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Effect of cyclic tensile load on the regulation of the expression of matrix metalloproteases (MMPs -1, -3) and structural components in synovial cells

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Abstract

Synovial cells are reported to colonize synthetic ligament scaffolds following anterior cruciate ligament (ACL) reconstruction but the process leading to ligamentization is poorly understood. The present study investigated the effect of cyclic tensile strain on the expression of genes involved in matrix remodelling in bovine synovial cells seeded onto an artificial ligament scaffold. Synovial cells were seeded and cultured on polyester scaffolds for 3 weeks and subsequently subjected to cyclic tensile strain of 4.5% for 1 hr at frequency of 1 Hz. Changes in the levels of expression of genes for major ligament components (type I and type III collagen) and also metalloproteinases (MMP-1 and MMP-3), and TIMP-1 were examined using RT-PCR. Additionally, metalloproteinase activity was measured using both zymography and collagenase assays. The gene expression of MMP-3 transcripts in the loaded group was almost 3-fold that observed in control group but no differences were observed in other transcripts. Consistent with these findings, MMP-3 activity increased by 85% under mechanical stimulus, and MMP-1 activity showed no changes. Over expression of MMP-3 under cyclic tensile load may mediate the proteolysis of certain substrates surrounding the ligament scaffold. This will play a critical role in facilitating cell migration, proliferation and tissue remodelling by breaking down the provisional tissue formed by the synovium, and by generating factors that induce angiogenesis and chemotactic cell migration.

Keywords: ligaments • mechanical strain • matrix metalloproteinase • tissue engineering • synovial cells

Introduction

Because of the anterior cruciate ligament (ACL's) well-recognized poor capacity for repair, its reconstruction remains an appropriate approach to the restoration of normal knee function. Continuous efforts have been made to devise a suitable method for the reconstruction of ACL using autografts, allografts or synthetic devices with variable degrees of success. However, none of these approaches is ideal for a variety of reasons. For example, following implantation, autografts are subject to histological and biochemical transformation during which their original characteristics are lost and those of ligaments are acquired. This process has been referred to as 'ligamentization' by Amiel et al. [1]. During this process, the ACL autograft undergoes necrosis followed by cell repopulation from extrinsic sources. On the other hand, studies using DNA analysis in a goat model have confirmed the replacement of allograft ACL tissue by native recipient tissue [2]. In that respect, the fate of an *in vitro* tissue-engineered ligament should not differ from that of an autograft or allograft (except that the tissue engineered ligament will most likely possess poorer mechanical properties than its natural counterpart). Rather the tissue-engineered ligament has to go through the same process of necrosis, revascularization and colonization by autogenous cells. Therefore, host cells appear to repopulate the graft, regardless of the source of the grafted material (autograft, allograft, tissue engineered ligament and, of course, nude graft), before vascular invasion, with subsequent transport of additional reparative cells once revascularization has been established.

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A previous animal study using a canine model [3] reported that the Leeds-Keio ligament, a synthetic scaffold type ligament, underwent different distinct stages when implanted. During the initial stage, the artificial ligament was invaded by fibrous tissue apparently originating from the synovial membrane. This was associated with rich vascularity and hypercellularity. Once the reactive fibrous tissue had totally covered the artificial ligament, proliferation of the fibrous tissue subsided, vascularity and cellularity decreased. Most notably the morphology of the cells was seen to change from an oblate shape to the spindle shape characteristic of fibroblasts observed in ligament tissue. Furthermore, mesenchymal-like cell differentiation and tissue remodelling and maturation occurred. The entire process of remodelling and maturation only happened when the scaffolds were implanted taut consequently they experienced the stimulatory effect of cyclic tensile strains during the animal/patient activities. However, when the scaffolds were implanted slack (or slackened subsequent to implantation), no tensile strains were transmitted to the ingrown tissue. Consequently, neither remodelling nor maturation of the tissue occurred and in some cases, the scaffold structure remained exposed. Identical observations were made in a clinical study [4] albeit that the maturation process of the induced tissue around the implant took much longer in the human (about 2 years *versus* 9 months) than in the canine model.

The above *in vivo* studies have highlighted the contribution of synovial cells in ligament reconstruction and the importance of cyclic mechanical strain in tissue induction, remodelling and maturation. Several other studies have reported that cyclic strain influences a number of aspects of cellular metabolism including cell proliferation, matrix gene expression and protein synthesis, synthesis and activity of metalloproteinases and their inhibitors, and expression and activation of various growth factors and cytokines [5–9]. In our two previous *in vitro* studies, bovine synovial cells were seeded onto plasma-treated polyester scaffolds and then subjected to short term (1 day) or medium term (5 and 9 weeks) cyclic tensile strain [5, 6]. The short-term tests showed that the DNA synthesis was affected by the period of strain application and that 1 hr of strain application was the optimal period. Furthermore, it was shown that there is a threshold of 1% for the amplitude of strain above which cell proliferation was triggered [5]. The medium term tests monitoring the tissue ingrowth has confirmed the same threshold for the amplitude of strain below which the amount of tissue was small and the scaffold filaments effectively remained exposed. However, when the strain amplitude was larger than the threshold the amount of tissue was comparatively substantial and the scaffold filaments were completely covered. Both short and medium-term studies established that cell proliferation and tissue ingrowth increase with the amplitude of strain. The stimulatory effect was maximal at an exercise period of 1 hr and an amplitude of strain of about 4.5%. This is the strain amplitude is that experienced by the ACL during normal activities [10].

The aim of the present *in vitro* study was to gain insight into the contribution of cyclic tensile strain to the remodelling and maturation processes of the provisional induced tissue around ligament grafts after implantation. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a major role in tissue regeneration and remodelling in both physiological and pathological situations [11–16]. These enzymes are synthesized and secreted by a variety of cell types [17–19] including synovial cells[18, 19]. Depending on their different substrates, MMPs can be divided into four subgroups, including collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11) and membrane-type MMPs (MMP-14, MMP-15, MMP16, MMP-17) [20]. The balance between MMPs and their tissue inhibitors (TIMPs) is decisive for the catalytic activity of MMPs [21], and appears to be crucial in directing successful wound repair [22]. MMP-1 and MMP-3 in particular are thought to play a major role in remodelling, because MMP-1 is the only enzyme capable of efficiently degrading interstitial collagens by cleaving their triple helical domains, and MMP-3 has a wide spectrum of substrates including non-collagenous proteins, gelatin and laminin [12]. The activities of MMP-1 and MMP-3 are regulated by a family of TIMPs [23–26]. The major inhibitor of MMP-1 and MMP-3 is TIMP-1, a 28.5-kDa glycoprotein, which forms 1:1 stoichiometric complexes with these proteases [27]. In this present study, we focused on determining the mRNA level of MMP-1, MMP-3 and TIMP-1, and the activities of MMP-1, MMP-3. We also investigated the expression of type I and type III collagen, because (i) these major ligament components are reported to be expressed by synovial cells [28], and (ii) during remodelling, degradation and re-synthesis of collagen is important.

Materials and methods

Ligament scaffold materials

The ligament scaffolds (Xiros PLC, Leeds UK) used were made from polyester (polyethylene terephthelate) and had an identical weave to that currently used in ligament and tendon implants. They had a maximum tensile strength of 320N and an average stiffness of 15N/mm and comprised nine yarns, each consisting of 96 monofilaments, each 20 μm in diameter. The scaffolds were treated with electronic discharge plasma prior to use [29].

Cells and Cell culture

Synovial cells were harvested from the synovium of metatarsophalangeal joints from young bovine animals (three animals) within 1 hr of slaughter. The synovium specimens were rinsed three times with Dulbecco's phosphate buffer saline (pH 7.2) (Sigma Aldrich, Ltd., Poole) and cut into small pieces. These were then digested at 37°C for 3 hrs using 0.25% (w/v) Collagenase type IA (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamate (Sigma). The digest was then centrifuged at 500 g for 10 min. and the pellet suspended in serum-free DMEM before passing through a 70 μm nylon filter to remove undigested residue. Cells were isolated by centrifugation at 500 g for 10 min. and re-suspended in DMEM. Primary cells were seeded into 25 cm^2 flasks at 10^4 cells/cm^2 in DMEM, supplemented with 10% foetal bovine serum (FBS), penicillin,

streptomycin and amphotericin. The culture medium was changed three times per week and cells were passaged after 7–9 days. Only passages three were used in the study.

Scanning electron microscopy (SEM)

The cells, cultured on a scaffold for 2 days and three weeks, were fixed in 2.5% glutaraldehyde (in 0.1M phosphate buffer saline, pH 7.4) for 24 hrs at 4°C, then dehydrated in a series of absolute ethanol dilutions (50%, 70%, 90% and 100%). The samples were dried under vacuum in a desiccator for 16 hrs and then attached to aluminium stubs for support and sputter coated using an E5000 sputter coater (Polaron, Watford, UK). The samples were examined using a Jeol JSM35 SEM (Japan Electron Optics Laboratory Co., Tokyo, Japan)

Scaffold seeding and application of mechanical strain

A specialized apparatus was designed, manufactured in house and subsequently commissioned for this investigation. It had eight test stations accommodating four test and four control specimens, to operate within the confines of an incubator. A full description of the apparatus is given elsewhere [6].

Each experiment was performed three times using cells harvested from different animals. For each experiment, the cells (10^5 cells) were seeded onto each of eight different ligament scaffolds, (each 2 cm long and 0.5 cm wide already gripped in its respective clamps). Four of these were subjected to cyclic tensile strain the remaining four were not and served as controls. Before the application of strain, the cell seeded scaffolds were incubated in DMEM with 10% FBS until cells completely covered the scaffold (3 weeks [see SEM assay]). The culture medium was changed twice a week. The culture medium was removed and replaced with 0.5% FBS for 24 hrs. Just before stretching, the culture medium was replaced with fresh 1% FBS and 25 μg/ml ascorbic acid. Synovial cells were then subjected to a single episode of cyclic tensile strain of 4.5% for 1 hr at a frequency of 1Hz.

Gene expression

Total RNA was extracted from Synovial cells, 3 hrs after the application of cyclic tensile strain, according to manufacturer's instructions (RNeasy, QIAGEN), and then treated with two units of DNase I (DNA-free; Ambion, Austin, TX, USA). Total RNA (100 ng) was reverse transcribed (RT) with 2.5 μM random decamers and 20 units of Enhanced Avian Reverse Transcriptase (Sigma-Aldrich) in a 20 μl reaction mixture for 15 min. at 25°C and for 50 min. at 42°C. An aliquot (0.6–1.5 μl) of RT product was then used for polymerase chain reaction (PCR) in a 30 μl reaction mixture containing 1x FailSafe PCR (Epicentre Technologies, Madison, WI, USA), 0.75 units of DNA polymerase, and 1 μm of each primer (Table 1). Coamplification of the glyceraldehyde-phosphate-dehydrogenase (GAPDH) gene was performed in the same PCR tubes as the internal standard [30], to facilitate semi-quantitative determination of targeted gene mRNA levels. The thermocycle included one cycle at 94°C for 3 min. of initial denaturation, and 30–40 cycles at 94°C for 1 min., at 60°C for 1 min., and at 72°C for 1 min., followed by a final extension at 72°C for 3 min. The PCR products were electrophoresed on a 1.8% agarose gel, stained with 0.25 μg/ml ethidium bromide, and the intensity of the bands was analysed using Multi-Analyst software (Bio-Rad, Hemel Hempstead, UK). The signal levels for each targeted gene were expressed as ratios to the signal levels for GAPDH.

Table 1 Sequence of primers used in PCR amplification reactions (bp: base pair)

Gene	Sequence (5' to 3')	Product size (bp)
GAPDH	For CCACGGCAAGTTCAACGG Rev AGACACGTTGGGAGTGGG	566
Type I Collagen	For GTTCCCCTGGAAATATCG Rev CTCTTGCACCAGCAGG	405
Type III Collagen	For GGAAACGTGCATAAGTGC Rev TGCGTGTTTGATACTGG	407
MMP-1	For ACAAACCTGACCTACAGG Rev GAACATCACCACTGAAGG	406
MMP-3	For GCAGTTAGAGAACATGG Rev GTCATTAGGGGAAACTGG	327
TIMP-1	For CATCAGGGCCAAGTTCG Rev TGTGAGCAGCTGGTCC	406

SDS-Page zymography

MMP enzyme expression was assessed by SDS-PAGE zymography using a method similar to that previously described [31]. For each sample, conditioned media, collected 20 hrs after the end of mechanical strain, were adjusted to represent the same quantity of cellular protein (15 μg) and subjected to substrate-gel electrophoresis on a 10% (wt/vol) polyacrylamide gel impregnated with 12% gel containing 0.1% casein. Samples containing equal amounts of protein were mixed with sample buffer [10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.0025% (wt/vol) bromphenol blue, and 0.06 M Tris, pH 8.0] and loaded into wells of a 12% (wt/vol) gel without boiling or reduction. In order to provide a reference, Kaleidoscope prestained molecular weight standards (Bio-Rad) were run alongside the samples. After electrophoresis, SDS was eluted from the gel with 2.5% (vol/vol) Triton X-100 washes (2 x 15 min at room temperature), and the gel was incubated overnight at 37°C in 50 mM Tris HCl, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.5 μg/ml NaN_3, pH 7.2, with and without a gelatinase inhibitor (10 mM ethylenediaminetetraacetic acid [EDTA]). Gels were rinsed in distilled water, stained with 0.5% co-omassie brilliant blue R-250 in 40% methanol and 10% acetic acid. Proteolytic activities in the samples were detected as cleared bands against the dark background of stained casein.

Quantitative collagenase activity assay

The quantification in each sample was carried out by incubation with telopeptide-free collagen extracted from rat skin and labelled with [^{3}H]-acetic anhydride according to protocol described by Dean and Woessner [32] and Li et al. [33]. TIMPs in the samples were inactivated by reduction in 2-mmol/l dithiothreitol at 37°C for 30 min., followed by alkylation in

5-mmol/M iodoacetamide at 37°C for 30 min. The samples were chilled on ice and dialysed for 4 hrs against the assay buffer (50 mM tris-HCl, 200-mM NaCl, 10 mM $CaCl_2$, and 0.005% Brij-35, pH 7.5). The latent procollagenase was activated by addition of aminophenylmercuric acetate (APMA) to a final concentration of 0.5 mM. The blanks were prepared by replacing the conditioned culture medium with the assay buffer. [^{3}H]-acetic collagen (1.2 x 10^5 cpm per 16 µg / 5 µl) was added to each sample and the final volume adjusted to 110 µl with the assay buffer. After incubation at 30°C for 18 hrs the sample were placed on ice to stop the reaction. After adding 120 µl of the assay buffer supplemented with 200 µg acid-soluble intact collagen as a cold carrier, 20 µg trypsin, 20 µg chymotrypsin and 30 mM EDTA, the samples were incubated at 31°C for 90 min. for a second digestion. The indigested collagen was precipitated by addition of equal volume of ice cold 20% trichloroacetic acid (TCA). After centrifugation at 18,000 g for 5 min., 100 µl of the digested fraction of each sample were counted by liquid scintillation for 3 min. The collagenase activity reported as units per millilitre, with one unit of activity being defined as that amount of enzyme required to degrade 1 µg of collagen / min per ml of sample at 30°C.

Statistical analysis

All experiments were performed three times using cells harvested from three animals. Data were subjected to univariate analysis of variance (ANOVA). Tukey's post-hoc comparisons were derived to test the significance of differences between individual loaded test results and their respective unstretched controls for each group of three animals. Further, for each assay, the replicated data from each animal (four loaded samples and four controls) were first normalized by expressing them as ratios of test to control means, which were then averaged for each animal. The values of these averages for loaded groups were then compared with those obtained in the control groups (n = 3). Critical levels were set at $P < 0.05$.

Results

Culture of synovial cells on scaffold

In order to approximate the *in vivo* situation, in which the implant is invaded with the synovial tissue during the early weeks after implantation, we carried out this preliminary study to determine the culture time required for cells to completely cover the scaffold before the application of mechanical strain. The plasma-treated polyester scaffold was seeded and kept in culture for three weeks. As shown in SEM micrographs two days after seeding, the synovial cells readily adhered, spread and grew on the plasma treated polyester scaffold (Fig. 1A) and formed cellular extensions to bridge neighbouring fibres. Cell growth, monitored on an optical microscope twice a week (results not shown), showed that more than two weeks was needed for the cells to fill the scaffold's intra-yarn space (average surface area 0.3 mm^2). As shown in SEM micrographs (Fig. 1B), a uniform cell sheet and possible extracellular matrix completely covering the fibres was observed by 3 weeks of culture. Therefore, for each experiment, cells were cultured on the scaffolds for three weeks prior to the application of cyclic tensile strain.

Effect of cyclic tensile strain on gene expression

The mRNA of GAPDH was well expressed in all samples including the loaded group and control group. On the basis of the analysis of 1µg of total RNA from each sample, the GAPDH mRNA levels at 24 PCR cycles (Linear range) showed no significant differences between loaded and control groups (result not shown). This suggested stable expression of the GAPDH gene and justified its use as housekeeping gene for synovial cells in the current experimental conditions.

The major components of ligament, namely type I and type III collagen, were well expressed by synovial cells, however no significant differences were observed between loaded and control group (Fig. 2).

The expression of metalloproteinases MMP-1, MMP-3 and TIMP-1 were all detected in both loaded and unloaded samples (Fig. 3). The densitometric analysis of the PCR products for MMP-1 and TIMP-1 revealed that cyclic tensile load induced no changes in the level of expression (Fig. 3B). Nevertheless, cyclic tensile strain of 4.5% consistently and significantly increased the expression of MMP-3 mRNA (Fig. 3A). The densitometric analysis of the PCR products for MMP-3 revealed that, in comparison with the control group, cyclic tensile load induced 3-fold increases in MMP-3 mRNA synthesis (Fig. 3B).

In order to ascertain whether changes in mRNA levels for a gene such as MMP-3 would reflect changes in activity levels, a zymography test and collagenase activity assay were carried out.

Zymography for caseinolytic activities of MMP-3

Zymography was performed to verify the caseinolytic activity in mechanically conditioned culture media (Fig. 4). The different forms of the enzymes were identified by their molecular masses [34].

The casein zymogram (Fig. 4A) disclosed a predominant 59 kd form which might be the glycosylated form of stromelysin (MMP-3) [34]. Three other less active MMP-3 forms were barley detectable including 63 kD, 56 kD and 54 kD forms. In three separate experiments using four replicates per group, the increase of MMP-3 activity was statistically significant ($P<0.05$) in samples subjected to cyclic tensile strain of 4.5% for 1 hr (loaded) compared to control groups. Densitometry of casein zymogram gels (Fig. 4B) indicated an 85% increase in 59 kD MMP-3 fraction. This finding was consistent with the transcript of MMP-3, indicating that synovial cells subjected to cyclic tensile strain of 4.5% (loaded) for 1 hr produced more MMP-3 than when unloaded (control).

Quantitative collagenase activity assay for MMP-1

The collagenolytic activity in each sample was determined using [^{3}H] radio-labelled telopeptide-free collagen. As shown in Figure 5, the collagenase activity in loaded and control (unloaded) group

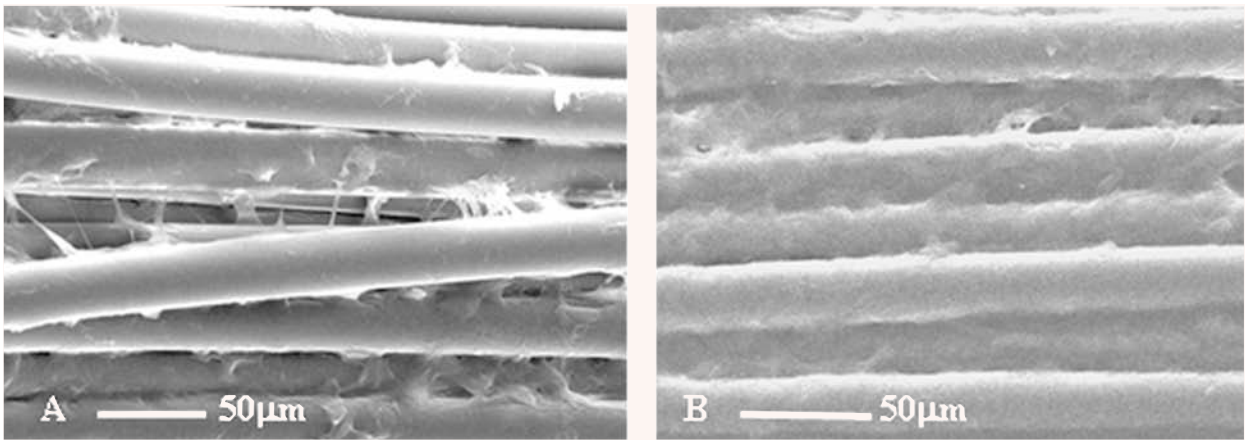

Fig. 1 SEM micrographs of the synovial cells-seeded ligament scaffold. (**A**): Ligament scaffold seeded with synovial cells and cultured for 2 days. (**B**) Ligament scaffold seeded with synovial cells and cultured for 3 weeks.

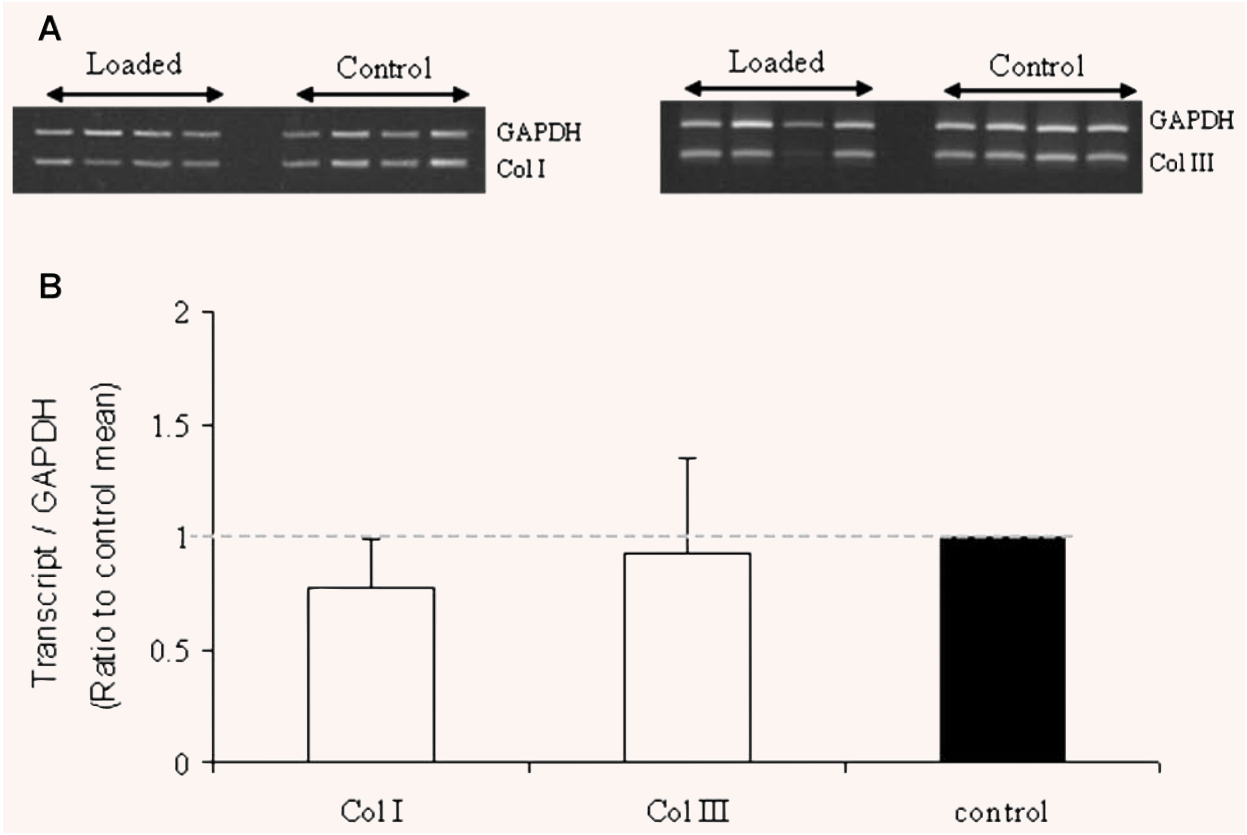

Fig. 2 Effect of cyclic tensile strain on type I collagen (Col I) and type III collagen (Col III) transcription. **A**: Ethidium bromide-stained agarose gel analysis of 4 representative reverse transcriptase chain reaction (PCR) samples in each group. **B**: The columns represent the weighted-mean and standard deviation (3 experiments) of ratio of band intensity of each transcript to GAPDH PCR product normalized to the control (unloaded samples) mean in each group.

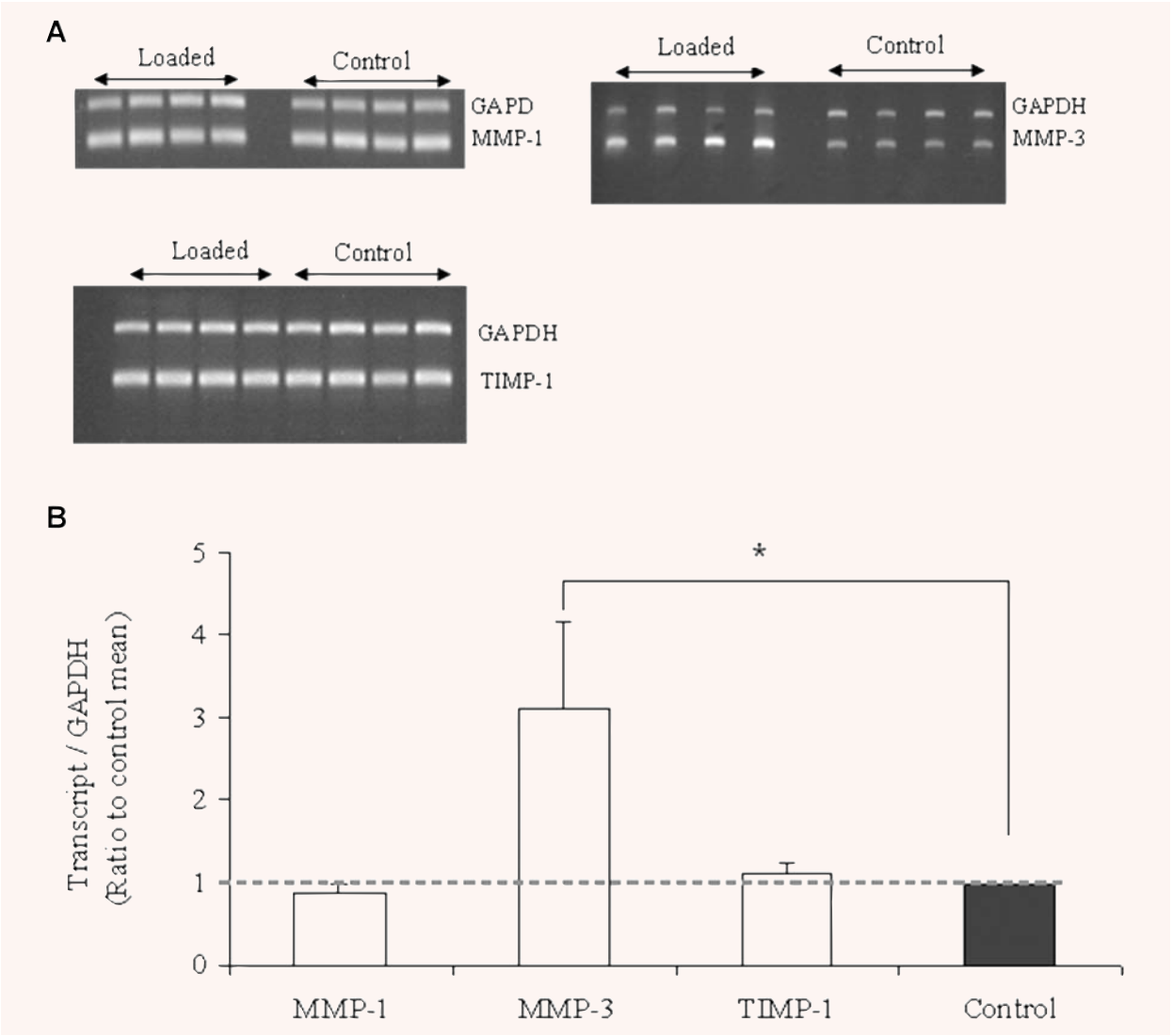

Fig. 3 Effect of cyclic tensile strain MMP-1, MMP-3 and TIMP-1transcription. **A**: ethidium bromide-stained agarose gel analysis of 4 representative reverse transcriptase chain reaction (PCR) samples in each group. **B**: The columns represent the weighted-mean and standard deviation (3 experiments) of ratio of band intensity of each transcript to GAPDH PCR product normalized to the control (unloaded samples) mean in each group. The asterix (*) indicates statistically significant difference ($P<0.05$) from the controls.

was 0.093±0.02 and 0.076±0.03. However, the difference between the two groups was not statistically significant.

Discussion

ACL reconstruction with bioactive synthetic implant works not only as a prosthesis but also as a scaffold onto which natural tissue grows. At the initial stage, the fibrous tissue from the synovial membrane invades the implant and proliferates within its structure [3, 4]. Tissue maturation occurs in the next stage once reactive fibrous tissue has totally covered the artificial ligament. During this stage the load acting along the implant is transferred to the new tissue, causing reorganisation of the fibres, which become well aligned along the direction of the tensile load. Furthermore, the cells are reduced in number and acquire the spindle shape typical of ligament cells. In a final remodelling stage the reconstructed ligament becomes dense with ligamentous

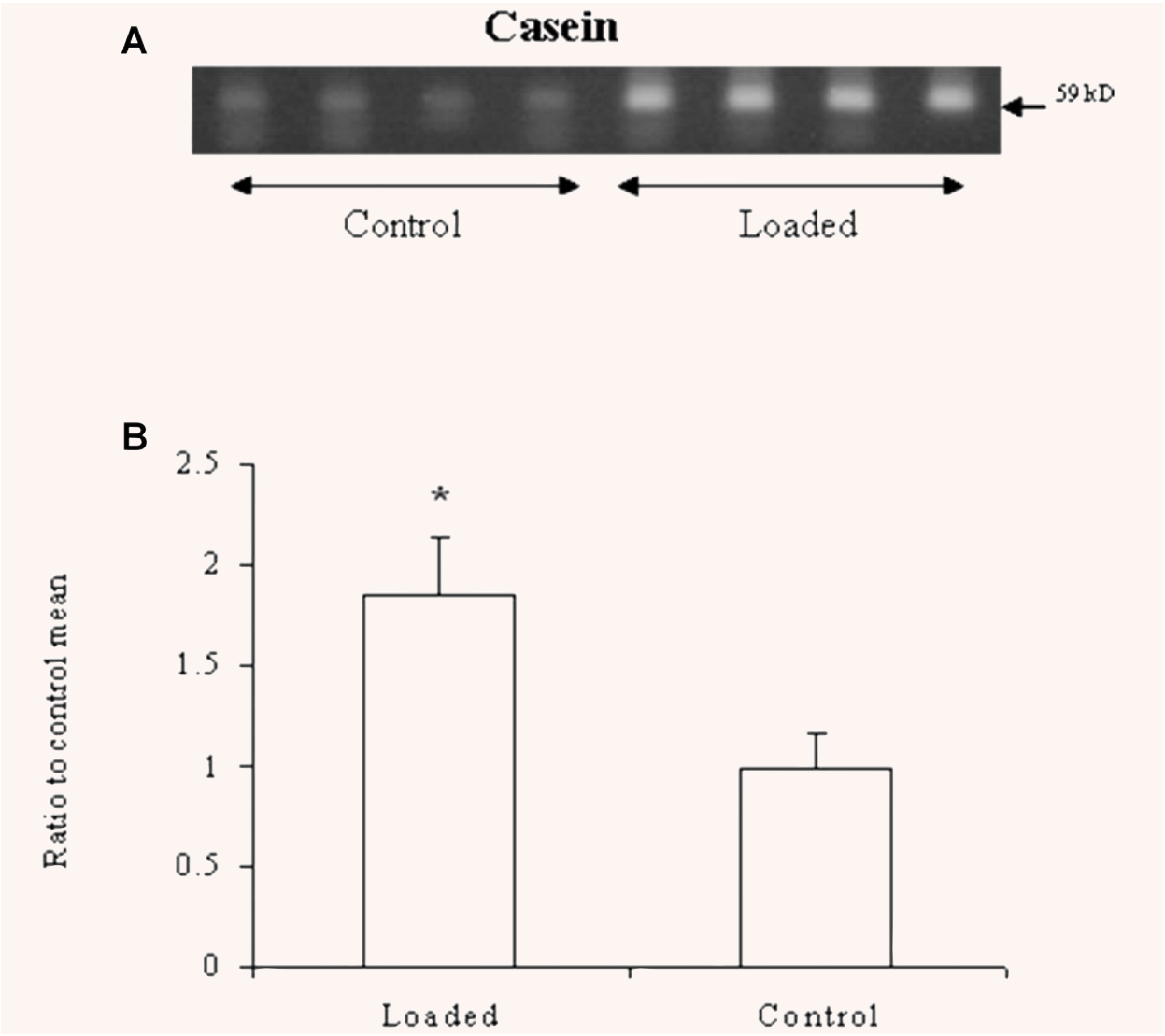

Fig. 4 Effects of mechanical strain on matrix metalloproteinase activity as determined by zymography. **A**: *Zymographic gel.* Casein zymograms show stromelysin (MMP3) activity from four strained specimens and four controls is demonstrated. **B**: The columns represent the weighted-mean and standard deviation (3 experiments) of band intensity normalized to the control mean. The asterix (*) indicates statistically significant difference ($P<0.05$) from the controls.

collagen-like fibres, which mature while the implant acts as a ligament. These last two stages, tissue maturation and remodelling, are observed only when implants are implanted taut: as a consequence the tissue is subjected to mechanical strain. However, when the implant is slack the newly formed tissue remains immature and highly cellular with no organisation of the fibres.

The present *in vitro* study was designed to gain insight into the effect of mechanical strain on the remodelling process of tissue induced around scaffolds used for ligament reconstruction. In order to approximate the *in vivo* situation, in which the implant is invaded with the synovial tissue during the early weeks after implantation, scaffolds were seeded with synovial cells and left in culture until completely covered (see SEM study Fig. 1), with a layer of cells and perhaps extracellular matrix. The short-term application of cyclic tensile strain of 4.5% at frequency of 1 Hertz induced no apparent changes to the expression of the type I and type III collagen genes. The products of these genes are associated with the extracellular matrix of tendon and ligament, and may be important for tissue engineering.

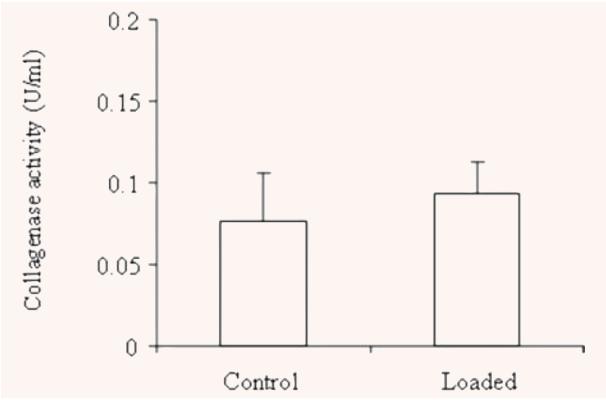

Fig. 5 Effects of mechanical strain on collagenase activity of MMP-1. The columns represent mean and standard deviation of the collagenase activity (units per millilitre). 1 unit of activity being defined as that amount of enzyme required to degrade 1 μg of collagen / min per ml of sample at 30°C.

This present study has clearly demonstrated that cyclic tensile strain of 4.5% induced up-regulation of the expression of MMP-3 in bovine synovial cells. In contrast to this there appeared to be no change in MMP-1 and TIMP-1 expression. Additionally, corroborating the RT-PCR data, it was shown that cyclic tensile strain caused an increase in MMP-3 enzyme activity but no change in the activity of MMP-1. The increase of MMP-3 enzyme activity was manifest in the casein zymogram which disclosed a predominant 59 kD form which might be the glycosylated form of stromelysin (MMP-3). Three other less active stromelysin forms were barley detectable in both the loaded and control groups including a 63kD (glycosylated prestromelysin), a 56kD (unglycosylated prestromelysin) and a 54kD (unglycosylated stromelysin). The finding in this study is in accordance with previous studies which have shown a correlation between the alteration of cellular morphology through the cytoskeleton and MMP gene expression by rabbit synovial fibroblasts, where agents that altered the actin-based cytoskeleton produced induction of MMP-3 expression [35, 36]. Whether and in what fashion MMP-3 can be involved in the process of tissue remodelling, still remains unclear. A review of some of literature on MMP and its function appears to shed light on this issue.

MMP-3 is a member of MMPs, which are a family of proteolytic enzymes implicated in many processes of extracellular matrix turnover. Due to their degradative nature, the uncontrolled expression of MMPs has been associated with many pathological conditions such as rheumatoid arthritis and osteoarthritis [14, 15]. However, their controlled expression has been associated with many physiological processes of extracellular matrix turnover in normal tissue [16]. Many past studies have reported that MMP-3 covers a wide range of biologic activities. In skin, MMP-3 has been suggested to play a major role in tissue response to injury. A number of MMPs, in particular MMP-3 was not expressed in normal skin, but showed a maximum expression within hours of injury, and then followed the pattern of the wound repair process [37–39]. While the MMP-3 levels return to normal in dermal wounds that heal, those in chronic non-healing ulcers remain elevated. In *MMP-3 knockout mouse mutants*, Mukherjee *et al.* [40] established that MMP-3 deficiency exacerbated the early increase in scar volumes which may impair post-radiofrequency myocardial wound healing. Thereby, MMP-3 plays a putative beneficial role during the early phases of myocardial remodelling following injury. Thus MMP-3 appeared to be requisite for the progression of a normal wound healing response, but its exact involvement in the process is still not clear. However, MMP-3 has been reported to have many functions, including degradation of a wide range of extracellular matrix components, apoptosis, morphogenesis, angiogenesis and activation of other MMPs [41–43]. Moreover, it has been hypothesized that MMP-3 is a key factor in wound repair processes by helping the migration and recruitment of various cell types to the site of repair. In a study examining the resorption of intervertebral discs, it has been demonstrated that MMP-3 elaboration was absolutely necessary for macrophage infiltration [42]. The authors also hypothesized that MMP-3 plays a contributory role in tissue wound healing response and tissue resorption by facilitating and recruiting myofibroblasts and inflammatory cell types to the site of injury. Mukherjee *et al.* [40] also reported that the early increase in scar volume in the *MMP-3 knockout mice mutants* was associated with an absence of macrophages, lymphocytes, and myofibroblasts at the site of injury. Whether or not MMP-3 plays a direct chemotactic role is not proven. However, it has been hypothesized that, by mediating the proteolysis of certain substrates, MMP-3 generates factors that induce chemotactic migration of various cell types involved on the process of tissue repair [44–46].This hypothesis is supported by other studies which reported that MMP-3 deficiency reduced the migration of appropriate cells at the site of injury [47, 48].

The present study demonstrates that once synovium-derived tissue has established residence around the ligament scaffold *in-vitro* synovial cells subjected to mechanical stimuli will produce MMP-3. In light of the above past studies, the increase of MMP-3 synthesis by synovial cells under cyclic tensile strain may be related to the formation of a new ligament around the implanted scaffold by breaking down the provisional tissue formed by the synovium and mediating the proteolysis of certain substrates. These play a critical role in facilitating cell migration and generate factors that promote angiogenesis and induce chemotactic migration of various cell types involved in the process of ligamentization.

To further understand the process of tissue remodelling and maturation around a ligament scaffold, it will be necessary to investigate the medium and long term effect of cyclic tensile strain on synovial cells. This might shed light on the role played by synovial cells in 'ligamentization', and whether they are central to the elaboration of the characteristic features of ligament. This possibility is supported by the knowledge that synovial cells are known to be a source of multi-potent mesenchymal stem cells (MSC's) which can be induced consistently into a range of differentiated cell types in vitro [49], and are documented to share similar phenotypic and functional properties with bone marrow MSC's [50].

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