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ORIGINAL ARTICLE

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Cyclic mechanical stretching modulates secretion pattern of growth factors in human tendon fibroblasts

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Abstract The objective of the study was to investigate whether the response profile of the growth factor of human tendon fibroblasts could be beneficially influenced through the application of mechanical stretch. It was considered that this would elucidate structural and functional problems, often seen after tendon and ligament healing. The secretion pattern of transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) was determined in mechanically stretched fibroblasts and compared to non-stretched controls. Human tendon fibroblasts were experimentally stretched for 15 and 60 min at a frequency of 1 Hz and an amplitude of 5%. The secretion of TGF-β, PDGF and bFGF was measured by enzyme-linked immuno-sorbent assay. All the growth factors investigated were indeed secreted by human tendon fibroblasts both in stretched cells and controls. Mechanical stretch increased the secretion pattern of the growth factors. The increased concentrations of TGF-β, bFGF and PDGF after cyclical mechanical stretching may have a positive influence on tendon and ligament healing through stimulation of cell proliferation, differentiation and matrix formation.

Keywords Cytokines · Human tendon fibroblasts · Secretion pattern

Introduction

The response profile of transforming growth factor (TGF-β), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) in healing tissue is thought to play an important role in the healing of tendons and ligaments. The TGF-β, PDGF and bFGF influence fibroblast growth performance (Dennison et al. 1994; Haase et al. 1998; Hamilton et al. 1994; Mihara et al. 1995). The TGF-β induces proliferation of connective tissue cells via a complex control of an autocrine PDGF loop (Battegay et al. 1990). The bFGF controls growth and differentiation of fibroblasts, epithelial and neuroectodermal cells and plays an important role in wound healing and angiogenesis (Ibelgaufts 1992). The TGF-β also plays an important role in influencing programmed cell death (Liu et al. 1999; Petrucci et al. 1999; Wakisaka et al. 1998; Wyllie 1997). The FGF and TGF-β also control the morphogenesis of digits in the embryo, indicating possible involvement of these cytokines in tendon repair (Merino et al. 1998). In mature tendon fibroblasts, FGF and PDGF were shown to stimulate proliferation in a serum-free medium in a rat in vitro model (Stein 1985).

In the transduction of mechanical forces/stretch, the pleiotropic function of cytokines is considered to play a physiological role in the fate of fibroblasts in terms of cell proliferation, differentiation and matrix formation. Progressive rehabilitation is widely used following tendon and ligament injuries and surgery (Gamble et al. 1984; Noyes 1977; Woo et al. 1982). However, the amount of loading necessary to improve/accelerate the healing process without causing damage to the healing tissue remains unclear (Steadman et al. 1989; Zeichen et al. 2000). It has been demonstrated by Banes et al. (1999) that a mechanical load stimulates the expression of genes in chicken tendon cells. Whether mechanical stretching directly influences cytokine secretion is, however, unclear. Furthermore, the possibility of ligament/tendon fibroblasts being able to secrete cytokines is also unknown.

To elucidate this matter, the aim of the present in vitro study was to investigate whether a defined biaxial mechanical stretch of human fibroblasts would influence the secretion pattern of PDGF, TGF-β and bFGF in a patient-dependent model.

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Methods

Source of cells

Topographic tendon samples were taken from healthy human patellar tendons during reconstruction of the anterior cruciate ligament. All patients [$n = 6$, mean (SEM) age 31.5 (7.8) years] had sustained a traumatic tear of the anterior cruciate ligament. Only excess material from the trimmed patellar tendon transplant was used. Samples, 1 mm^2 in area, were cut and human fibroblasts were cultivated in Dulbecco's modified Eagle medium + 10% fetal calf serum. After the second passage, cells were trypsinized and 150,000 cells were seeded into silicone dishes. After 3 days growth, serum reduction to 1% was performed to arrest the cells in the G0/G1 phase. Cell cultures were investigated to confirm their purity using specific fibroblast antibodies (Saalbach et al. 1996). Furthermore, fibronectin detection was performed (data not shown).

Cell stimulation device

Cyclic biaxial mechanical stretching of a physiologically relevant magnitude was carried out for 15 and 60 min, using an electromechanical stimulation device (Fig. 1) (Baskin et al. 1993; Neidlinger-Wilke et al. 1994). The device applied cyclical biaxial stretch to the silicone dish on which the cells were grown to simulate physiological loading conditions (Duncan and Turner 1995). Constant factors were the temperature (37°C), an atmosphere containing 5% CO_2 having a relative humidity of 90%, and the frequency (1 Hz) and amplitude (5%) of stretching. As controls, non-stretched fibroblasts were used. The experiment was concluded 2, 4 and 8 h after the end of the mechanical stretching.

Quantification of TGF-β, PDGF, and bFGF

For quantification of the cytokines human immunoassays (TGF-β1, PDGF-AB, and bFGF, R + D Systems, Minneapolis, USA) were used. A specific monoclonal antibody against the cytokine was precoated on to a microtiterplate. Standards and samples were pipetted into the wells and any cytokine present was specifically bound by the immobilized antibody. After washing out any unbound substances, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, 3,3',5,5'-tetramethylbenzidine solution was added to the wells after which a blue colour developed in proportion to the amount of the cytokine bound during the initial step. The reaction was stopped using sulphuric acid and the optical density of the yellow solution so formed was measured spectrophotometrically at 450 nm. The coefficients of variation (CV) for cell culture supernate intra-assay precision were 5.2% (TGF-β), 2.8% (PDGF) and 5.7% FGF-basic.

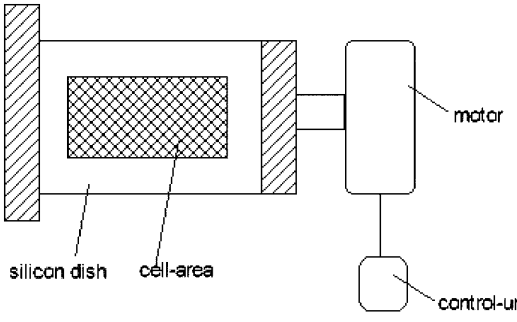

Fig. 1 Cell stimulation device. Cyclical biaxial stretch was applied to the silicone dish on which the cells were grown to simulate physiological loading conditions. Throughout all experiments, a frequency of 1 Hz and an amplitude of 5% were used

Statistical analysis

Results are expressed as the mean and SEM. A summary has been displayed in the figures using box plots. Each box shows the median, the interquartile range and the extreme values within each group. Specific stretch-induced cytokine release was calculated by dividing the measured value from the sample of stretched cells by the corresponding value from the control cells. To compare the means of the stretched cells with those of the controls the Kruskal-Wallis test was used, and a significance level of $P < 0.05$ was accepted.

Results

Effects of mechanical stretching on cytokine production in vitro by human tendon fibroblasts

The cytokines TGF-β, bFGF and PDGF were indeed constitutively secreted by human tendon fibroblasts as was observed in the control sample. Stretch was able to alter this secretion. There was a time-dependent increase or decrease in the cytokines secreted, implying a stretch-dependent change in the secretion pattern. The differences between the stretched cells and the controls were marked, but they were not, however, statistically significant ($P > 0.05$).

Secretion of bFGF

The mean concentrations of bFGF at 2 h after 15 min of cyclic mechanical stretching averaged 10.59 (10.12) $pg \cdot ml^{-1}$ in the stretched cells and 14.26 (19.29) $pg \cdot ml^{-1}$ in the unstretched cells. Concentrations increased with time (Fig. 2A), however, the specific stretch-induced cytokine release decreased with time (Fig. 3). After 60 min of stretching a similar pattern was observed (Fig. 2B). The specific stretch-induced bFGF release was increased after 2 h and remained high with time (Fig. 3).

Secretion of TGF-β

Secretion of TGF-β after 15 and 60 min of stretching was increased compared to unstretched controls at all times (2,4 and 8 h, Fig. 4 A, B, respectively). The specific stretch-induced release of TGF-β at 2 h and after 15 min of stretching was increased by a factor of 1.26 (0.25) and after 60 min of stretching by a factor of 1.40 (0.23) (Fig. 5).

Secretion of PDGF

Initially, the secretion of PDGF after 60 min of stretching was lower compared to that in nonstretched controls; however it did increase with time (Fig. 6). The specific stretch-induced release of PDGF was increased after 60 min of stretching at 2,4 and 8 h and increased with time (Fig. 7). After 15 min of stretching, however,

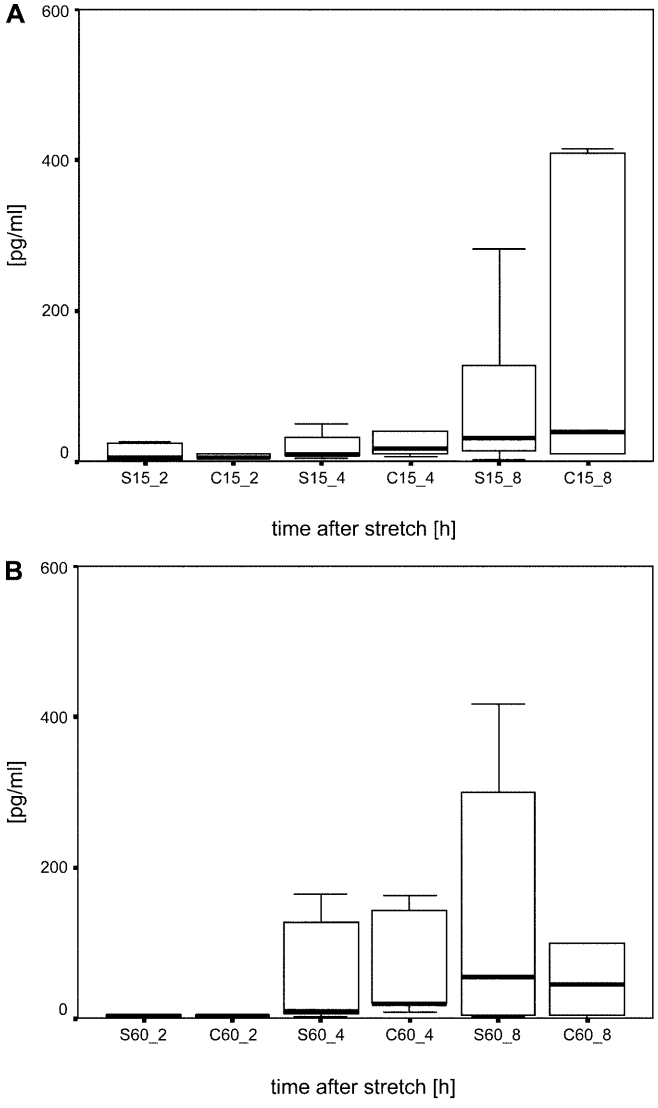

Fig. 2 Summary of basic fibroblast growth factor concentration after 15 min **A** and 60 min **B** of cyclic mechanical stretching in stretched cells (*S15* and *S60*, respectively) and controls (*C15* and *C60*, respectively), measured 2, 4, and 8 h after the end of stretching. *Boxes* show the median, interquartile range and extreme values in the two groups

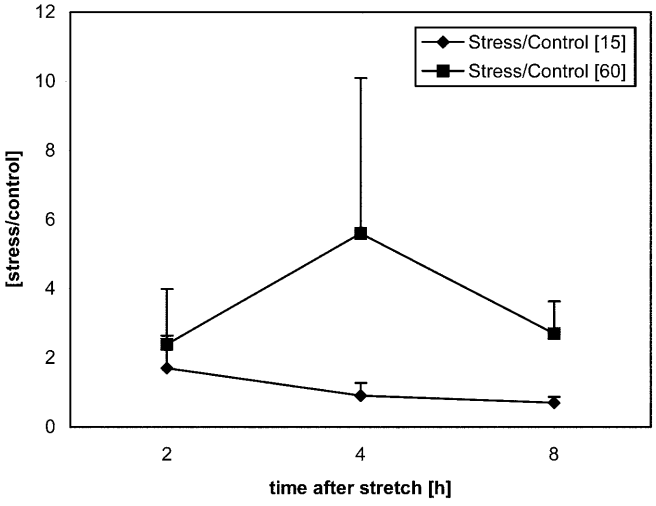

Fig. 3 Specific stretch-induced release of basic fibroblasts growth factor at 2, 4, and 8 h after 15 and 60 min of cyclic mechanical stretching compared to non-stretched controls. Stretch/control mean and SEM

the specific stretch-induced cytokine release was lower than in the controls at all observation times.

Discussion

It was intended to determine if a defined mechanical stretching would experimentally influence the cytokine secretion pattern in human tendon fibroblasts. Cyclic biaxial stretching was therefore applied for 15 and 60 min to cultured fibroblasts from six patients using an electromechanical stimulation device.

The design of the study employed arbitrary stress cycles, used an artificially constructed medium and therefore isolated the human tendon fibroblasts from the benefits of their normal in vivo environment. However, culture conditions were continually controlled, and the setup offered the possibility of altering the intensity and duration of the stretching. Furthermore, the isolation of the tendon fibroblasts from the effects of systemic metabolic changes allows the findings to be correlated with the fibroblasts examined, for the medium did not contain any growth factors.

The fact that mechanical stretching was seen to alter the secretion-pattern of growth factors in human tendon fibroblasts represented a novel observation. Moreover, the increased concentrations of TGF-β, bFGF and PDGF after stretching may have a positive influence on tendon and ligament healing through the stimulation of cell proliferation. The individual differences in the responses of the six different fibroblast samples to stretching in terms of the cytokine secretion might be one reason for the lack of significance in this study and this is the subject of further investigations. It was found in earlier studies, that TGF-β, PDGF and FGF influence fibroblast growth performance (Dennison et al. 1994; Haase et al. 1998; Hamilton et al. 1994; Mihara et al. 1995). The secretion of TGF-β, bFGF and PDGF from human fibroblasts has been previously demonstrated. Wilson et al. (1993) found that mechanical strain induced growth of smooth muscle cells via autocrine PDGF stimulation. An increased proliferation was detected using ^{3}H-thymidine incorporation. Cell proliferation was observed after 48 h (Wilson et al. 1993). Bishop et al. (1998) reported that mechanical load enhanced the stimulatory effect of PDGF on procollagen synthesis of pulmonary artery fibroblasts. It was shown by Hamilton et al. (1994) that a combination of bFGF and PDGF resulted in increased DNA synthesis in synovial fibroblasts. The TGF-β was shown to promote proliferation of cultured smooth muscle cells via both PDGF-AA-dependent and PDGF-AA-independent

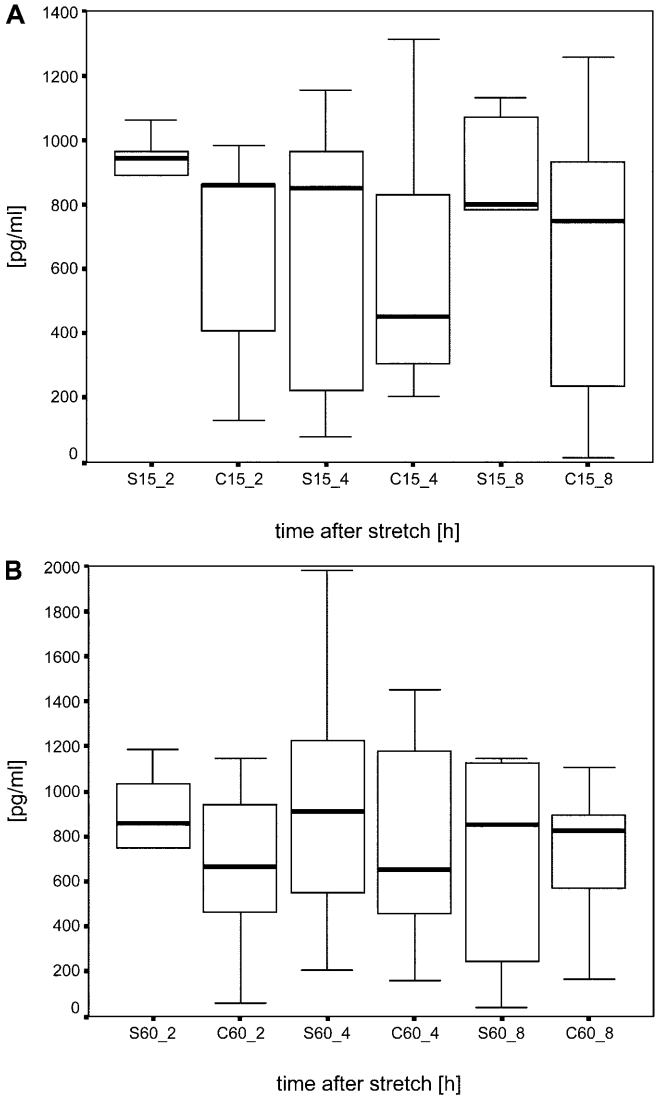

Fig. 4 Summary of transforming growth factor beta concentration after 15 min **A** and 60 min **B** of cyclic mechanical stretching in stretched cells (*S15* and *S60*, respectively) and controls (*C15* and *C60*, respectively), measured 2, 4, and 8 h after the end of stretching. *Boxes* show the median, interquartile range and extreme values in the two groups

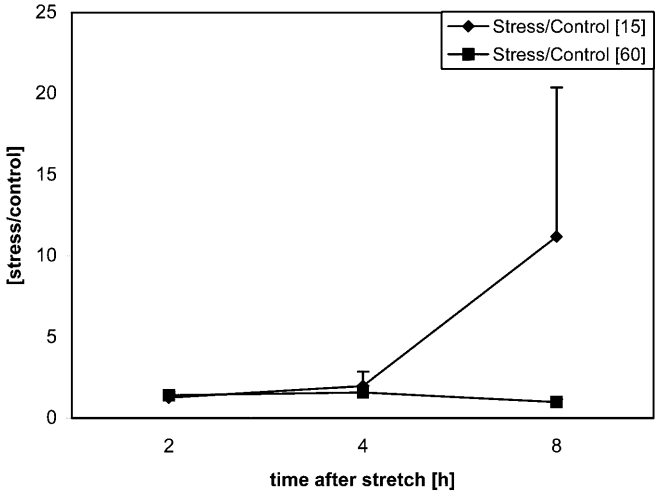

Fig. 5 Specific stretch induced release of transforming growth factor beta at 2, 4, and 8 h after 15 min and 60 min of cyclic mechanical stretching compared to non-stretched controls. Stretch/control mean and SEM

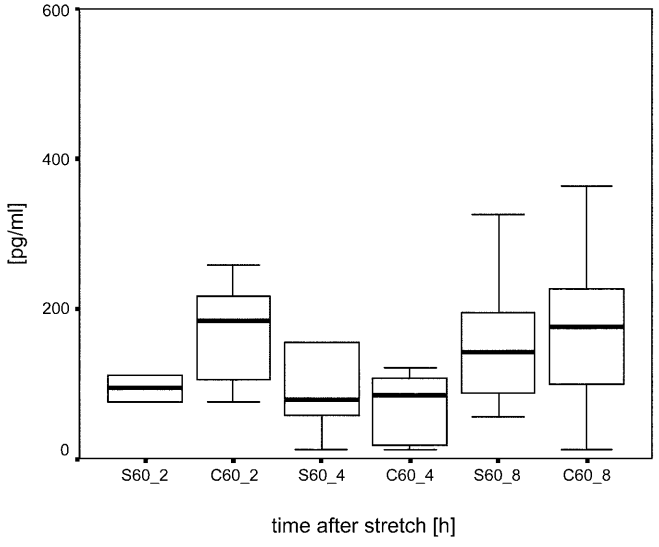

Fig. 6 Summary of platelet derived growth factor concentration after 60 min of cyclic mechanical stretching in stretched cells (*S60*) and controls (*C60*), measured 2, 4, and 8 h after the end of stretching. *Boxes* show the median, interquartile range and extreme values in the two groups

mechanisms (Stouffer and Owens 1994). The TGF-β and bFGF also influenced positively wound healing via cell differentiation and proliferation (Pierce et al. 1992). Seko et al. (1995) demonstrated the influence of bFGF as a potent growth stimulator and TGF-β as a growth regulator on scleral chondrocytes and scleral fibroblasts in vitro. There is also some evidence that a variety of growth factors are present in uninjured and healing digital canine flexor tendons (Duffy et al. 1995).

In the present study, TGF-β, bFGF and PDGF were indeed secreted by the cultured human tendon fibroblasts. There was a change in the secretion pattern induced by stretching compared to that seen in the non-stretched controls. These increased concentrations of the cytokines investigated may contribute to tendon and ligament healing. The decreased concentrations of PDGF as seen after 15 min and partially after 60 min of stretching also represent an interesting finding. This might cause a delayed cell proliferation and consequently slow down the healing process.

Clinical relevance

Mechanical stretching stimulated release of TGF-β, bFGF and partially PDGF from human fibroblasts in vitro. The observation that mechanical stretching has a positive influence on tendon and ligament healing in

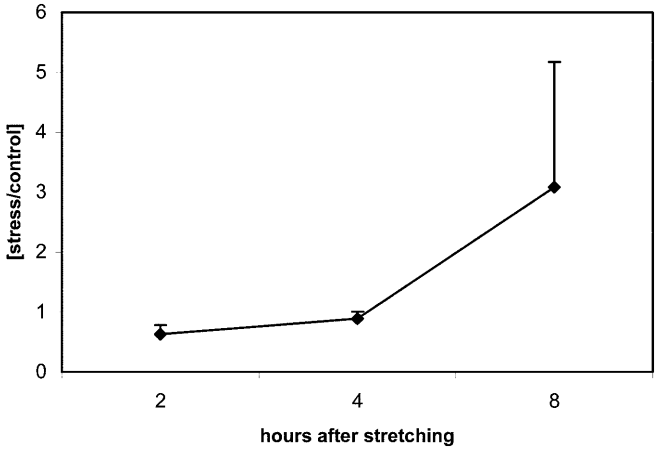

Fig. 7 Specific stretch-induced release of platelet derived growth factor at 2, 4, and 8 h after 60 min of cyclic mechanical stretching compared to non-stretched controls. Stretch/control mean and SEM

vivo may be explained in part by the direct release of growth factor from the tendon fibroblasts. The TGF-β, bFGF and PDGF measured in the synovial fluid of injured joints might be an important predictive parameter to monitor and to improve therapeutic strategies in terms of tendon/ligament healing and is the subject of further investigations.

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