EXTRINSIC FIBROBLASTS INFILTRATING THE IN SITU FROZEN PATELLAR TENDON SYNTHESIZE TYPE-III COLLAGEN EVEN WHEN A PHYSIOLOGICAL STRESS IS APPLIED TO THE TENDON

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**Introduction** Many studies have shown that, in the patellar tendon autograft for ligament reconstruction, extrinsic cell infiltration and revascularization subsequently occur in the tendon after intrinsic fibroblast necrosis. Amiel et al. performed ACL reconstruction with the free patellar tendon graft in the rabbit, and reported that the type-III collagen content is significantly greater in the tendon autograft than in the normal tendon [1]. Their result suggests that extrinsic fibroblasts infiltrating the patellar tendon autograft predominantly produce type-III collagen. This leads to the following question has arisen: Do extrinsic fibroblasts predominantly produce type-III collagen when an non-physiologically low stress is applied to the tendon graft, or even when a physiological stress is applied? No studies have answered this question. We have established the in situ freeze-thaw treatment for the patellar tendon to necrotize intrinsic fibroblasts without disturbing the mechanical environment surrounding the tendon. The purpose of the present study using this technique is to clarify if extrinsic fibroblasts infiltrating the in situ frozen patellar tendon synthesize type-III collagen even when a physiological stress is applied to the tendon.

**Materials and Methods** This study was conducted with 41 male 16-week-old Wistar-King rats weighing 340 ± 30 g (mean ± SD). Five rats were used for tissue culture to evaluate the necrotizing effect of the in situ freeze-thaw treatment on intrinsic fibroblasts of the patellar tendon. The remaining 36 rats were used for immunohistochemical (IHC) evaluation and RT-PCR analysis. In 27 of the 36 rats, the right patellar tendons underwent the in situ freeze-thaw treatment with liquid nitrogen [2] and the left knees underwent a sham-operation. To maintain the physiological tension in the patellar tendon, each animal was allowed unrestricted activity in the cage. Nine animals were killed at 3, 6, and 12 weeks, respectively. In the remaining nine, the right patellar tendon was harvested without any treatment to obtain normal control data. Of each nine rats, four and five rats were used for IHC and RT-PCR examination, respectively. The right patellar tendons underwent the in situ freeze-thaw treatment and the left tendon did not undergo any treatment. Bilateral patellar tendons were harvested immediately after treatment. Then, each tendon was divided into 20 explant pieces of approximately 1 mm³ each, and each piece was placed in a culture well containing DMEM with 10% FBS. After 7 days of incubation at 37 degrees centigrade in 5% carbon dioxide, the number of explants with fibroblast outgrowth was counted. IHC analysis: Paraffin sections were stained with the ABC method using the monoclonal antibody specific for rat type-III collagen and counterstained with Mayer's hematoxylin to investigate the relationship between the immunolocalization of type-III collagen and the cellular distribution. For a quantitative analysis of expression of type-III, sections were stained with the double immunofluorescence technique using antibodies to type-I collagen and type-III collagen. The fluorescence microscopic image was taken by the CCD camera and analyzed with NIH Image 1.6. The ratio of the type-III collagen positive area to the area where type-I collagen or type-III collagen was expressed was defined as a Type-III ratio. The two-way ANOVA was performed for statistical analysis. RT-PCR analysis: Total RNA of 2 micrograms extracted from each tendon was reverse-transcribed using a pdN6 primer. PCR was performed with use of primers specific for rat type-III procollagen and GAPDH as an internal standard. Then, signals of the amplified products by 2% agarose gel electrophoresis with ethidium bromide were quantified by the MCID image analyzer. The ratio of type-III procollagen mRNA to GAPDH mRNA was analyzed using the Wilcoxon's signed rank test.

**Results** 1) Tissue Culture: Cell outgrowth was not observed around each explant harvested from the in situ frozen tendon. Around the non-treated patellar tendon, fibroblast outgrowth was observed in 16.6 ± 3.5 wells of the 20 wells per each tendon. 2) IHC analysis: At 3 and 6 weeks, expression of type-III collagen was localized around cells with an ovoid nucleus in the anterior and posterior parts of the in situ frozen tendon (Fig 1-a). At 12 weeks, expression of type-III collagen was widely distributed over the entire depth of the in situ frozen tendon. For the sham-operated tendon, expression of type-III collagen was not detected in the tendon substance throughout the experimental period (Fig 1-b). The Type-III ratio was significantly greater in the in situ frozen tendon than in the sham-operated tendon (p<0.01, Fig. 2). The Type-III ratio significantly increased with time in the in situ frozen tendon (p<0.01). 3) RT-PCR analysis: Expression of type-III procollagen mRNA was significantly higher in the in situ frozen patellar tendon than in the sham-operated tendon at 6 and 12 weeks (p<0.05), while there were no significant differences at 3 weeks (Fig. 3).

**Discussion** The immunohistochemical analysis showed that type-III collagen was expressed around the extrinsic cells infiltrating the in situ frozen patellar tendon, which was physiologically stressed during cage activities. In addition, the RT-PCR analysis indicated that type-III procollagen mRNA was overexpressed in the extrinsic cells at 6 and 12 weeks. These results demonstrated that the extrinsic cells infiltrating the in situ frozen patellar tendon synthesize type-III collagen even when a physiological stress is applied to the tendon. As to clinical relevance for ligament reconstruction, the type-III collagen synthesis by extrinsic fibroblasts infiltrating the graft, which results in deterioration of the tendon graft, may be unavoidable by application of physiological stress to the graft.