The responses of extrinsic fibroblasts infiltrating the devitalised patellar tendon to IL-1 β are different from those of normal tendon fibroblasts

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In order to clarify the role of cytokines in the remodelling of the grafted tendon for ligament reconstruction we compared the responses to interleukin (IL)-1 β , platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)- β 1 of extrinsic fibroblasts infiltrating the frozen-thawed patellar tendon in rats with that of the normal tendon fibroblasts, in regard to the gene expression of matrix metalloproteinase (MMP)-13, using Northern blot analysis. We also examined, immunohistologically, the local expression of IL-1 β , PDGF-BB, and TGF- β 1 in fibroblasts infiltrating the frozen-thawed patellar tendon.

Northern blot analysis showed that fibroblasts derived from the patellar tendon six weeks after the freeze-thaw procedure *in situ* showed less response to IL-1 β than normal tendon fibroblasts with respect to MMP-13 mRNA gene expression. The immunohistological findings revealed that IL-1 β was over-expressed in extrinsic fibroblasts which infiltrated the patellar tendon two and six weeks after the freeze-thaw procedure *in situ*, but neither PDGF-BB nor TGF- β 1 was over-expressed in these extrinsic fibroblasts. Our findings indicated that IL-1 β had a close relationship to matrix remodelling of the grafted tendon for ligament reconstruction, in addition to the commencement of inflammation during the tissue-healing process.

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It has been shown that in tendon autografts used for the reconstruction of ligaments, repopulation by fibroblasts from an extrinsic origin occurs during revascularisation after the intrinsic fibroblasts in the tendon have died.¹⁻³ Numerous studies have shown that various types of cytokine, including interleukin-1 (IL-1), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF- β), are over-expressed in fibroblasts in the tissue during healing.⁴ These factors are known to regulate the synthesis and degradation of collagen by the fibroblasts.^{5,6} It is therefore possible that extrinsic fibroblasts infiltrating necrotised tendons over-express IL-1, PDGF and TGF- β . We have previously observed that extrinsic fibroblasts infiltrating necrotic tendons have significantly different biological characteristics from those of intrinsic fibroblasts in normal tendon as regards proliferation and invasive migration into patellar tendon.⁷ There is also a possibility that extrinsic fibroblasts infiltrating the necrotised tendons respond to cytokines differently from the intrinsic fibroblasts in the normal tendon. No studies have compared the responses to cytokines of the two types of fibroblast derived from tendon.

We compared the responses to IL-1 β , PDGF-BB, and TGF- β 1 of extrinsic fibroblasts infiltrat-

ing necrotic patellar tendon and intrinsic fibroblasts in normal tendon as regards the gene expression of matrix metalloproteinase (MMP)-13 using Northern blot analysis, and examined immunohistologically the expression of IL-1 β , PDGF-BB, and TGF- β 1 in the extrinsic fibroblasts infiltrating the necrotic tendons after freeze-thaw treatment *in situ*.

Materials and Methods

We used 15 male 16-week-old Wistar-King rats with a mean weight of 350 g (330 to 370). In order to compare the responses to IL-1 β , PDGF-BB, and TGF- β 1 of the extrinsic fibroblasts infiltrating the necrotic patellar tendon and the intrinsic fibroblasts, Northern blot analysis was carried out to evaluate the gene expression of MMP-13 in these two types of fibroblast after stimulation with IL-1 β , PDGF-BB or TGF- β 1. An immunohistological study examined the local expression of IL-1 β , PDGF-BB, and TGF- β 1 in the patellar tendon after fibroblast necrosis. All the surgical procedures were carried out in the Institute of Animal Experimentation at Hokkaido University School of Medicine under the Rules and Regulations of the Animal Care and Use Committee.

Experimental design. For Northern blot analysis, we used nine rats. Freeze-thaw treatment was performed *in situ* on the right patellar tendons, while the left knees were left untreated. These rats were killed six weeks later and the patellar tendons harvested. The extrinsic fibroblasts and the normal fibroblasts for Northern blot analysis were obtained from both patellar tendons. The remaining six rats were used for immunohistological evaluation. In these we performed freeze-thaw treatment *in situ* on the right patellar tendon and a sham operation on the left knee. Three were killed at two weeks and the other three at six weeks after surgery, and the patellar tendons on both sides were evaluated immunohistologically.

In situ freeze-thaw procedure. The rats were operated on under sterile conditions and anaesthetised with pentobarbital (50 mg/kg, interperitoneally). The patellar tendon was frozen *in situ* for one minute by liquid nitrogen using a silicone rubber sheet.⁸ The frozen tendon was then thawed using physiological saline. Our previous study had shown that there was no cell outgrowth from explants of the tendons which had undergone this freeze-thaw treatment during incubation for two weeks in the culture medium.⁸

Isolation of fibroblasts. After freeze-thaw treatment of the right patellar tendon, each rat was allowed unrestricted activity in its cage. In a previous study, using an identical procedure, we found that fibroblasts were observed in the anterior and posterior parts of the tendon after three to six weeks, while they were widely distributed throughout the thawed tendon at 12 weeks.⁸ We therefore selected a time point of six weeks because a number of extrinsic fibroblasts would have infiltrated the necrotic patellar tendon by then and there was a possibility that cellular infiltration would be complete by 12 weeks. For each rat, the extrinsic fibroblasts and the normal fibroblasts were obtained from the right patellar tendon six weeks after freeze-thaw treatment and from the left untreated tendon. The outer synovial layer was removed by sharp dissection and the tendons cut into five pieces, 2 mm to 5 mm in size, and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. A confluent monolayer formed for 14 days or less. When the cells became confluent in the primary tissue-culture dishes, we seeded the cultured fibroblasts from the primary tissue culture into a single 60 mm × 15 mm dish at 10⁵ cells at the first passage. They were subcultured at the initial number of 2.5 × 10⁵ cells through the second and third passages. Cells from the third passage were used for all experiments.

Northern blot analysis. To examine the effect of IL-1β, PDGF-BB, and TGF-β1 on the mRNA expression of MMP-13 cultured fibroblasts from the third passage were used throughout the experiment. We used 60 mm × 15 mm dishes for all Northern blot experiments. After reaching confluence, the fibroblasts were rinsed with phosphate-buffered saline, serum-starved for 24 hours, and challenged with recombinant rat IL-1β (R & D Systems, Minneapolis,

Minnesota), recombinant human PDGF-BB (R & D Systems) or recombinant human TGF-β1 (R & D Systems) in 10 ml of serum-free DMEM. Northern blot analyses were performed using the templates of rat MMP-13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA which were obtained from a cDNA library (Takara Bio Inc., Ohtu, Japan) of rat synovial fibroblasts. After hybridisation the RNA was transferred on to a nylon membrane, the radioactive bands were then visualised by autoradiography on Kodak X-AR5 film and quantitatively analysed using the NIH Image system (National Institute of Health, Bethesda, Maryland).

Immunohistological examination. The right patellar tendons of six rats underwent freeze-thaw treatment *in situ* to devitalise it,⁸ while their left knees had a sham operation. These rats were killed at two and six weeks after the freeze-thaw treatment and both patellar tendons were evaluated immunohistologically. At two or six weeks the rats were perfused transcardially with 100 ml of 10% neutral buffered formalin under anaesthesia. Immunohistochemical staining was performed using Histofine Simple Stain Kit (Rat PO Multi; Nichirei, Tokyo, Japan) with anti-IL-1β antiserum (Yanaiharu, Shizuoka, Japan), anti-PDGF-BB polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California), and anti-TGF-β monoclonal antibody (Genzyme, Cambridge, Massachusetts).

Statistical analysis. For each Northern blot procedure, all the intensities of the radioactive bands were standardised with respect to those without cytokine stimulation for normal fibroblasts. The relative intensities of MMP-13 to GAPDH were then compared as an internal control between extrinsic and normal tendon fibroblasts using paired *t*-tests at each condition. We also compared the relative intensities of MMP-13 six hours after cytokine stimulation with those without stimulation using paired *t*-tests for each cytokine. The significance limit was set at *p* = 0.05.

Results

Effects of cytokine stimulation on MMP-13 mRNA expression. We carried out Northern blot analysis for the dose dependency and time course using different cell sources. Stimulation with IL-1β and PDGF increased the MMP-13 gene expression of extrinsic fibroblasts depending on the amount of cytokine up to 10 ng/ml and 100 ng/ml respectively, while TGF-β stimulation decreased the MMP-13 gene expression for up to 10 ng/ml (Fig. 1a). The time-course study showed that the MMP-13 gene expression of fibroblasts increased or decreased with time for up to six hours after cytokine stimulation (Fig. 1b). Therefore, we compared the MMP-13 gene expression of extrinsic fibroblasts with that of normal tendon fibroblasts six hours after stimulation with IL-1β of 10 ng/ml, PDGF of 100 ng/ml and TGF-β1 of 10 ng/ml (Fig. 1c).

The relative levels of intensity of MMP-13 mRNA were significantly higher in the extrinsic fibroblasts than in the normal patellar tendon fibroblasts (paired *t*-test, *p* = 0.010;

Fig. 1 Northern blot analysis of MMP-13 mRNA levels showing a) the effects of the cytokine concentration on MMP-13 mRNA of extrinsic fibroblasts, b) the time course of MMP-13 mRNA of extrinsic fibroblasts after cytokine stimulation and c) the comparison of MMP-13 between normal patellar tendon fibroblasts (n) and extrinsic fibroblasts (e).

Table I). The induction level of MMP-13 mRNA expression by IL-1 β stimulation was significantly lower in extrinsic fibroblasts than in the normal patellar tendon fibroblasts (paired *t*-test, $p = 0.005$), while IL-1 β stimulation significantly increased the MMP-13 mRNA expression in both types (paired *t*-test, $p = 0.001$). Stimulation with PDGF-BB did not have a significant effect on the MMP-13 mRNA expression of either type of fibroblast, while the relative level of MMP-13 mRNA after PDGF-BB stimulation was significantly higher in extrinsic fibroblasts than in the normal tendon fibroblasts (paired *t*-test, $p = 0.005$). Stimulation with TGF- β 1 significantly decreased the MMP-13 mRNA expression in both types of fibroblast. There was no significant difference in MMP-13 mRNA expression after

TGF- β 1 stimulation in extrinsic and normal fibroblasts (paired *t*-test, $p = 0.252$).

Local expression of cytokines in extrinsic fibroblasts infiltrating the necrotised tendons. At two weeks after freeze-thaw treatment, no cells were seen in the deep portion of the midsubstance, while cellular infiltration was observed at the superficial portion close to the infrapatellar fat pad (Fig. 2a) and IL-1 β -positive cells were found here. At six weeks, a number of fibroblasts were scattered in the tendon and some of these cells expressed IL-1 β (Fig. 2b). Tendons with a sham procedure had a minimal amount of staining for IL-1 β (Fig 2c and d). Concerning the local expression of PDGF-BB (Fig. 3) and TGF- β (Fig. 4), few positive cells were found in the tendons after the freeze-thaw treatment or the sham operation.

Table I. Comparison of the MMP-13 gene expression between extrinsic and normal tendon fibroblasts six hours after cytokine stimulation

	Mean ($\pm$ SD) relative intensity ratio of MMP-13 mRNA/GAPDH mRNA		
	Extrinsic fibroblasts	Normal fibroblasts	p-value
No cytokines	2.01 $\pm$ 0.48	1.00	0.010
IL-1 β (10 ng/ml)	13.88 $\pm$ 5.84*	18.48 $\pm$ 7.34*	0.005
PDGF-BB (100 ng/ml)	2.13 $\pm$ 0.76	1.41 $\pm$ 0.49	0.005
TGF- β (10 ng/ml)	0.36 $\pm$ 0.19*	0.39 $\pm$ 0.14*	0.252

* significantly different from the values without cytokine stimulation ($p < 0.05$)

Fig. 2a

Fig. 2b

Fig. 3a

Fig. 3b

Fig. 2c

Fig. 2d

Fig. 3c

Fig. 3d

Photomicrographs showing the local expression of IL-1 β in the patellar tendon a) two weeks and b) six weeks after freeze-thaw treatment and c) two and d) six weeks after the sham operation (streptavidin-biotin and haematoxylin $\times$ 100).

Photomicrographs showing the local expression of PDGF-BB in the patellar tendon a) two weeks and b) six weeks after freeze-thaw treatment, and c) two and d) six weeks after the sham operation (streptavidin-biotin and haematoxylin $\times$ 100).

Discussion

Fibroblasts from an extrinsic origin repopulate the transplanted patellar tendon after fibroblast necrosis during the early phase after ligament reconstruction. These extrinsic fibroblasts remodel the graft matrix.¹⁻³ IL-1, PDGF, and TGF- β are known to regulate the synthesis and degradation of collagen by the fibroblasts.^{5,6,9} Our study was undertaken to clarify the intrinsic response of the fibroblasts which infiltrate the necrotic patellar tendon to IL-1 β , PDGF-BB, and TGF- β 1 by assessing the gene expression of MMP-13. Our findings showed that fibroblasts derived from the patellar tendon six weeks after the freeze-thaw procedure had less response to IL-1 β than normal tendon fibroblasts with respect to the regulation of MMP-13 mRNA. IL-1 β was found to be over-expressed in the extrinsic fibroblasts which had infiltrated the necrotic patellar tendon.

We have previously compared the characteristics of extrinsic fibroblasts with those of normal tendon fibroblasts in a rabbit model.⁷ In the present study we selected the rat model to obtain the antibodies for immunohistology and the probe for Northern blot analysis. Size and species differences may have affected the reliability and validity of this model. Our previous study in rats using the freeze-thaw technique *in situ* showed that this devitalised all the fibroblasts in the patellar tendon. Extrinsic fibroblasts then entirely infiltrated the patellar tendon by six weeks after freeze-thawing and they expressed more type-III collagen than the fibroblasts in the normal patellar tendon.⁸ In the present study, for the Northern blot analysis we used cells derived from the patellar tendon six weeks after the freeze-thaw treatment. Immunohistological assessment showed that no cells were seen in the deep portion of the midsub-

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

Photomicrographs showing the local expression of TGF- β in the patellar tendon a) two weeks and b) six weeks after freeze-thaw treatment and c) two and d) six weeks after the sham operation (streptavidin-biotin and haematoxylin $\times 100$).

stance two weeks after freeze-thaw treatment, while cellular infiltration was observed at the superficial portion close to the infrapatellar fat pad. Therefore, it was difficult to isolate extrinsic fibroblasts from the patellar tendon two weeks after freeze-thaw treatment, but by six weeks, a number of fibroblasts were scattered in the tendon and we easily obtained extrinsic fibroblasts by using our tissue-explant technique.

With the Northern blot analysis, we evaluated the mRNA of MMP-13 six hours after cytokine stimulation. As shown in Figure 1b, we observed the peak of MMP-13 mRNA in response to IL-1 β or PDGF stimulation six hours after stimulation. We therefore used six hours as the time point for evaluation of MMP-13 with Northern blot analysis.

Collagenases are MMPs which can initiate the cleavage of collagen fibrils at neutral pH.⁹ Three collagenases have been described: collagenase 1 (MMP-1), collagenase 2 (MMP-8), and collagenase 3 (MMP-13).⁹⁻¹¹ MMP-1 and MMP-13 degrade type-1 collagen fibrils with similar efficiency.⁹ Unlike in man, the rat expresses only one interstitial collagenase, now named MMP-13, because it has a high degree of homology of function and sequence (86%) with human MMP-13, but not with human MMP-1.¹² Consequently, MMP-13 is considered to be central to the process of collagen degradation and matrix breakdown in the rat. The present study demonstrated that IL-1 β induced the gene expression of MMP-13 in extrinsic fibroblasts which were derived from the devitalised patellar tendon as well as

in normal tendon fibroblasts. It is thought that tendon cells would express receptors and respond to IL-1 in an initial 'molecular inflammation' cascade, the connective tissue-cell expression of cytokines which induce matrix-destructive enzymes.^{6,13} For example, Tuszaki et al¹³ reported that IL-1 β stimulated the MMP-1, -3, and -13 gene expression of normal human tendon cells. Their findings were consistent with those of our present study. Therefore, interstitial collagenases could be induced by IL-1 expressed by the fibroblasts infiltrating the grafted tendon, and such collagenases could then destroy the matrix of the graft during the remodelling of the grafted tendon. A previous study has shown that the expression of mRNA for collagenase in cultured fibroblasts from hypertrophic scars after skin wounds was lower than that from normal dermis.¹⁴ Another found that no change in MMP-1 or MMP-3 could be shown with stimulation by IL-6 in hypertrophic scar fibroblasts, although mRNA and protein for MMP-1 and MMP-3 were increased by IL-6 in normal skin fibroblasts.¹⁵ We have previously shown that extrinsic fibroblasts are phenotypically different from normal tendon fibroblasts in terms of proliferation, migration abilities and expression of integrin.⁷ In the present study, we found that extrinsic fibroblasts were less stimulated by IL-1 β than normal tendon fibroblasts in terms of the induction of interstitial collagenase mRNA.

Our study has shown over-expression of IL-1 β in the tendon graft model, although several other authors have reported that IL-1 β was over-expressed in the surrounding tissues of tendons with tendonitis.¹⁶⁻¹⁹ It is recognised that the level of IL-1 β released by neutrophils begins to rise rapidly after wounding and reaches a peak after several hours, suggesting that IL-1 β plays an important role in the commencement of inflammation.^{20,21} However, Kondo and Ohshima²² observed a second increase in IL-1 β at the proliferative stage after wounding in the rat skin, with migration of fibroblasts and the formation of new granulation tissue. We found that the extrinsic fibroblasts which were infiltrating the patellar tendon continued to over-express IL-1 β for at least six weeks after necrosis of the intrinsic fibroblasts. These experimental findings indicated that IL-1 β had another function with a close relationship to matrix remodelling, in addition to the commencement of inflammation during the process of tissue healing.

IL-1 is a polypeptide which is produced by macrophages, fibroblasts and neutrophils as a response to infection, injury, or antigenic challenges.²³ After trauma, IL-1 β is initially produced by activated macrophages and plays an important role in the initiation of the inflammatory response. It has a close relationship to wound remodelling as well as inflammation.²² In general, stimulation of IL-1 increases its synthesis in the autocrine and paracrine fashion and decreases the expression of IL-1 receptor.²⁴ Based on these findings in previous studies and the results of the present investigation, we suggest the following mechanism of the cellular response to graft integration with a cytokine/cellular control cascade for extrinsic fibroblasts during the

remodelling of the tendon after fibroblast necrosis. First, IL-1 β is produced by macrophages, fibroblasts, and neutrophils surrounding the patellar tendon as a response to the invasive effects of the surgical procedure. Then the secreted IL-1 β stimulates the production of collagenase by extrinsic fibroblasts during the infiltration of fibroblasts into the tendon after necrosis of the intrinsic cells. The secreted IL-1 β from extrinsic fibroblasts also stimulates the production of IL-1 β by the extrinsic fibroblasts themselves in the autocrine and paracrine fashion. IL-1 β may reduce IL-1 receptor expression, resulting in the decrease of the responsiveness of extrinsic fibroblasts to IL-1 β during the infiltration of extrinsic fibroblasts into the necrotic patellar tendon. Further study should be conducted to verify this hypothesis.

A few limitations are apparent in our study. First, we obtained infiltrative fibroblasts from the patellar tendon after a freeze-thaw treatment which had killed the intrinsic fibroblasts. However, this treatment is not identical to the conditions at tendon grafting and hence the cellular characteristics of infiltrative fibroblasts obtained in the current study may not have corresponded completely to those of fibroblasts in a grafted tendon. Secondly, we evaluated the cellular characteristics at approximately three weeks after harvesting of the patellar tendon. Although we performed only a third passage in the present study because serial passaging diminishes the differences in the phenotypical characteristics of fibroblasts, some cellular characteristics may have changed during the three weeks of culture. Previous studies have shown that the differences in the characteristics of different fibroblasts become insignificant with the serial passaging of the cells.^{25,26} Therefore, the difference in the cellular response to cytokines between extrinsic and normal tendon fibroblasts may have been underestimated in this study. However, many investigators have used the cultured tendon fibroblasts from the third passage or more,^{9,27,28} and we believe that our usage of cultured fibroblasts from the third passage is acceptable. Thirdly, we starved the fibroblasts of serum in the culture medium. This may have affected their differentiation, although the morphological appearances of cells before cytokine stimulation were similar to those before FBS starvation in the present experiments. In the present study we did not apply FBS 24 hours before cytokine stimulation because the existence of cytokines in FBS probably affected the response to additional cytokine stimulation. Based on previous studies,²⁹⁻³¹ the mean concentration of IL-1 β , PDGF and TGF- β 1 in human serum is approximately 2 pg/ml, 2 ng/ml and 40 ng/ml, respectively. Therefore the amounts of PDGF and TGF- β 1 in FBS are not considered to be negligible compared with the amounts which we applied as cytokine stimulation. The fourth limitation is that we compared the response of extrinsic fibroblasts to cytokine stimulation in terms of MMP-13 under the condition which we had observed to have the most significant effect on cytokine stimulation of gene expression of extrinsic cells in the time-course and the

dose-dependency experiments. We had no such information concerning the effect on gene expression in normal tendon fibroblasts. There is therefore, a possibility that we have overestimated the response of the extrinsic fibroblasts to cytokine stimulation in terms of MMP-13 mRNA compared with that of normal fibroblasts. In spite of this, we observed less response of the extrinsic fibroblasts to IL-1 β stimulation in terms of MMP-13 mRNA than in normal tendon fibroblasts.

An anti-IL-1 β strategy has attracted interest as a clinical treatment for inflammatory diseases such as a rheumatoid arthritis.³² The findings of our study indicate that IL-1 β is significantly involved in the remodelling of the grafted tendon after reconstruction of the ligament. Inhibition of IL-1 β may prevent degradation of the graft and our study highlights a possible application of anti-IL-1 β strategy for reducing the mechanical deterioration of grafted tendons after ligament reconstruction.

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