Influence of cyclical mechanical loading on osteogenic markers in an osteoblast–fibroblast co-culture in vitro: tendon-to-bone interface in anterior cruciate ligament reconstruction

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Abstract

Purpose We aimed to evaluate the influence of cyclical mechanical loading on osteoblasts and fibroblasts, and co-cultures of both in vitro, simulating the conditions of the tendon-to-bone interface in anterior cruciate ligament reconstruction.

Methods Osteoblast-like cells (OBL) and tendon-derived rodent fibroblasts (TDF) were cultured alone or in co-culture to simulate the tendon-to-bone interface. Cyclical loading was applied for one hour twice a day for three days, with a frequency of 1 Hz and 3 % strain. Alkaline phosphatase (AP), osteocalcin (OC), collagen type 1 (COL1A1), and bone morphogenetic protein 2 (BMP-2) gene expression and protein deposition were detected by real-time polymerase chain reaction (qPCR) and immunocytochemical analysis.

Results Mechanical loading significantly decreased AP, OC, and COL1A1 gene expression in both OBL and TDF,

compared to non-loaded culture. However, mechanical load increased gene expression of the same marker genes including BMP-2 during co-culture. Immunocytochemistry demonstrated increased deposition of corresponding proteins in the same range, independent of culture conditions. Higher depositions of BMP-2 were shown under loading conditions for osteoblast and TDF monocultures. Prolongation of mechanical loading resulted in cell detachment and spheroid formation.

Conclusion Cyclical mechanical loading caused downregulation of genes involved in osteointegration and osteoinduction, such as OC, ALP, and COL1A1 in monocultures of osteoblasts and fibroblasts; co-cultures lacked this phenomenon. Immunocytochemistry and qPCR analysis showed slight upregulations of marker genes and corresponding proteins. This might be due to the potential stabilising effects of osteoblast-fibroblast cross talk in the co-culture environment, simulating fibrocartilage formation at the tendon-to-bone interface.

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Integration

Introduction

Injuries to the anterior cruciate ligament (ACL) are quite common in most impact sports. More than 100,000 ACL reconstructions are performed in the United States yearly with rising trend since the mid 1990s [1]. Modern techniques of arthroscopic reconstruction and individualised postoperative rehabilitation programs are essential for therapeutic success. However, there is no clear consensus among clinicians [2, 3].

Up to 10 % of patients require operative revision after primary ACL reconstruction due to graft failure with resulting joint instability [4, 5]. Therefore, the success of ACL reconstruction hinges on initial graft fixation and biological integration. Firm attachment, proper graft remodelling, and incorporation at the tendon-to-bone interface is vital to the quality of newly reconstructed ACLs [6, 7].

Recent research has focused on integration of common ACL grafts. In experimental animal models and clinical studies, integration of the fibrovascular interface and bone tunnel remodelling have been shown to promote the progressive attachment of the tendon graft to its surrounding bony tunnel [8, 9]. The success of ACL reconstruction depends on initial graft fixation and subsequent positive biological integration [10]. Based on current literature growth factors, especially the BMPs are important in this process by acting at the tendon-to-bone interface, thus promoting healing and improvement of mechanical strength [11]. The attachment of the native ACL insertion point to the subchondral bone is achieved by a unique fibrocartilage interface, characterised by a defined spatial distribution of different cell types within the extracellular matrix [8]. Successful graft incorporation of the newly formed tendon insertion must be completed after reconstruction to provide tensile strength comparable with that of native ACL insertions [12, 13].

The native tendon-to-bone interface is composed of three distinct tissue types: cortical bone, tendon, and fibrocartilage. Fibrocartilage is further divided into non-mineralised and mineralised zones. The native ACL tendon matrix consists of types I and III collagen with fibroblasts in between layers. Non-mineralised fibrocartilage has a region rich in ovoid chondrocytes, surrounded by types I and II collagen, within a proteoglycan-rich matrix. Mineralised fibrocartilage is rich in hypertrophic chondrocytes, surrounded by a type X collagen matrix that carries hydroxyapatite. Subchondral bone consists of osteoblasts, osteocytes, and osteoclasts, embedded in a matrix primarily composed of type I collagen [8]. It is important to understand that ACL reconstruction does not generate a native tendon-to-bone interface, nor does it re-establish the anatomical insertion, even if performed according to the latest available techniques. Fibrocartilage-like regenerative tissue can fill the bony tunnel and might bridge the free space between tendon graft and cancellous bone. Other authors argue that the type of bone-to-tendon healing seems to depend on the fixation technique and device [14]. Interactions between tendon and bone-derived cells (e.g. fibroblasts and osteoblasts) play an important role in formation of this fibrocartilage regenerative tissue, by initiating trans-differentiation via cross talk [10, 15]. However, the distinct mechanism of this interface formation, and the impact of mechanical loading on tendon-to-bone incorporation remains unclear.

Mechanical loading substantially influences cell proliferation and differentiation *in vitro* and also induces growth factors

that direct tissue formation *in vivo* [16, 17]. In the current literature, cyclical mechanical loading of osteoblast and fibroblast cell lines may induce biological responses, including remodelling of the actin cytoskeleton, changes in cell proliferation, differentiation, gene expression (bone morphogenetic proteins, alkaline phosphatase), and protein biosynthesis [18, 19]. However, these mechanisms have not yet been investigated in co-culture models of osteoblasts and fibroblasts, simulating conditions after common tendon repair (e.g. ACL reconstruction).

We aimed to evaluate the influence of cyclical mechanical loading on osteoblast-like cells (OBL), fibroblasts, and co-cultures of both “*in vitro*”, simulating the tendon-to-bone interface after ACL reconstruction. We postulated that mechanical loading leads to a higher expression of osteointegration and osteoinduction marker genes and their corresponding proteins, such as bone morphogenetic protein 2 (BMP-2), osteocalcin (OC), alkaline phosphatase (AP), and collagen type 1 (COL1A1). The benefits of osteoblast and fibroblast co-culturing gives evidence of superior graft incorporation. We anticipate that our findings will provide new insight into the mechanism of fibrocartilage formation.

Methods

Osteoblast-like cells and tendon derived fibroblast (TDF) isolation and culture

All cells were obtained from three ten-week-old male Sprague–Dawley rat cadavers. Femora and tibiae were harvested under aseptic conditions. The metaphyses were removed and the remaining long bone was flushed with NaCl 10 % via the medulla several times to obtain osteoblast-like cells from the bone marrow. Suspensions were centrifuged for ten minutes at 10,000 rpm, the supernatant removed, and pellets resuspended with growth medium DMEM (Biochrom AG, Berlin, Germany).

Tendon derived fibroblasts were isolated from rat tails, cut into small pieces, and digested with collagenase (0.3 % CLS II) in phosphate buffered saline (PBS) for two hours at 37 °C. The remaining tendon material was sieved and cells were transferred for culture.

Within this setup three different cultures of each group (1, osteoblast non-loading; 2, osteoblast loading; 3, fibroblast non-loading; 4, fibroblast loading; 5, co-culture non-loading; 6, co-culture loading) were cultivated over a period of three days. Potential effects of loading and non-loading conditions onto the process of tendon-to-bone integration were evaluated by generation of these subgroups. Cells were seeded in standard culture flasks (75 cm²) in DMEM, with low glucose and glutamine (Biochrom AG, Berlin, Germany), supplemented with foetal calf serum (10 % FCS) and penicillin/streptomycin (1 % pen/strep). After reaching

subconfluency, cells were split at a ratio of 1:3. For experiments, cells were used after passage three.

Cyclical loading of cell culture

Experiments were performed using a Flex Cell 3000 apparatus (Flexcell, Hillsborough, USA). Cells were seeded onto a special collagen-coated six-well plate and cultured to subconfluency prior to mechanical loading. Cyclical loading was performed for one hour, twice a day for three days, with a frequency of 1 Hz and 3 % strain at 37 °C and 5 % CO₂. To increase detectable differences in protein deposition, loading conditions were altered. Continuous, instead of cyclical, loading was applied for one or three days with the same frequency and strain as before.

Immunocytochemical staining

After three days, the culture media were removed and cell culture slips were fixed with 4 % buffered formalin, and washed with PBS 1 % for immunocytochemical staining. Cell culture slips were blocked with normal horse serum (Santa Cruz, Heidelberg, Germany) and incubated overnight with polyclonal IgG antibodies against either OC, diluted 1:100 (FL-100), BMP-2, diluted 1:100 (N-14), AP, diluted 1:100 (H-300) or COL1A1 (D-13). They were then incubated with a biotinylated secondary antibody diluted 1:50 for 30 minutes at room temperature. An avidin-biotin-complex detection system coupled with DAB as a chromogen was used to visualise antibody binding after ten minutes of incubation time, also at room temperature. Finally, all slips were counterstained with Gill's haematoxylin solution, diluted 1:2 for ten seconds (all reagents from Santa Cruz, Heidelberg, Germany). Negative controls, incubated without primary antibody, were treated accordingly.

Immunocytochemical and morphometric cell analysis

Morphometric analysis was performed with transmitted light at ten-fold magnification using a digital microscope DM5000B (Leica Microsystems, Bensheim, Germany) and QUIPS analysis software (Leica Microsystems, Bensheim, Germany). Color detection was performed in five fields (ROI) per specimen using the respective module of the QUIPS package to visualise the DAB stained area in relation to the total area of the ROIs for each specimen.

Polymerase chain reaction

Ribonucleic acid was extracted from cell layers after three days of cyclical loading or non-loading using Peq Gold total RNA Kit (PeqLab, Erlangen, Germany), according to the manufacturer's instructions, and quantified spectrometrically. Then, 18 s was set as the housekeeping gene. Starting with

1 ng RNA, 20 µl of cDNA were synthesised using a Verso cDNA Kit (Thermo Fisher Scientific, Lafayette, USA) with oligo-dT primer in the presence of dNTP. qPCR reactions were performed and monitored using a Mastercycler® ep Realplex Detection System (Eppendorf, Hamburg, Germany) and Absolute Blue qPCR Sybr Green Mix (Thermo Fisher Scientific, Lafayette, USA). Genes of interest were analysed using CyberGreen and the DeltaDeltaCt method. Primers (TIB Biomol, Berlin, Germany) used included AP, COL-I, OC, BMP-2, and 18 s rRNA.

Statistical analysis

The Mann–Whitney U-tests were used to evaluate differences between groups with or without cyclical loading using SPSS version 20.0 (IBM, Chicago, USA). Data were expressed as the median with according range. A p-value of 0.05 was considered to be statistically significant.

Results

This co-culture model was designed to analyse the in vitro conditions of soft tissue graft placed directly adjacent to bone tissue within the bone tunnel. This biomimetic model permits both physical contact and paracrine interaction between both cell types under cyclical mechanical loading.

Immunocytochemistry and morphometric cell analysis

As shown in Table 1, proteins of corresponding marker genes were detected in the same range, independent of culture conditions. Even higher depositions of BMP-2 could be measured under loading conditions for osteoblast and TDF monocultures. As shown in Fig. 1, detachment of some cells as well as early spheroid formation was also noted under cyclic loading conditions.

Continuous, instead of cyclical, loading resulted in significant detachment of cells, as shown in Fig. 2. However, the remaining cells were insufficient for further analysis (Fig. 2).

Polymerase chain reaction

As shown in Table 2, mechanical loading decreases gene expression in both OBL and TDF as compared to non-loaded culture. In contrast, mechanical loading increases the same gene expression during co-culture.

Discussion

The overall success of ACL reconstruction depends on initial graft fixation and subsequent biological integration, proper

Table 1 Relative antibody positive area as determined by histomorphometry [%]

Cells	Marker	Non-loading condition (range)	Loading condition (range)	<i>p</i> -value
Osteoblast	AP	5.3 (21.0)	4.3 (36.1)	0.60
	OC	0.5 (6.6)	1.2 (4.7)	0.07
	COL-I	2.0 (7.2)	1.7 (5.1)	0.65
	BMP-2	0.2 (7.5)	0.5 (34.9)	0.03
TDF	AP	3.7 (7.4)	3.9 (9.4)	0.71
	OC	1.5 (6.1)	1.3 (6.4)	0.69
	COL-I	0.3 (0.5)	2.9 (5.1)	0.37
	BMP-2	0.7 (4.9)	0.9 (18.9)	0.02
Co-culture	AP	2.6 (7.0)	5.8 (6.8)	0.07
	OC	0.9 (3.7)	1.2 (6.3)	0.21
	COL-I	1.0 (1.2)	1.1 (4.1)	0.46
	BMP-2	1.0 (3.0)	0.4 (4.4)	0.36

Values are given as median (range)

graft remodelling, and incorporation at the tendon–bone interface. The long-term goal is to elucidate the exact mechanism for regeneration of the ligament-to-bone interface in order to optimise operative reconstruction and postoperative rehabilitation [4, 5]. In particular, individualised postoperative rehabilitation is vital for overall therapeutic success. In particular, the extent and intensity of loading within the direct postoperative interval is still a matter of debate [16, 17]. Therefore, we aimed to simulate the tendon-to-bone healing environment after ACL reconstruction, specifically, cyclical mechanical loading on osteoblast and fibroblast differentiation, as well as the heterotypic cellular interactions in this process.

The most important finding in this study was that cyclical mechanical loading leads to downregulation of genes involved in osteointegration and osteoinduction, such as OC, AP, and COL1A1 in mono-cultures of OBL and fibroblasts. On the other hand, no downregulation of osteoinductive markers was observed under co-culture conditions. In contrast, immunocytochemistry and qPCR analysis showed slight but insignificant upregulation. This may be due to the potential stabilising effects of osteoblast-fibroblast cross talk in the co-culture environment.

Focusing on osteoblastic differentiation alone, this biometric culture model showed a downregulation of various osteoblast marker genes. Our results are in agreement with

those of Kaspar [20] and Stanford [21], who showed decreased ALP and OC deposition in mechanically loaded osteoblasts. However, this phenomenon might be dependent on the source of the osteoblasts, as Jiang et al. showed in a recent investigation of spinal cord-injured rats. They demonstrated that isolated osteoblasts responded less to mechanical loading compared to osteoblasts obtained from hindlimb-immobilised rats [22]. Obviously this preconditioning of cells can have an impact on their response to experimental loading.

This phenomenon was also observed in unloaded TDF-cultures, although the basic expression level of respective marker genes was lower for ALP (25-fold) and OC (5-fold) than in osteoblast cultures, while expression levels for COL and BMP-2 were nearly unaffected (data not shown). With respect to OC, similar results were shown by Pauly et al. in a study comparing tenocytes, osteoblasts and chondrocytes [23], as well as AP [24], on enzymohistochemical analysis. Tendon-derived fibroblasts may therefore have the potential to differentiate into the osteoblastic lineage to a certain extent [25].

In this study, mechanical loading of TDFs also resulted in downregulation of AP, OC, and COL, comparable to that observed in osteoblast-like cells, indicating that the underlying mechanism may be identical.

The applied strain may loosen the cell and collagen coated surface interaction and thus attenuate the osteoinductive effect

Fig. 1 Influence of cyclical mechanical loading on all three culture types on BMP-2 deposition as detected by immunocytochemistry (10× magnification). **a** Osteoblast-like cells. **b** Fibroblasts. **c** Co-culture

Fig. 2 Influence of prolonged continuous mechanical loading on all three culture types, for one or three days, on BMP-2 deposition as detected by immunocytochemistry. Significant detachment of cells from collagen-coated surfaces were found in all specimens (10× magnification). **a** Osteoblast-like cells. **b** Fibroblasts. **c** Co-culture

of collagen via the $\alpha 2\beta 1$ integrin, as described previously [26, 27]. In fact, AP and OC expression was comparable to that of cells cultured on plastic surfaces (data not shown). This speculation was detectable.

In contrast to our results other study groups have recently shown that co-culturing of fibroblasts and osteoblasts

decreases AP activity, as well as mineralisation, as compared to corresponding mono-cultures [8, 28]. On the other hand these experiments were performed without mechanical loading which does not correspond to tendon-to-bone interface as it is found in vivo after ACL reconstruction. Furthermore, the authors rely on much longer cultivation periods. Therefore our

Table 2 X-fold increase of osteoblast marker genes with or without loading

Cells	Marker	Non-loading condition (range)	Loading condition (range)	<i>p</i> -value
Osteoblast	AP	1.017 (0.031)	0.438 (0.808)	0.05
	OC	1.014 (0.025)	0.405 (0.400)	0.05
	COL-I	1.011 (0.127)	0.617 (0.992)	0.51
	BMP-2	1.004 (0.009)	0.936 (0.887)	0.51
TDF	AP	1.024 (0.084)	0.432 (0.040)	0.05
	OC	1.039 (0.058)	0.309 (0.416)	0.05
	COL-I	1.025 (0.072)	0.436 (0.431)	0.05
	BMP-2	1.024 (0.146)	0.911 (2.590)	0.51
Co-culture	AP	1.083 (0.092)	1.275 (1.873)	0.51
	OC	1.900 (0.152)	2.029 (1.116)	0.05
	COL-I	1.083 (0.237)	3.626 (2.642)	0.13
	BMP-2	1.012 (0.082)	1.191 (5.307)	0.05

Values are given as median (range)

results provide further information simulating a more realistic setting with regard to “native” ACL reconstructions. Moreover the expression of interface-relevant marker proteins such as collagen type II and aggrecan was detected within these respective studies, indicating that osteoblast/fibroblast interaction may lead to cell transdifferentiation [8]. However, our study indicates that the application of mechanical load increases AP, OC, COL1A1, and BMP-2 expression. Therefore, the downregulation observed in monocultures was completely compensated. This suggests possible cross-talk between fibroblasts and osteoblasts, or intercellular interactions. We may speculate in the direction of Pirraco et al., who suggested that these interactions could be particularly regulated through gap junctions [28]. Mechanical load may thus enforce this type of intercellular communication, resulting in upregulation of marker genes as mentioned above. Another support for this hypothesis is the observed formation of spheroids in co-cultures under mechanical loading. However, downregulation of marker genes was only detectable via qPCR. With regards to immunocytochemistry, mechanical loading did not appear to impact subsequent deposition of corresponding proteins. In order to rule out the possibility that these findings were only due to insufficient loading, the loading conditions were altered. Prolonged mechanical loading resulted in the radical detachment of cells. These findings suggest an alteration of cross-talk between fibroblasts and osteoblasts, or intercellular interactions under continuous loading.

Our study has several limitations. In the model a random mixture of cells involved in the interface formation was used. Currently, the distinct mechanism of the interface formation is not known. A co-culture model permitting osteoblast–fibroblast communications and cell migration using cell dividers to form mono-cell regions and an additional interface region in combination with mechanical load would be more favourable. Furthermore, the influence of inflammatory cell such as various interleukins was not addressed and should be evaluated in future studies. With regard to the immunohistochemistry the results are limited due to a great inter-essay variability, therefore only trends could be detected. On the other hand our results suggest that co-culturing induces significant changes in cell phenotype, leading to the expression of matrix relevant markers under cyclical loading. This study focused on osteoblast–fibroblast interactions, while the interface consists of chondrocytes embedded within a fibrocartilage matrix. Similar to our findings, osteoblast mineralisation potential was significantly reduced due to heterotypic cellular interactions [8, 29]. It is likely that osteoblast–fibroblast and osteoblast–chondrocyte interactions are key modulators of cell phenotypes at the graft-to-bone junction. If in vitro results transfer to in vivo cruciate ligament surgery, osteoblast–fibroblast interactions under mechanical loading would result in phenotypic changes and expression of matrix-relevant markers. Thus, it is likely that a postoperative treatment

regimen including balanced mechanical loading might not only prevent arthrofibrosis, but also enhance graft incorporation in vivo. However, an excessive treatment regimen should be scrutinised as continuous mechanical loading results in cell detachment and alteration of intercellular cross-talks under in vitro conditions. With regard to the clinical setting, this might contribute to critical tendon-to-bone healing at the tendon–bone interface in the bony tunnel in ACL reconstruction, and therefore a potential increase in chronic inflammation, tunnel widening, and finally recurrent instability.

Conclusion

We designed and optimised a biomimetic co-culture model to evaluate the role of osteoblast–fibroblast interaction and the expression of marker genes under cyclical loading conditions. Cyclical mechanical loading causes downregulation of genes involved in osteointegration and osteoinduction, such as OC, AP, and COL1A1 in mono-cultures of osteoblast-like cells and fibroblasts. No downregulation of osteoinductive markers was observed under co-culture conditions. Immunocytochemistry and qPCR analysis showed slight upregulations of marker genes and their corresponding proteins. This may be due to potential stabilising effects of osteoblast–fibroblast cross talk in the co-culture environment, simulating fibrocartilage formation, demonstrating the utility of in vitro co-culture models for the investigation of mechanisms governing the formation of the soft tissue-to-bone interface.

Conflicts of interest There are no conflicts of interest to report.

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