Growth kinetics and integrin expression of fibroblasts infiltrating devitalised patellar tendons are different from those of intrinsic fibroblasts

Y. Ikema, H. Tohyama, H. Nakamura, F. Kanaya, K. Yasuda

From Hokkaido University School of Medicine, Sapporo, Japan

We compared the biological characteristics of extrinsic fibroblasts infiltrating the patellar tendon with those of normal, intrinsic fibroblasts in the normal tendon in vitro. Infiltrative fibroblasts were isolated from the patellar tendons of rabbits six weeks after an in situ freeze-thaw treatment which killed the intrinsic fibroblasts. These intrinsic cells were also isolated from the patellar tendons of rabbits which had not been so treated.

Proliferation and invasive migration into the patellar tendon was significantly slower for infiltrative fibroblasts than for normal tendon fibroblasts. Flow-cytometric analysis indicated that expression of α5β1 integrin at the cell surface was significantly lower in infiltrative fibroblasts than in normal tendon fibroblasts. The findings suggest that cellular proliferation and invasive migration of fibroblasts into the patellar tendon after necrosis are inferior to those of the normal fibroblasts. The inferior intrinsic properties of infiltrative fibroblasts may contribute to a slow remodelling process in the grafted tendon after ligament reconstruction.

Previous studies have shown that in tendon autografts used for reconstruction of ligaments, repopulation by fibroblasts from an extrinsic origin occurs during revascularisation after intrinsic fibroblasts in the tendon have died. 1-3 We have reported that infiltration of extrinsic fibroblasts results in mechanical deterioration of the extracellular matrix of the grafted tendon. 4 Thus, infiltrative fibroblasts have an important role in the remodelling of the autogenous tendon graft. 1-3 Infiltrative fibroblasts repopulating in skin wounds are phenotypically distinct from normal dermal fibroblasts. 5-7 Nagineni et al, 8 Amiel et al 9 and Hannafin et al 10 have shown that the proliferation and migration characteristics of the ligament fibroblasts depended on their origin. Therefore, there is a high possibility that extrinsic fibroblasts infiltrating the necrotised tendons have significantly different biological characteristics from those of intrinsic fibroblasts in the normal tendon. In order to understand the remodelling of the tendon autograft in ligament reconstruction, it is necessary to clarify such differences since the characterisation of both types of fibroblast is also important in future technology in tissue engineering. No studies have compared the two types of fibroblast derived from tendon. It has been difficult to isolate the intrinsic and infiltrative fibroblasts from the grafted tendon. In situ frozen-thawed tendons and ligaments with intrinsic fibroblast necrosis have been developed to create an idealised tendon autograft model, which exists in physiologically mechanical conditions. 11-15 By using this model we have succeeded in isolating intrinsic and infiltrative fibroblasts from patellar tendon. 16

Our aim was to compare the rate of cellular growth and of cellular migration into the tendon matrix, and the expression of α5β1 integrin at the cell surface between the extrinsic fibroblasts infiltrating the necrotised patellar tendon and the intrinsic fibroblasts in the normal tendon.

Materials and Methods

We used 36 skeletally-mature female Japanese White rabbits with a mean weight of 3.0 kg (SD 0.1). From 18 rabbits we isolated infiltrative and normal fibroblasts and from the other 18 we obtained patellar tendons for the evaluation of invasive migration into the tendon matrix. We measured the rate of cell proliferation, the rate of migration into the tendon matrix and the expression of integrin at the cellular surface for infiltrative and normal fibroblasts. All the surgical procedures were carried out in the Institute of Animal Experimentation at the University School of Medicine under the Rules and Regulations of the Animal Care and Use Committee.
Isolation of infiltrative fibroblasts. To obtain infiltrative fibroblasts, we performed in situ freeze-thaw treatment on the right patellar tendon to kill the intrinsic fibroblasts. This was done under sterile conditions using intravenous pentobarbital anaesthesia (0.05 mg/kg). The subcutaneous retinacula were incised longitudinally along the patellar tendon after the anterior part of the right knee had been exposed through a longitudinal incision. The posterior surface of the patellar tendon was separated from the infrapatellar fat pad. A silicone rubber sheet was inserted between the patellar tendon and the fat pad to make a trough around the tendon (Fig. 1). The trough was filled with liquid nitrogen for one minute. The frozen tendon was then thawed using warm physiological saline. We have previously shown that this procedure kills 97% to 100% of intrinsic fibroblasts. Therefore, only the extrinsic fibroblasts were available to repopulate in the tendon. Six weeks later the tendons were harvested and placed individually in Dulbecco’s modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The outer synovial layer formed in two weeks. For comparison, the untreated patellar tendons, harvested from a different group of 18 rabbits, were immersed in liquid nitrogen for one minute and then thawed in saline solution at 37˚C to kill the intrinsic fibroblasts. This procedure was repeated five times. Each tendon was then stored at -80˚C for ten days to minimise its immunogenicity. Before it was cut, each tendon was partially thawed overnight at 4˚C and then thawed at room temperature. It was divided into three portions along the direction of the fibres and each portion was trimmed into 3 x 1 x 15 mm³ blocks.

Confluent fibroblasts from the first passage were detached from the dishes by treatment for two minutes with trypsin-EDTA. Each type of fibroblast was seeded into collagen gel (Cellgen; Koken Co, Tokyo, Japan) at a final concentration of 5 x 10⁵ cells/ml. The devitalised tendon was placed in the cell-containing gel with DMEM and 10% FBS. The dishes were incubated at 37˚C in a humidified, 5% CO₂ atmosphere for one, three, or six weeks (n = 6 for each). The tendons were washed with 37˚C PBS three times and then fixed with the optimum cutting temperature compound to make frozen tissues. They were cut sagittally into slices 25 to 30 µm thick. These were stained in 1 ml of FBS containing 0.5 µg of propidium iodide (Molecular Probes, Eugene, Oregon) for 30 minutes. We evaluated the cellular distribution in the tendon by confocal laser microscopy (MRC-1024; Bio-med Laboratories, Tokyo, Japan) for both types of fibroblast derived from six animals. The total number of infiltrated cells was counted in a 1000-µm square central area of the section for 25 µm in the vertical direction using image-analysis software (LaserSharp 2000; Bio-med Laboratories) which was installed in the confocal laser microscopic system. The counting was performed for two slices of each tendon at each period for each type of cell and the mean of these two experimental values was defined as a representative value at each period for each tendon.

Evaluation of integrin expression by flow cytometry and immunofluorescence. We evaluated the integrin expression of cultured fibroblasts at ten to 14 days after the harvesting of the tendons. Fibroblasts derived from a monolayer were placed in suspension after digestion with trypsin for two minutes. The suspension was diluted with medium containing 10% FBS to inhibit the trypsin, centrifuged and washed.
with fluorescence-activated cell-sorter buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). Pellets containing 1 x 10^6 cells were suspended in the buffer, passed through a nylon cell strainer (pore size 40 µm) and incubated at 4°C for 30 minutes with 20 µg/ml of mouse monoclonal antibody against extracellular domains of human α5β1 integrin (Chemicon, Temecula, California). The cells were washed three times with the buffer, suspended in 0.1 ml of the buffer, incubated with phycoerythrin-linked secondary antibody at 4°C for 30 minutes, and washed three times with the buffer. The cell pellet was then suspended in 1 ml of the buffer and analysed for fluorescence with a FACScan flow cytometer and Cell Quest software (Becton-Dickinson Immunocytometry System, San Jose, California). We examined cells derived from five animals. The analyses were performed twice at each cell type for each animal and the mean of these two experimental values was defined as a representative value for each cell type of each animal.

**Statistical analysis.** In the analyses of cellular proliferation and migration into the patellar tendon matrix, two-way analysis of variance (ANOVA) was performed to assess the effects of cell type and time. We also compared the fluorescence intensities of integrin between infiltrative fibroblasts and normal fibroblasts using a paired t-test. The limit of significance was set at p = 0.05.

**Results**

**Proliferation rates of infiltrative cells and normal patellar tendon fibroblasts.** The numbers of both types of fibroblast increased with time (Fig. 2). The infiltrative fibroblasts had a slower rate of growth than the normal patellar tendon fibroblasts from days 2 to 7. At day 2, the difference was 2.3-fold and at day 7, 2.5-fold. Two-way ANOVA showed statistically significant differences in the rate of proliferation by cell type (p < 0.001), time (p < 0.001), and interaction (p < 0.001).

**Ex vivo invasive migration of infiltrative cells and normal patellar tendon fibroblasts into the patellar tendon matrix.** Confocal laser-microscopic observations showed that cultured normal tendon fibroblasts infiltrated the acellular tendon matrix over time (Fig. 3a). They were scattered around the core of the tendon matrix by six weeks while cultured infiltrative fibroblasts were observed only superficially in the same period. Figure 3b – Graph showing that the total number of fibroblasts in a 1000-µm square central area of the specimen section for 25 µm in the vertical direction (n = 6 for each group at each period).
Integrin expression at cellular surfaces of infiltrative cells and normal tendon fibroblasts. Flow cytometric analysis showed that the mean fluorescence intensity of the infiltrative fibroblasts was 3.2 (SD 1.6) times lower than that of normal tendon fibroblasts ($p = 0.006, n = 5$ for each; Fig. 4).

**Discussion**

It is well recognised that necrosis of fibroblasts in the transplanted patellar tendon occurs sequentially during the early phase after ligament reconstruction. Our in vitro study was undertaken to clarify the characteristics of fibroblasts which infiltrate the necrotised patellar tendon, by using an in situ freeze-thaw procedure. Our findings showed that fibroblasts derived from the patellar tendon six weeks after the in situ freeze-thaw procedure had a slower rate of proliferation and a slower rate of migration into the patellar tendon matrix than normal patellar tendon fibroblasts. In addition, the expression of $\alpha_5\beta_1$ integrin on the cell surfaces was significantly lower in infiltrative fibroblasts than in normal patellar tendon fibroblasts.

Several studies have compared the proliferative activities of wound fibroblasts with those of normal dermal fibroblasts.\(^5\)\(^7\) The wound fibroblasts were obtained from polyvinyl alcohol sponges implanted subcutaneously in mice. They showed decreased proliferative properties compared with those of normal dermal fibroblasts. We similarly found that the rate of proliferation of infiltrative fibroblasts from the rabbit patellar tendon after necrosis was less than that of normal tendon fibroblasts. Schaffer et al.\(^7\) have also shown that wound fluid has a stimulatory effect on proliferation of fibroblasts. Therefore, a growth-stimulating wound environment may suppress the responsiveness of infiltrative fibroblasts to growth-stimulating growth factors in FBS culture medium.

There have been studies on cell migration using Boyden chambers for various types of cell including tendon and ligament fibroblasts.\(^10\)\(^19\) In our study we attempted to mimic the conditions of tendon grafting for ligament reconstruction. Our findings showed that infiltrative fibroblasts had less invasiveness into the patellar tendon matrix than normal patellar tendon fibroblasts which contributes to their reduced invasiveness into the patellar tendon matrix. Other factors such as the interaction between infiltrative fibroblasts and the extracellular matrix may slow the migration of fibroblasts into the tendon matrix.

Cell-extracellular matrix interactions are mediated by cell-surface molecules including integrins which are transmembrane proteins which connect extracellular matrix molecules to the cytoskeleton and convey information about the structure of the extracellular matrix to cells. They form heterodimeric receptors composed of alpha and beta subunits. The classical fibronectin receptor, $\alpha_5\beta_1$ integrin, recognises a sequence of three amino acids, Arg-Gly-Asp (RGD), in each fibronectin molecule.\(^20\) The expression levels of $\alpha_5\beta_1$ integrin are reported to vary among different types of fibroblast. For example, the damaged medial collateral ligament showed a striking increase in $\beta_1$ and $\alpha_5$ subunits of integrin on fibroblasts within the site of repair, while the damaged anterior cruciate ligament had no detectable changes in these subunits of integrin. Hakkinen et al.\(^21\) found that the quantity of $\alpha_5$ and $\beta_1$ integrin subunits expressed on cell surfaces by granulation-tissue fibroblasts was elevated relative to the quantity expressed by normal gingival fibroblasts. Conversely, our results showed that infiltrative fibroblasts had a decreased expression of $\alpha_5\beta_1$ integrin on the cell surfaces compared with normal tendon fibroblasts. The receptor $\alpha_5\beta_1$ integrin is considered to mediate cell migration into fibronectin.\(^22\) Therefore, low expression of $\alpha_5\beta_1$ integrin on the cell surfaces of infiltrative fibroblasts may cause low invasiveness of the infiltrative fibroblasts into the patellar tendon matrix.

Several limitations are apparent in our study. First, we obtained infiltrative fibroblasts from the patellar tendon after an in situ freeze-thaw treatment which killed the intrinsic patellar tendon. However, this treatment was not identical to the conditions at tendon grafting and therefore the cellular characteristics of infiltrative fibroblasts obtained in our study may not correspond to those of fibroblasts in a grafted tendon. Secondly, the present in vitro examination was performed in medium with 10% FBS and the presence of serum in the medium may have had complicating effects. Thirdly, we evaluated cellular characteristics at approximately two weeks after the harvesting of the patellar tendon. Although we performed only a single passage because serial passaging diminishes the differences in phenotypical characteristics of fibroblasts,\(^18\) some cellular characteristics may have changed during the two weeks in culture.

The process of the remodelling of the grafted tendon is considered to be very slow after intrinsic fibroblast necrosis.\(^23\) Delay, McGrath and Mindell\(^24\) reported that there
were still necrotic portions in patellar tendon after 18 months.

Our findings suggest that the cellular proliferation and invasive migration of infiltrative fibroblasts are inferior to those of normal tendon fibroblasts. The slow remodelling process in a tendon graft may be attributed to these inferior potentials of infiltrative fibroblasts. Therefore, we may be able to accelerate the remodelling process of the grafted tendon after ligament reconstruction if we restore the potentials of infiltrative fibroblasts to the levels of normal tendon fibroblasts.

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References