

Tissue engineering of tendons and ligaments by human bone marrow stromal cells in a liquid fibrin matrix in immunodeficient rats: Results of a histologic study

Stefan Hankemeier · Martijn van Griensven · Marco Ezechieli ·
Tanja Barkhausen · Matthew Austin · Michael Jagodzinski ·
Rupert Meller · Ulrich Bosch · Christian Krettek · Johannes Zeichen

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Abstract

Introduction The original complex structure and mechanical properties are not fully restored after ligament and tendon injuries. Due to their high proliferation rate and differentiation potential, Bone Marrow Stromal Cells (BMSC) are considered to be an ideal cell source for tissue engineering to optimize the healing process. Ideal matrices for tissue engineering of ligaments and tendons should allow for homogenous cell seeding and offer sufficient stability.

Material and methods A mixture of human BMSC and liquid fibrin glue was injected into a standardized full-thickness window defect of the patellar tendon of immunodeficient rats (BMSC group). The histology of the tissue was analysed 10 and 20 days postoperatively and compared to four control groups. These groups consisted of a cohort with a mixture of human fibroblasts and fibrin glue, fibrin glue without cells, a defect group without treatment, and a group with uninjured patellar tendon tissue.

Results Tendon defects in the BMSC group revealed dense collagen fibres and spindle-shaped cells, which were mainly orientated along the loading axis. Histologic sections of the control groups, especially of untreated defects and of defects filled with fibrin glue only, showed irregular patterns of cell distribution, irregular formed cell nucleoli and less tissue maturation. Compared to healthy tendon tissue, higher numbers of cells and less intense matrix staining was observed in the BMSC group. No ectopic bone or cartilage formation was observed in any specimen.

Conclusions Injection of human BMSC in a fibrin glue matrix appears to lead to more mature tissue formation with more regular patterns of cell distribution. Advantages of this “in-vivo” tissue engineering approach are a homogenous cell-matrix mixture in a well-known and approved biological matrix, and simple, minimally-invasive application by injection.

Keywords BMSC · Tissue engineering · Tendon · Ligament · Fibrin glue · Histology · Healing

S. Hankemeier (✉) · M. Ezechieli · T. Barkhausen ·
M. Jagodzinski · R. Meller · C. Krettek · J. Zeichen
Trauma Department, Hanover Medical School (MHH),
Carl-Neuberg-Str. 1, 30625 Hanover, Germany
e-mail: hankemeier.stefan@mh-hannover.de

M. van Griensven
Ludwig Boltzmann Institute, Research Centre for Traumatology,
Vienna, Austria

M. Austin
Rothman Institute, Orthopaedic Surgery, Philadelphia,
PA, USA

U. Bosch
Orthopaedic Department, International Neuroscience Institute,
Hanover, Germany

Introduction

Ligament and tendon tears are among the most common injuries seen by the orthopaedic surgeon. However, the biological and biomechanical properties of ligaments and tendons are incompletely restored after trauma. Histologic studies of healing tissue have shown higher cell numbers and less parallel collagen fibre organisation [33, 34]. These altered structural properties may contribute to reduced joint stability, degenerative changes, inferior function, and increased risk of re-rupture [6, 24, 31, 33, 36]. Therefore, innovative treatment options to improve ligament and tendon healing are of great interest.

Tissue engineering is a complex process involving the acquisition and cultivation of adequate cells, growth-inducing stimuli, and scaffolds supporting tissue formation. In many laboratory and clinical studies bone marrow stromal cells (BMSC) have been used to stimulate tissue healing. BMSC are undifferentiated, pluripotent cells of the bone marrow which offer high proliferation potential, differentiate into different cell lineages, and are capable of producing mesenchymal tissues such as bone, cartilage, fat, muscle, ligament, and tendon [1, 11, 20, 22, 26, 32, 37, 43]. BMSC can be isolated from bone marrow aspirates and cultured in-vitro under tissue-specific conditions. Long-term vitality of autologous and allogeneous BMSC in-vivo have been demonstrated by Ouyang et al. [30] and Awad et al. [2]. However, it is still unknown whether BMSC represent the ideal cell source for the tissue engineering of ligaments and tendons. Alternatively, fibroblasts might be useful, however their potential in the tissue engineering of ligaments and tendons has not been elucidated yet.

In several areas of medicine such as cardiac surgery, plastic surgery, urology, and abdominal surgery the indications for tissue engineering have arisen [13, 21, 22, 29, 39]. In orthopaedic surgery, long bone defects have been reconstructed with the help of tissue engineering [35]. Cartilage defects have been successfully treated by autologous chondrocyte transplantation to form “hyaline-like” cartilage [7]. In regards to ligaments and tendon injuries, clinical application of tissue engineering approaches can be expected in the future. In addition to potential stimulation of the healing process, tissue engineering might offer an opportunity to reduce donor-site morbidity.

Whereas histologic analysis of uninjured ligaments and tendons demonstrate homogenous distribution of cells between dense collagen fibres, homogenous seeding of dense matrices with sufficient biomechanical properties remains to be challenging, since cells are distributed mainly on the surface of the matrix [3, 9, 12, 18, 40]. Cartmell et al. [12] demonstrated an average maximum penetration depth of 74 μm of allogeneous fibroblasts 14 days after seeding rabbit ligaments. If the average radius of rabbit anterior cruciate ligament is 1.2 mm, a maximum cell penetration depth of 6% of the ligament can be calculated [42]. Considering the larger dimensions of human ligaments and tendons, one can assume a much lower relative penetration depth of cells.

In contrast, with liquid matrices like fibrin glue or collagen gels, homogenous cell-matrix constructs can be produced [2, 24]. However, some studies have shown that constructs of collagen gel and cells provide only 0.5% of the material properties of healthy structures [2, 10, 17, 18].

The use of fibrin glue as a matrix could offer several advantages for the tissue engineering of ligaments and tendons. The liquid matrix allows for a homogenous mix-

ture for injection into the injury site. Several studies demonstrated long term vitality and proliferation of BMSC in fibrin glue in vivo [30] and of BMSC, fibroblasts, and osteoblasts in vitro [5, 23, 41]. The application mode is user-friendly and can be done in a minimally-invasive fashion. Fibrin glue is bioresorbable and long-term clinical results are available [38]. Some studies have shown a stimulative effect on tendon healing [13, 25]. However, there remains a very low risk of disease transmission, since fibrin glue is produced from pooled human plasma.

The aim of this animal study was to analyse whether a homogenous mixture of human BMSC and fibrin glue improves the healing process in a standardised patellar tendon window defect when compared to untreated defects, defect filled with fibrin glue alone, treatment with a mixture of fibroblasts plus fibrin glue, and uninjured patellar tendons. The hypothesis was that treatment of the tendon window defect with an injection of BMSC and fibrin glue leads to more mature tissue formation 10 and 20 days postoperatively. Another aim was to analyse the range of motion of the knee joint treated by BMSC and fibrin glue injection in comparison to the uninjured contralateral side. Furthermore, one aim of the study was to analyse, whether treatment of patellar tendon defects with BMSC leads to ectopic bone or cartilage formation, as described by Awad et al. [2] and Dressler et al. [15].

Material and methods

Isolation and cultivation of human BMSC

Bone marrow aspirates from the iliac crest were collected from six donors undergoing dorsal instrumentation and fusion because of vertebral fractures. The donors were otherwise healthy and their age ranged from 23 to 51 years. All procedures were approved by the institutional ethics committee, and informed consent was obtained from all donors.

The bone marrow aspirates were washed in Dulbecco's Modified Eagle's Medium (DMEM)–Ham's F12 (1:1) (Biochrom, Berlin, Germany) supplemented with 10% human serum (institutional blood bank), penicillin–streptomycin (200 U/ml; GIBCO, Karlsruhe, Germany), amphotericin B (2.5 $\mu\text{g}/\text{ml}$; Biochrom), buffered with HEPES buffer (pH 7.0; Roth, Karlsruhe, Germany), and centrifuged for 5 min at 2,000g. Each cell pellet was then centrifuged over a Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) for 15 min at 2,000g. Supernatants were resuspended in the medium mentioned above and centrifuged. After calculating cell density, the obtained cell pellets were plated in 75 cm^2 culture flasks (Nunc, Berlin, Germany) at 2×10^5 cells per flask, and incubated at 37°C

and 5% CO₂ in humidified atmosphere. The medium was changed three times per week. After reaching confluence on days 14–21, the cells were harvested with 0.25% trypsin (GIBCO, Karlsruhe, Germany), counted, and subcultured in 75 cm² cell culture flasks. Cells of the third passage were used for the experiments.

Isolation and cultivation of human fibroblasts

Tendon samples were taken from healthy human patellar tendons during reconstruction of the anterior cruciate ligament. All patients ($n = 7$, age 21–40 years) had sustained a traumatic tear of the anterior cruciate ligament. Only excess material of the trimmed patellar tendon graft was used. All procedures were approved by the institutional ethics committee, and informed consent was obtained from all donors. The tendon samples were cut into 1 mm² pieces with a sterile scalpel.

The samples were cultivated in DMEM plus 10% fetal calf serum (Biochrom, Berlin, Germany), 2.5 µg/ml Amphotericin B, 200 U/ml Penicillin/Streptomycin and HEPES buffer (pH 7.0) and incubated in silicon dishes (Nunc, Berlin, Germany) at 37°C and 5% CO₂ in humidified atmosphere. The medium was changed three times per week. After reaching confluence on days 14–21, the cells were harvested with 0.25% trypsin, counted, and 150,000 cells subcultured in 75 cm² cell culture flasks. Cells of the third and fourth passage were used for the experiments [44].

Operative procedure

Forty-eight immunodeficient male Lewis RNU-rnu rats with a body weight of 191–261 g were used for the experimental study. All operations and procedures were approved by the district veterinary administration and complied with the Animal Protection Act of Germany.

After the animals were anesthetised, the soft tissue over the right patellar tendon was dissected via a ventral longitudinal incision. A custom-made metal template was placed over the central portion of the patellar tendon. Using the template, two parallel longitudinal incisions with a distance of 1.4 mm were made at the central portion of the patellar tendon. The central tissue between the incisions was carefully excised from the distal pole of the patella to the insertion of the tibial tuberosity. Thus, standardized full-thickness, full-length defects of the patellar tendons were created.

The rats were randomly divided into four groups. In the defect group, neither cells nor fibrin glue was injected. In the fibrin glue group, 5 µl of the thrombin and fibrin component were injected into the defect without cells. In the fibroblast group and in the BMSC group, 10⁵ fibroblasts

or 10⁵ BMSC were mixed with 5 µl of the thrombin component of the fibrin glue (Tissu Duo-s, Baxter, Heidelberg, Germany). The mixture was injected into the defect with 5 µl of the fibrin component. Throughout the whole postoperative course the rats moved around freely without any restrictions.

Clinical examination

The animals were killed by an intraperitoneal injection of Eutha77 (Pentobarbital, Essex, Muenchen, Germany) at either 10 or 20 days postoperatively, according to the protocol. The timing of tissue removal was chosen according to data of other rat studies analysing healing of mesenchymal tissue [4, 19, 27]. The range of motion of the treated knee joint was measured with a goniometer and compared to the uninjured contralateral knee joint.

The template was placed over the patellar tendon and the tissue samples taken from the mid-portion of the former defect. The mid-portion of six contralateral patellar tendons of the uninjured side served as additional controls. Thus, 54 tissue samples were available for histology.

Histology

After fixation in 4% neutral buffered formalin, the tissue samples were processed through a gradient of alcohols and embedded in paraffin blocks. The tendon portions were cut at regular intervals into 8 µm thick longitudinal sections spanning the thickness and length of the tendon. These were then stained with hematoxylin and eosin. The central section was chosen for each tendon sample for histologic analysis. Using a light microscope (Zeiss, Jena, Germany), the results were compared between the different treatment groups under 63×, 100× and 200× magnification. Histologic sections were examined for the morphology and number of cells, amount and orientation of collagen fibres, and extracellular matrix staining.

Results

Clinical examination

On the day of surgery the activity of the animals was reduced. The animals did not display any difference in activity from preoperative levels after the day of surgery. All animals were allowed unrestricted range-of-motion of the knee joint. Macroscopically, no signs of adhesions or inflammation were noted. After sacrifice on day 10 or 20, the range of motion of the operated knee joint was free and revealed no differences compared to the uninjured contralateral knee joint.

Histology

10 days postoperative

The defect group and the fibrin glue group showed similar histologic results. In both groups, irregular patterns of cell distribution and irregular formed cell nucleoli were noted. The matrix was moderately stained and the collagen fibres were irregularly oriented (Fig. 1a, b).

Histologic sections in the fibroblast group showed that the cells were mainly spindle-shaped and more orientated along the collagen fibres. These findings were in contrast to the defect group and fibrin glue group (Fig. 1c).

Cells in window defects treated by BMSC and fibrin glue revealed a more homogenous, spindle-shaped morphology. Collagen fibres were clearly seen, and the healing tissue appeared to be more organised. The cells were mainly arranged along the collagen fibres (Fig. 1d).

Sections of uninjured patellar tendon tissue on the contralateral side were dominated by strictly parallel, dense, wavy collagen fibres. Only a few fibroblasts were noted, which were orientated between thick collagen fibre bundles (Fig. 1e).

20 days postoperative

20 days after surgery the histologic sections in the defect group and fibrin glue group still revealed high number of cells, which now developed a more spindle-shaped morphology. Collagen fibres were visible, which were partially orientated parallel to the cells (Fig. 2a, b).

In the fibroblast group, spindle-shaped cells were most abundant, which were mainly orientated parallel to the collagen fibres and surrounded by matrix. Compared to the uninjured patellar tendon tissue, cell density appeared to be higher and the matrix seemed to be less stained (Fig. 2c).

Histologic sections in the BMSC group demonstrated more mature tissue dominated by dense collagen fibre bundles. Spindle-shaped cells were orientated parallel to the collagen fibres. The matrix was stained homogenously and the number of cells was lower when compared to the defect group, fibrin glue group, and fibroblast group. In contrast to normal patellar tendon tissue, matrix staining was less intense and cell density was higher (Fig. 2d).

None of the specimens including those of the BMSC group developed ectopic tissue formation (bone or cartilage) in the patellar tendon window defect.

Fig. 1 a–e H&E staining 10 days after operation, $\times 63$ magnification

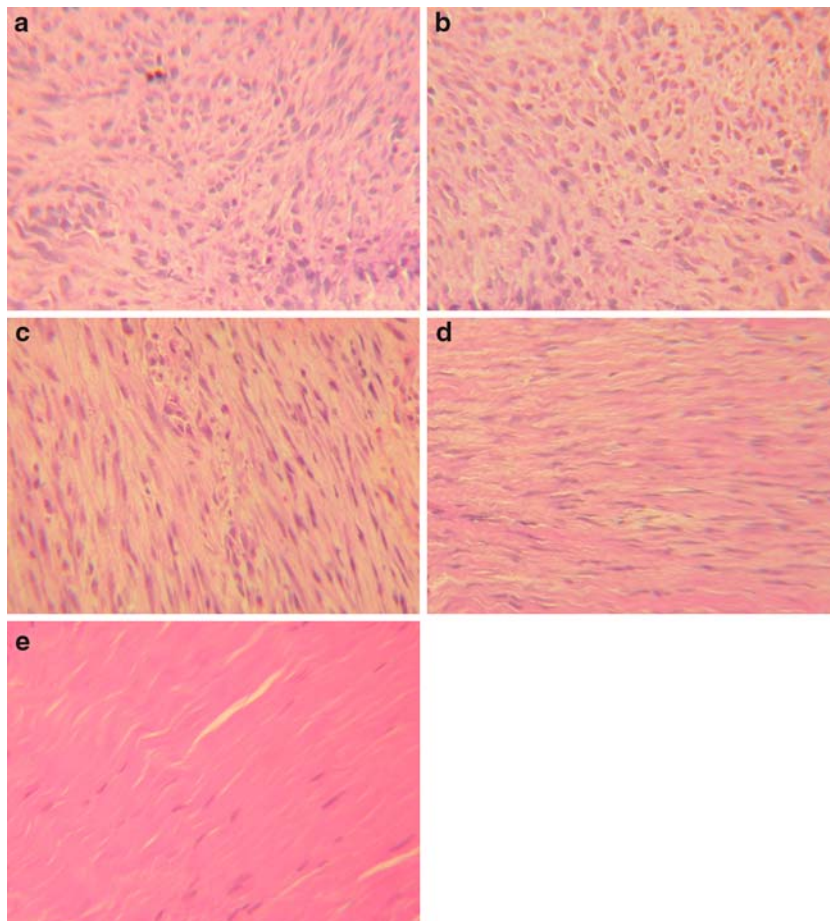
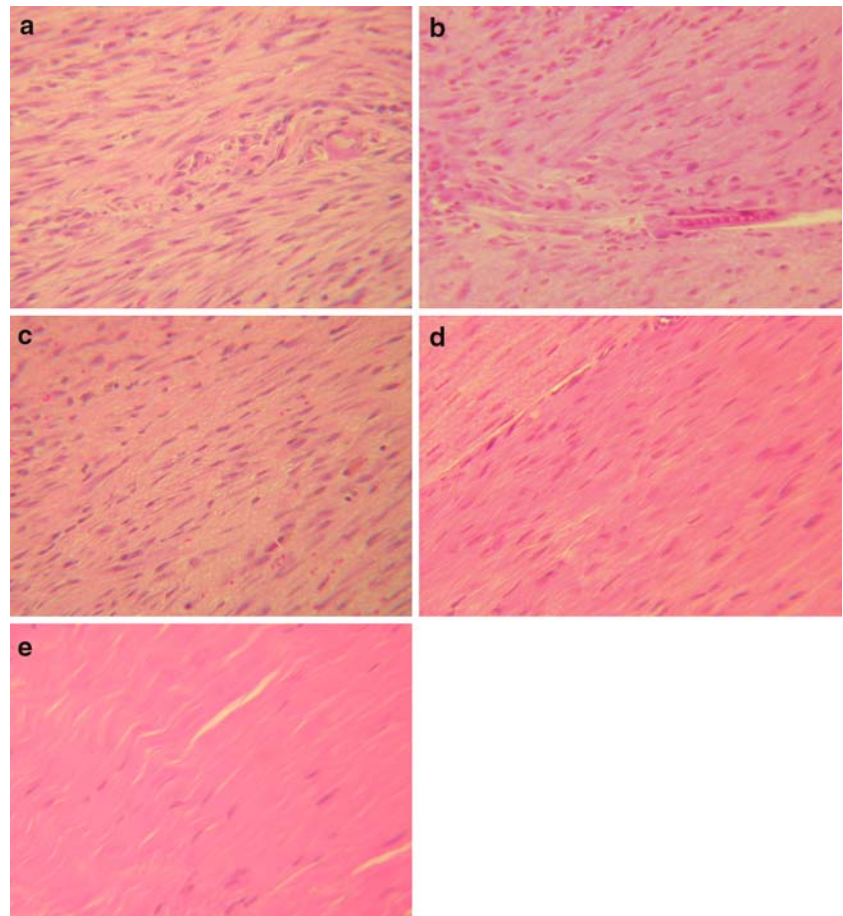


Fig. 2 a–e H&E staining
20 days after operation, $\times 63$
magnification



Discussion

This animal study analyses the histologic results of an injection of BMSC in a liquid fibrin glue matrix in a partial patellar tendon defect, and compares the observations to treatment groups with fibroblasts with fibrin glue, treatment with fibrin glue only, and a defect group. To the best of our knowledge, no studies have investigated the effect of a mixture of BMSC and liquid fibrin glue on the healing process of tendon injuries so far. Furthermore, no studies have compared the treatment with BMSC or fibroblasts in an animal study yet. One main concern of the treatment of tendon and ligament injuries with BMSC is ectopic tissue formation after local treatment with BMSC [4, 15].

In our study, partial patellar window defects treated with a mixture of BMSC and liquid fibrin glue appeared to reveal more mature tissue formation with denser matrix staining than in untreated defects or defects filled with fibrin glue only. The cells had a more spindle-shape morphology and were mainly uniformly orientated. Tissue healing appeared to be more advanced in the BMSC group than in the fibroblast group.

The results of our study are in keeping with those of other authors, who found a higher cellularity, relatively irregular shape of the cells, less matrix staining and less organization in untreated tendon injuries or defects [8, 14, 16, 24, 33]. Young et al. [43] suspended BMSC in a collagen gel, contracted the cell-gel composite on a pre-tensioned suture and implanted the resulting tissue into a 1 cm-long gap defect in the rabbit Achilles tendon. Collagen fibres appeared to be better aligned than those in the untreated controls with a more mature tissue structure and more spindle-shaped cells. Furthermore, the material properties of the group treated with BMSC and collagen gel were significantly increased. In our study, the differences between the defect group or fibrin glue group and the BMSC group differences concerning cell morphology, cell density and collagen fibre orientation were observed. In comparison to collagen gel, better initial stability, well-known long-term results and the use of a human biological matrix seem to be beneficial.

One main concern of injection of undifferentiated BMSC into injured tendons and ligaments is undesired tissue differentiation. In the studies of Awad et al. [2] and Dressler et al. [15], ectopic bone formation was noted after

implantation of a collagen gel with BMSC in patellar tendon defects. In contrast, cell differentiation into osteocytes or chondrocytes and ectopic tissue formation was not detected in any histologic specimen of our study. Different cell culture conditions in the studies of Awad et al. and Dressler et al. are one possible explanation [11, 20, 28, 32]. Furthermore, Awad et al. and Dressler et al. created bony defects in the patella and tibial tuberosity with an oscillating saw and some scattered bone tissue might have induced bone formation. Dressler et al. [15] discussed whether marked contraction of the collagen gel in vitro before implantation might have influenced tissue differentiation.

Decreased joint mobility by adhesions is another concern when fibrin glue is injected into injured tendon of ligament tissue. However, in this study clinical post-mortem examinations did not provide any restrictions in comparison to the uninjured side.

Advantages of the cell-matrix construct of this study are homogenous cell seeding, minimally-invasive application, simple production, and the use of a human, biological matrix with well known long-term effects. Although histologic studies do not allow for quantitative statements, they may provide at least good overall impressions of the healing process. This animal model can be used for future biomechanical and ultrastructural studies [2, 15]. Biomechanical studies will have to prove, if the histologic observations of more mature tissue formation correspond with improvement of biomechanical characteristics, e.g. higher elastic modulus and ultimate load.

This animal study shows a new therapeutic approach for the treatment of tendon and ligament injuries and defects. According to the first histologic results, the complex process of tendon and ligament healing seems to be improved by the injection of BMSC in liquid fibrin glue. However, the histologic results have only a descriptive character, and further investigations have to quantify the biologic and biomechanical effects of BMSC/fibrin glue injection.

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