) staining for fibronectin (FN). Bars = 100 μm.

lead to orientation of the collagen fibers and the embedded cells [12,17,18]. As already known for human ACL fibroblasts [12], our data suggest that MSC repeatedly adopted a defined longitudinal orientation and organized the surrounding matrix in parallel, highly organized fibers in a wavy pattern with bundles of extracellular matrix when submitted to cyclic stretching, as shown by H&E, Masson-Goldner and Azan staining. This organization was particularly evident at the periphery of the gel close to the bone plugs, while the middle part of the gel showed much less wave-like collagen matrix organization. One reason for this observation might be a much higher stress occurring at the transition zone between the collagen gel and the bone plugs. This explanation is strengthened by the phenomenon that freshly molded constructs, as well as constructs that have been exposed to high stretching forces, break near the transition zone. How this transition zone really looks has to be investigated by histologic analysis of the bone–collagen gel connection in the future.

Immunohistochemical and RT-PCR analyses revealed that MSC showed an increased expression and synthesis of fibronectin and elastin, as well as collagen types I and III,

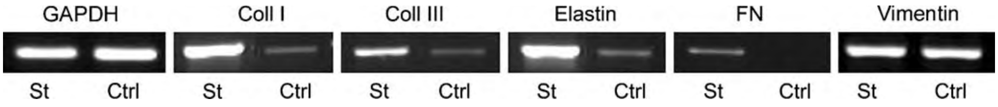

Figure 5. RT-PCR analysis of ligament-specific marker gene expression of constructs stretched for 14 days (St) compared with non-stretched controls (Ctrl). Coll I, collagen type I; Coll III, collagen type III; FN, fibronectin.

when submitted to cyclic stretching, compared with the non-stretched control group. Immunohistochemical analyzes of normal human ACL revealed the presence of the tested molecular markers *in vivo*. Thus, we speculate that cyclic stretching induced a complex structural organization of the extracellular matrix in the bioengineered ligament-like constructs made from MSC *in vitro*. The cell density appeared to be slightly higher in the stretched compared with the non-stretched constructs, probably because of more contraction of the gel, as a result of the more highly organized extracellular matrix. The cell density appeared not to be different between the stretched and the control group in the middle of the collagen gel.

The lack of strength of the construct is currently its major limitation as an eventual ACL prosthesis. Nevertheless, collagen type I scaffolds or gels provide the basic protein needed in the field of ACL engineering. Dunn *et al.* [19,20] reported the successful replacement of ACL by 200 cross-linked cell-free collagen fibers in the rabbit, with a tensile strength similar to those of the normal human ACL. Such cross-linked collagen type I fibers seeded with MSC and embedded in a collagen type I hydrogel might be a promising approach for future ACL prostheses and are currently being investigated in our group. Other scaffold materials for cell-based ACL engineering, such as fibronectin-coated braided poly-alpha-hxdroxyesters, biodegradable PLA/PGA materials and different silk fibers, have been described recently as potential cell carrier materials [6,9,21–24] but there is still a lot of work to do to define an optimal carrier material for a successful cell-based reconstruction of the ACL. Also, the application of growth factors to promote ACL healing by bridging the gap at the site of an ACL rupture *in vivo* or inducing ligamentous differentiation of an *in vitro* fabricated ACL construct from MSC has to be taken into consideration [25,26].

In conclusion, we have shown that it is possible to fabricate an ACL construct from MSC and a collagen type I hydrogel *in vitro*. Furthermore, cyclic stretching led to a highly organized extracellular matrix in a wavy pattern and induced protein and gene expression of ligament-specific markers. The fabricated constructs are still too weak for *in vivo* implantation and we are currently developing strategies to fabricate more stable and vascularized constructs by the incorporation of collagenous or synthetic fibers and coating the constructs with angiogenic progenitor cells. The presented data strongly suggest that MSC embedded in collagen type I hydrogels can respond to mechanical stimuli *in vitro*. The results are promising for the development of ligament-like constructs with improved mechanical properties by the application of cyclic stretching *in vitro*. They also give hope for functionally engineered ACL analogs made from autologous MSC in the future.

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