

Gene Expression of Type I and Type III Collagen by Mechanical Stretch in Anterior Cruciate Ligament Cells

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ABSTRACT. Mechanical stretch affects the healing and remodeling process of the anterior cruciate ligament (ACL) after surgery in important ways. In this study, the effects of mechanical stress on gene expression of type I and III collagen by cultured human ACL cells and roles of transforming growth factor (TGF)- β 1 in the regulation of mechanical strain-induced gene expression were investigated. Uniaxial cyclic stretch was applied on ACL cells at 10 cycles/min with 10% length stretch for 24 h. mRNA expression of the type I and type III collagen was increased by the cyclic stretch. TGF- β 1 protein in the cell culture supernatant was also increased by the stretch. In the presence of anti-TGF- β 1 antibody, stretch-induced increase in type I and type III mRNA expression was markedly ablated. The results suggest that the stretch-induced mRNA expression of the type I and type III collagen is mediated via an autocrine mechanism of TGF- β 1 released from ligament cells.

Key words: anterior cruciate ligament (ACL)/fibroblast/mechanical stretch/type I collagen/type III collagen/transforming growth factor (TGF)- β 1

Ligaments are dense bands of connective tissue that connect bones and mediate normal joint movement and stability. Ligaments contain rows of fibroblasts within parallel bundles of extracellular matrix composed mainly of type I collagen and a small proportion of type III (Amiel *et al.*, 1984). The anterior cruciate ligament (ACL) of the knee has a very important role for the restraint to anterior displacement of the tibia. It has been estimated that the ligament typically bears peak loads of about 169 N during normal ambulation, with a three-fold increase to 400 N to 500 N during strenuous athletic activity (Bessette and Hunter, 1990). Moreover, the posterolateral bundle of the ACL is stretched in extension of the knee and the anteromedial bundles become taut in flexion (Girgis *et al.*, 1975; Amis and Dawkins, 1991). Therefore, the ligament fibroblasts are repeatedly stretched in physiological activities. Stress deprivation alters the morphologic and biochemical characteristics of the ligament tissue. In immobilized rabbit knee, significant losses of

collagen mass were observed after 12 weeks (Harwood and Amiel, 1992). Stress-deprived rabbit ligament fibroblasts decreased the synthesis of fibronectin and increased the expression of β 1 and α 5 integrins (AbiEzzi *et al.*, 1997). These results suggest the importance of maintaining normal stress on ligaments to prevent the degradation of extracellular matrix components.

Rupture of the ACL often necessitates graft surgery due to the poor potential for healing and subsequent functional instability, meniscus injury and osteoarthritis. Mechanical stretch plays important roles in the remodeling process of the tendon graft. The effect of graft tension on remodeling of the autograft in ligament reconstruction was investigated using an in situ freezing model (Tohyama and Yasuda, 1998). Transmission electron micrographs revealed that the number of small diameter fibrils decreased in the stress-shielded tendons compared with non-stress-shielded ones.

Transforming growth factor (TGF)- β 1 is known to play an important role in the healing and remodeling process of connective tissue. Previous reports have shown the TGF- β 1 to enhance healing of incisional skin wounds (Miyazono and Heldin, 1992), and TGF- β 1 has been observed to be present in the early healing stage of the lacerated ligament and reconstructed ACL (Lee *et al.*, 1998; Kuroda *et al.*,

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Abbreviations: ACL, anterior cruciate ligament; TGF- β 1, transforming growth factor; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

2000). *In vivo* and *in vitro* studies show that exogenous TGF- β 1 is able to enhance collagen secretion by ligament fibroblasts (Murphy *et al.*, 1994; DesRosiers *et al.*, 1996; Marui *et al.*, 1997).

Although ligament fibroblasts are uniaxially stretched during normal activity, the response of ligament fibroblasts following *in vitro* uniaxial stretch has not been reported. The purpose of this study was to investigate effects on mRNA expression of type I and III collagen in the ACL fibroblasts after the application of cyclic stretch *in vitro*, and roles of TGF- β 1 in the regulation of mechanical strain-induced gene expression.

Materials and Methods

Cell culture

Isolation of human ACL fibroblasts was performed as described by Nagineni *et al.* (Nagineni *et al.*, 1992) with a modification of the explant culture technique. The ACLs were obtained by undergoing total knee arthroplasty in osteoarthritis. This study received the approval of the ethics committee of our institution and all patients gave their informed consent. The ligament was washed repeatedly in calcium- and magnesium-free phosphate buffered saline (PBS) and the synovial sheath was carefully scraped off. The tissue was cut into small pieces and placed in culture dishes containing growth medium. Growth medium consisted of Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), 10 mM NaHCO₃, 10 mM HEPES, 10 mM nonessential amino acids, and antibiotics (50 μ g/ml penicillin, 50 μ g/ml streptomycin and 100 μ g/ml neomycin). The culture dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The explant pieces were removed when adequate cell outgrowth had been observed. After cells reached confluency, cells were detached by treating with PBS containing trypsin (0.125%) and EDTA (0.2%). The ligament fibroblasts between passage one and four were used.

Mechanical stretch application

A system applied for uniaxial cyclic stretch to cultured cells on a silicon membrane was developed (Fig. 1). ACL fibroblasts were transferred onto 2.5 \times 10 cm rectangular silicon dishes coated with 0.05% type I collagen (Koken, Tokyo, Japan) and were cultured in DMEM with 10% FBS for 24 h. The subconfluent cells were then washed twice with PBS and the culture medium was replaced with DMEM containing 0.5% FBS. The chambers were mounted on a stretching stage that was driven by a computer controlled motor (Sigma Koki, Tokyo, Japan). Uniaxial cyclic stretch was applied on cells at 10 cycles/min with 10% length stretch for 24 h.

RNA isolation and RT-PCR

Total cellular RNA was isolated from fibroblasts after dissolving in TRIzol reagent (Gibco BRL, NY) according to the manufactur-

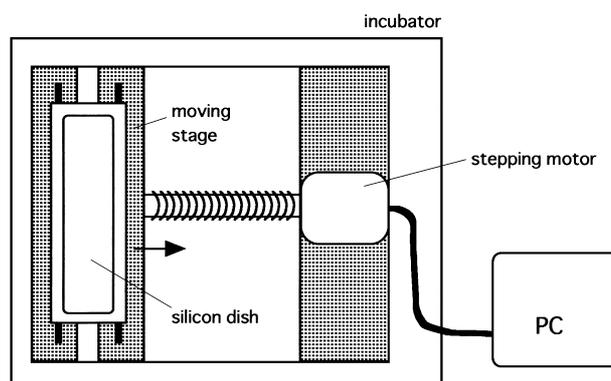


Fig. 1. The stretch system. Rectangular silicon dishes were held at one side by a fixed stage and the opposite side by a moving stage that was driven by a computer controlled motor.

er's instruction. The first strand cDNA was prepared from the RNA with reverse transcriptase (RTase) (M-MLV reverse transcriptase: Gibco BRL, Grand Island, NY). The cDNA was amplified by polymerase chain reaction (PCR) (Takara Z-taq: Takara Shuzo Co., Ltd., Shiga, Japan) using primers. The cyclic program was 94°C for 30 s, 60°C for 30 s, 72°C for 60 s and 20 min in the last cycle and comprised 22 cycles. The cycle number was selected so that all primers fell in the linear part of the curve and not toward the plateau region. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers were derived from GenBank (<http://www.ncbi.nih.gov>) sequences and were designed by GeneWorks sequence analysis software (IntelliGenetics, Inc., Mountain View, CA). Primers specific for type I collagen (α 1(I) chain) (sense: 5'-CCAGCAGATCGA-GAACATCC-3'; antisense: 5'-ATCGACAGTGACGCTGTAGG-3'), type III collagen (sense: 5'-AACGGTCTCAGTGGAGAACG-3'; antisense: 5'-CCACTCTTGAGTTCAGGATGG-3') and GAPDH (sense: 5'-AACAGCCTCAAGATCATCAGC-3'; antisense: 5'-GGATGATGTTCTGGAGAGCC-3') were used and resulted in amplified products of 523, 869 and 199 bp, respectively. Densitometric analysis was performed using NIH imaging after resolving on 2% agarose gel stained with ethidium bromide. The resulting data were expressed as a ratio to the control value denoted as one.

Enzyme-linked immunosorbent assay (ELISA) for TGF- β 1 protein

TGF- β 1 in the cell culture supernate after 24 h cyclic stretch stimulation was measured by ELISA (R&D Systems, Minneapolis, MN). This assay employs the quantitative sandwich enzyme immunoassay technique. The cell culture supernate (0.5 ml) was mixed with 0.1 ml of 1 N HCl, incubated at room temperature for 10 min, and neutralized by 1.2 N NaOH and 0.5 M HEPES. TGF- β 1 protein was measured by ELISA using polyclonal antibody (R&D Systems, Minneapolis, MN).

Bioassay of TGF- β 1

The relationship between TGF- β 1 and mRNA expression of col-

lagen was investigated. Ligament fibroblasts were cultured in DMEM with 10% FBS. When the cultured cells became confluent, the culture medium was removed and the cells were washed with PBS. The culture medium was replaced with DMEM containing 0.5% FBS and treated with recombinant human TGF- β 1 (WAKO, Osaka, Japan) at a final concentration of 200 or 1000 pg/ml. The cells were cultured for 24 h and the mRNA expression was evaluated.

The activation of TGF- β 1 in the culture medium was estimated by bioassay. The ligament fibroblasts were placed on tissue culture plates in DMEM with 10% FBS for 24 h. The cells were washed with PBS and further incubated for 24 h with the conditioned medium which had been collected from either culture chambers of 24 h, 10% stretched or non-stretched cells. Then, mRNA expression of the type I and III collagen was examined by RT-PCR.

Effect of neutralizing antibody

Anti-TGF- β 1 antibody (R&D Systems, Minneapolis, MN) was used to determine if mechanical stretch-induced mRNA expression of collagen by ligament fibroblasts was mediated by TGF- β 1. The stretch experiments were repeated in the presence and absence of a neutralizing antibody. The ligament fibroblasts were stretched with anti-TGF- β 1 antibody (100 ng/ml) (Tsang *et al.*, 1995) or equal amounts of a control antibody (IgY).

Statistical analysis

Student's *t* test was used for statistical analysis. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Effects of stretch on cell alignment and proliferation

The ligament fibroblasts did not align microscopically in response to the axial strain for 24 h (data not shown). There were no significant differences in the cell numbers counted with a hemocytometer between control and stretched fibroblasts. The ratio of stretched fibroblasts to control was 1.03 ± 0.06 (mean \pm SEM).

Effect of stretch on collagen mRNA expression

Fig. 2 shows RT-PCR analysis of type I and type III collagen mRNA according to mechanical stretch. The results indicated that the mRNA expression of type I and type III collagen was increased by the cyclic stretch. The ratios of stretch to control values of type I and type III collagen were 1.63 ± 0.21 and 2.69 ± 0.39 , respectively.

Effect of stretch on TGF- β 1 protein

TGF- β 1 has been shown to induce collagen synthesis (Kresse and Schonherr, 2001). Since the mRNA expression of type I and type III collagen was increased by the cyclic stretch, TGF- β 1 secretion after the stretch was investigated.

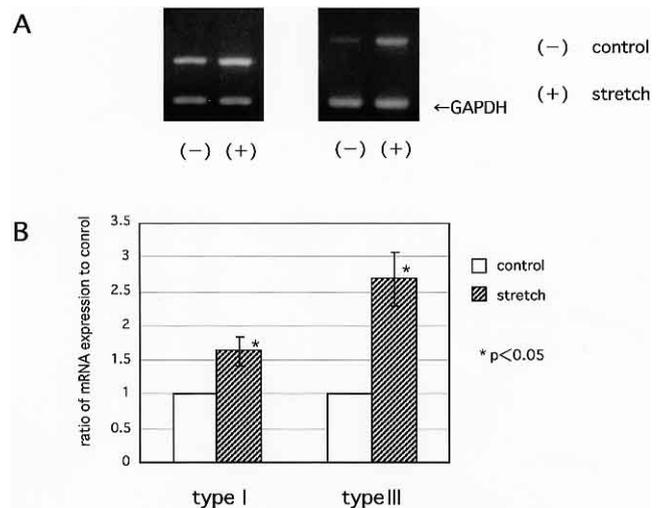


Fig. 2. (A) RT-PCR of type I and type III collagen mRNA in response to mechanical stretch. Integrated density values for polymerase products were normalized to the values for GAPDH. (B) The resulting data were then expressed and illustrated as a ratio of the control. Each bar represents mean \pm standard error of the mean (SEM) ($n=7$). The mRNA expression of type I and type III collagen was increased by the cyclic stretch. $*p < 0.05$.

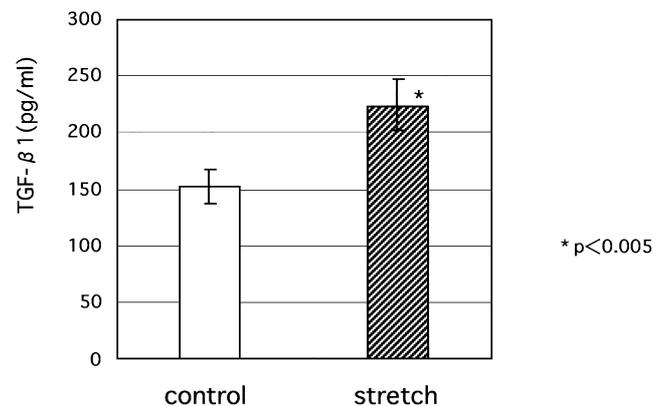


Fig. 3. Effect of stretch on the TGF- β 1 protein in the supernate. TGF- β 1 was increased by the stretch. TGF- β 1 protein was assayed by ELISA. Non-stretched cultures served as controls ($n=7$, mean \pm SEM). $*p < 0.005$.

TGF- β 1 protein in the cell culture supernate was shown in Fig. 3. It was found that TGF- β 1 protein in response to the stretch was increased to $149 \pm 11\%$.

Effect of TGF- β 1 on collagen mRNA expression

To determine if TGF- β 1 induces mRNA expression of type I and type III collagen in ligament fibroblasts, the effect of TGF- β 1 on collagen mRNA expression was investigated. Fig. 4 shows that the mRNA expression of type I and type III collagen in ligament fibroblasts was increased in the presence of exogenous TGF- β 1 with a dose-dependent manner. The ratios of mRNA of type I and type III collagen to control were 1.23 ± 0.01 and 1.99 ± 0.31 at a concentration of

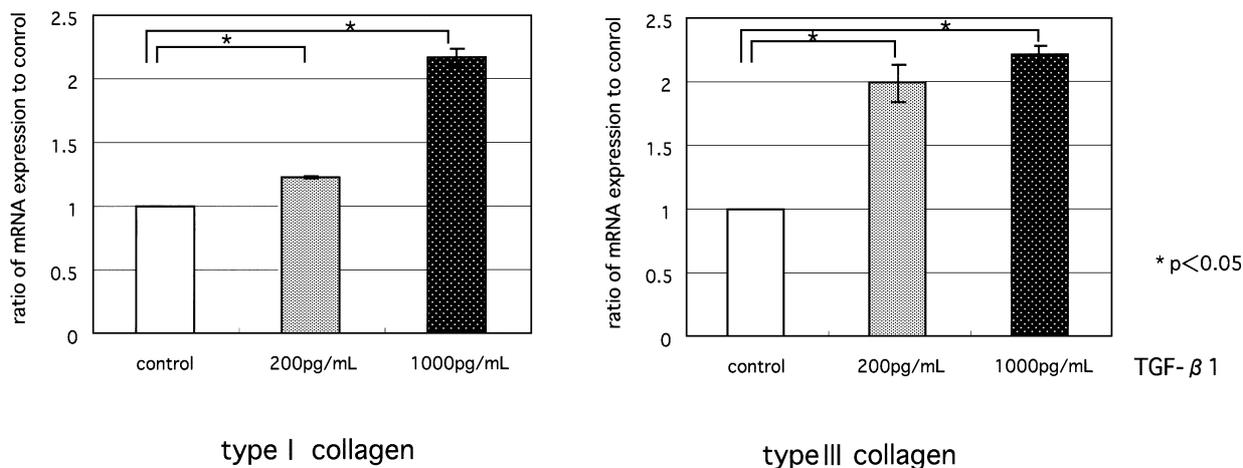


Fig. 4. TGF-β1 increased type I and type III collagen mRNA expression by ligament fibroblasts. The mRNA content was assayed by RT-PCR (n=3, mean±SEM). *p<0.05.

200 pg/ml, and 2.17±0.06 and 2.22±0.14 at 1000 pg/ml, respectively.

Bioassay by the conditioned medium

The bioassay by the conditioned medium was performed in order to evaluate whether stretch-induced TGF-β1 is active form. Differences of mRNA expression of the type I and type III collagen between control medium from non-stretched cells and conditioned one from stretched cells were shown in Fig. 5. The results indicated that the mRNA expression of the type I and type III collagen in the conditioned medium from stretched cells was increased in compared with that of control medium.

Effect of anti-TGF-β1 antibody

To determine whether the stretch-induced mRNA expression of type I and type III collagen is dependent on TGF-β1,

the TGF-β1 neutralizing antibody was added before the stretch. Effects of anti-TGF-β1 antibody on the stretch-induced mRNA expression of type I and type III collagen were shown in Fig. 6. It was found that the stretch-induced mRNA expression of type I and type III collagen was markedly inhibited in the presence of anti-TGF-β1 antibody whereas that of type I and type III collagen was increased by the stretch with IgY as a control.

Discussion

In the process of ligament healing, the synthesis of type I and type III collagen is stimulated by certain growth factors (Murphy *et al.*, 1994). Although a small amount of type I collagen is produced, most of the newly synthesized collagen is type III in the early phase of healing (Woo *et al.*,

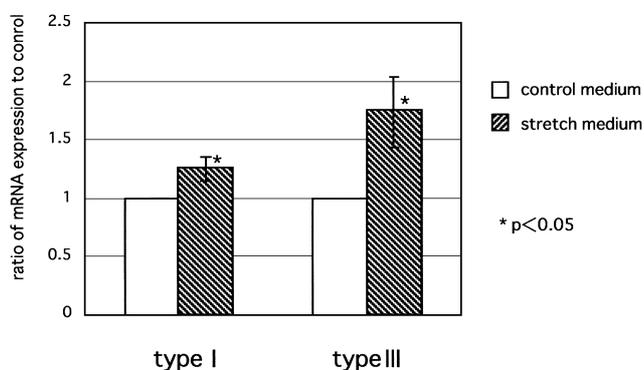


Fig. 5. Differences of mRNA expression of type I and type III collagen between control medium and stretch medium. The conditioned medium from stretched cells increased the mRNA expression. The mRNA content was assayed by RT-PCR (n=4, mean±SEM). *p<0.05.

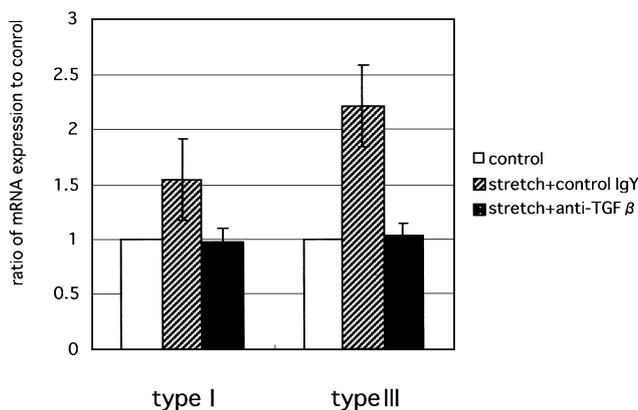


Fig. 6. Effect of anti-TGF-β1 antibody on the stretch-induced mRNA expression of type I and type III collagen. In the presence of neutralizing anti-TGF-β1 antibody, stretch-induced up-regulation of collagen mRNA expression was ablated. The mRNA content was assayed by RT-PCR (n=3, mean±SEM).

1999). The type III collagen is also increased in a remodeling process termed "ligamentization" after tendon graft (Amiel *et al.*, 1986). In our study, the mRNA expression of type III collagen was higher than that of type I. This result suggests that mechanical stretch of the ligament fibroblasts is a positive regulator in the healing and remodeling process.

The effect of equibiaxial strain on ligament fibroblasts was investigated (Hsieh *et al.*, 2000). The cells on a flexible circular membrane were stretched hemispherically by vacuum. ACL fibroblasts responded to cyclic strain by mRNA expression of higher levels of type I collagen with almost no significant increases in type III collagen. However, our study showed that mechanical stretch increased mRNA expression of both type I and type III collagen. Differences in the direction, duration, elongation rate and frequency of stretch may account for the inconsistency between them. Stretch direction may be especially important for the investigation of stretch response because the directions of mechanical loading are different among cell types *in vivo*. In order to mimic the physiological condition when ligament cells are stretched uniaxially, the system, which can apply uniaxial stretch to the cells *in vitro*, was developed in this study.

The alignments of a number of different type cells in response to cyclic surface deformation have been investigated (Naruse *et al.*, 1998; Neidlinger-Wilke *et al.*, 2001; Kato *et al.*, 1998). Skin fibroblasts and osteoblasts become oriented in response to axial strain. The cell orientation in response to cyclic culture dish deformation is primarily due to avoidance of large axial surface strains and is specific to the cell type. In our experiment, the ligament cells did not align in response to the stretch, thus ligament cells may be hard to align by such stretch. Because cells were stretched in subconfluent density in this study, the alignment might be affected by cell-cell contact.

TGF- β 1 is a multifunctional cytokine that has been shown to induce new matrix synthesis (Kresse and Schonherr, 2001). Intracellular signaling by TGF- β 1 occurs via serine/threonine kinase receptors. Smad2 and Smad3 are direct substrates of TGF- β kinase and interact with Smad4. Smad complexes then translocate from the cytoplasm into the nucleus and stimulate collagen transcription (Ghosh *et al.*, 2001). Several investigators have demonstrated that TGF- β 1 increases the collagen synthesis by ligament fibroblasts *in vitro* (DesRosiers *et al.*, 1996; Marui *et al.*, 1997). We showed that exogenous TGF- β 1 increased type I and type III collagen mRNA expression. The results of this study support previous investigators. Up-regulation of TGF- β 1 by mechanical stretch was observed in other cell types, including smooth muscle cells, cardiac fibroblasts and mesangial cells (Li *et al.*, 1998; Lee *et al.*, 1999; O'Callaghan and Williams, 2000; Gruden *et al.*, 2000). In ligament fibroblasts, we showed that cyclic stretch increased TGF- β 1 protein in the cell culture supernate.

Although some studies have shown increased extracellular matrix synthesis in response to stretch loading (Li *et al.*, 1998; Lee *et al.*, 1999; O'Callaghan and Williams, 2000), the signal pathway of mechanical stress remains unclear. This study showed that neutralizing anti-TGF- β 1 antibody ablated stretch-induced up-regulation of collagen mRNA expression. This result suggests that TGF- β 1 up-regulated by mechanical stretch stimulates gene expression of type I and type III collagen. Naruse *et al.* (1998) demonstrated that stretch-activated calcium influx through stretch-activated (SA) channels was essential in the molecular mechanism of converting physical forces to biochemical signals. Fluid-induced shear stress increased intracellular calcium concentration in ligament fibroblast (Gruden *et al.*, 2000). In human mesangial cells, stretch activates, via a protein kinase C-dependent mechanism, p38 mitogen-activated protein (MAP) kinase, which independently induces TGF- β 1 (Hung *et al.*, 1997). Schwachtgen *et al.* (1998) demonstrated that treatment of human endothelial cells with PD98059, a MAP kinase kinase inhibitor, inhibited shear stress activation of early growth response-1 (*egr-1*). However, Yamamoto *et al.* (1999) reported that neither PD98059 nor SB203580, a MAP kinase inhibitor, inhibited the effect of mechanical stretch on tenascin-C mRNA expression in rat cardiac myocytes.

TGF- β 1 is one of the most important regulators in the healing and remodeling process of the ligament. The temporal and spatial expression of TGF- β 1 in the healing rat patellar ligament was investigated by immunohistochemistry (Natsu-ume *et al.*, 1997). It was shown that wounding provoked a rapid rise in TGF- β 1 levels at the site of patellar ligament injury which persisted for at least 8 weeks. There was an expression of TGF- β 1 at the adjacent uninjured site for 4 weeks. This result suggests that TGF- β 1 is an important cytokine over the whole length of ligament tissue in the healing process. Localization of growth factors in the reconstructed ACL was examined in the canine model (Kuroda *et al.*, 2000). In the reconstructed graft, stained levels for TGF- β 1 were increased in early postoperative period. In our study, mechanical stretch increased production of TGF- β 1, which stimulated mRNA expression of type I and type III collagen. Temporal regulation of mechanical stretch to ligament fibroblasts *in vivo* and *in vitro*, which can be a controlled release system of autocrine TGF- β 1, will allow design of novel strategies for graft surgery and tissue engineering.

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