Effect of Recombinant Basic Fibroblast Growth Factor (bFGF) on Fibroblast-Like Cells from Human Rotator Cuff Tendon

Shu Takahashi, Mizuho Nakajima, Moto Kobayashi, Ikuko Wakabayashi, Naohisa Miyakoshi, Hiroshi Minagawa and Eiji Itoi

Department of Orthopedic Surgery, Akita University School of Medicine, Akita 010-8543

Takahashi, S., Nakajima, M., Kobayashi, M., Wakabayashi, I., Miyakoshi, N., Minagawa, H. and Itoi, E. Effect of Recombinant Basic Fibroblast Growth Factor (bFGF) on Fibroblast-Like Cells from Human Rotator Cuff Tendon. Tohoku J. Exp. Med., 2002, 198(4), 207-214 —— Rotator cuff tendon cells (RCC) derived from surgical samples showed fibroblast-like morphology. Histological staining demonstrated collagen secretion by RCC. Immunohistological findings revealed that RCC secreted type I and III collagen, but not type II collagen. In addition, the SDS-PAGE analysis suggested that RCC predominantly produced type I collagen. Basic fibroblast growth factor (bFGF) had a stimulatory effect on the proliferation of RCC dose-dependently up to 1 ng/ml. Administration of bFGF suppressed the secretion of collagens from RCC in a dose-dependent manner. —— basic fibroblast growth factor; rotator cuff tendon; collagen; proliferation; primary culture

© 2002 Tohoku University Medical Press

The tendons of the subscapularis, supraspinatus, infraspinatus, and teres minor muscles blend together to form the rotator cuff before inserting into the tuberosities of the humerus. The collagenous structure of the tendon changes into fibrocartilage before attaching to the bone. These tendons in humans, especially the supraspinatus, are more prone to develop lesions than any other deep tendon of the body (Frederick and Craig 1990).

In general, the tendon heals at a relatively slower rate than other connective tissue, even though they usually undergo similar stages during healing. Initial tendon healing includes inflammatory infiltration, cell proliferation and alteration in collagen phenotypes. At the early phase of tendon healing, there is an increased appearance of type III collagen. The tendon then enters a synthetic phase, in which the deposition of extracellular matrix starts. This is accompanied by the remodeling and maturation phases, in which the tensile strength of the injured tendon is regained. The restoration of mechanical strength is the goal of tendon

Received September 5, 2002; revision accepted for publication December 16, 2002.

Address for reprints: Shu Takahashi, M.D., Department of Orthopedic Surgery, Akita University School of Medicine, 1-1-1 Honjo, Akita 010-8543, Japan.
e-mail: shu@med.akita-u.ac.jp

207
healing. Type III which increased during the early phase of tendon healing is gradually replaced by type I as the mechanical strength of the tendon returns to normal (Hefti and Stoll 1995).

Growth factors, a major group of candidate of modulating agents, defined by their primary growth-stimulating functions, have proved to be involved in most stages of the healing process. Basic fibroblast growth factor (bFGF) has been identified to promote the proliferation of mesenchymal cells in vitro (Huang and Huang 1986; Gospodarowicz et al. 1987). Up-regulated expression of bFGF (Lee et al. 1998) and its receptors (Panossian et al. 1997) in healing of the medial collateral ligament also indicate an important role of bFGF in soft tissue healing. Few biochemical studies of the effect of bFGF on the healing of rotator cuff tendons have been performed despite the importance of these tendons in pathogenesis of painful shoulders which are commonly observed in the clinical settings. We hypothesized that bFGF affected the early phase of tendon healing when cell proliferation mainly take place. We administrated bFGF to the cells from the rotator cuff tendon and evaluated the cell proliferation and collagen expression.

**MATERIALS AND METHODS**

**Culture techniques for rotator cuff cells**

Human rotator cuff was obtained from patients undergoing rotator cuff repair. In this experimental procedure samples of rotator cuff were minced and placed into flasks in alpha Minimum Essential medium (α-MEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA), 50 mg/ml L-ascorbic acid 2-phosphate (Wako), 100 U/ml penicillin and 100 mg/ml streptomycin. Specimens were incubated at 37°C under a humidified 5% CO₂ atmosphere and fresh medium was added after 2 days. Cultures were maintained under these conditions, until the formation of a cell monolayer at 4-6 weeks, with media changes being performed twice weekly. At confluence, cells were used in the assays detailed below. Recombinant human bFGF was kindly provided by Kaken Pharmaceutical Co., Ltd. (Tokyo).

**Characterization assays**

**Cell morphology.** Rotator cuff tendon cells (RCC) derived from surgical samples were examined by phase contrast microscopy as they progressed to confluent cultures. Some cultures were stained with haematoxylin and eosin to demonstrate morphology.

**Growth curves.** Confluent cultures of RCC were trypsinized and RCC were seeded at a density of 1×10⁴ cells/well on to 24-well multiwell plates in α-MEM as above. Cultures were incubated at 37°C and maintained for 10 days. The media with or without bFGF were replaced every 2 days. RCC were trypsinized and counted in a haemocytometer at 48-hours intervals over the culture period; three replicate well were counted for each time point.

**MTT assays.** Confluent cultures of RCC were trypsinized and RCC were seeded at a density of 1×10⁴ cells/well on to 24-well multiwell plates in α-MEM supplemented with 10% FBS, 50 mg/ml L-ascorbic acid 2-phosphate (Wako), 100 U/ml penicillin and 100 mg/ml streptomycin. Cultures were incubated at 37°C and maintained for 48 hours. Then, the media were replaced in α-MEM with 0.1% FBS and cultured with or without bFGF for 48 hours. Three hours before the end of bFGF exposure, 125 μl tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, MTT) (Sigma Chemical Co., St. Louis, MO, USA) solution (2 mg/ml in phosphate-buffered saline) was added to each well. At the end of bFGF exposure the enzyme reaction was stopped by adding 1N HCl: isopropanol (1:24) (500 μl/well) followed by through mixing with a pipette. Then, 200 μl of the mixture were transferred to a 96-well microtiter plate, and the
plate was read on Benchmark (BIO-RAD) at 540 nm.

**Synthesis of matrix proteins**

Cultures were examined for the synthesis of type I and III collagen. RCC were cultured on chamber slides (Lab Tek II, Nunc) to subconfluence. RCC were then fixed in acetone for 2 minutes, air dried and stained total collagen using the staining technique (Lopez-De Leon and Rojkind 1983) and collagen type I or II or III using immunostaining techniques. The primary antibody (mouse anti-human collagen I or II or III; Sigma-Aldrich Co., Ltd., Poole, UK) was used at a dilution in 0.5% BSA; the secondary antibody (anti-mouse IgG, peroxidase labeled; Sigma) was used at a dilution of 1:1000 in buffer containing 0.5% BSA.

For collagen synthesis, RCC were seeded into 12-well plates at a seeding density of 1 x 10^4 cells/ml and incubated for a total of 10 days. At the end of culture period, cell layers formed under various conditions were washed with PBS for three times and incubated with elution buffer (Tris-buffered saline containing 0.1% NP-40). The insoluble materials which remained on the wells were used for determination of collagen. The insoluble materials were washed further twice with cold PBS, digested for 2 h at room temperature with 0.2% (w/v) pepsin in 0.5N acetic acid and centrifuged (15,000 x g for 10 minutes at 4°C). Pepsin-resistant mature collagen in the supernatant was determined for its components by SDS-polyacrylamide (7.5%) gel electrophoresis in the presence of (β-mercaptoethanol. After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue G-250. The gels were scanned in a scanner to determine peak area. Graphical representation of gels were performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://imaging.us.mnh.gov/nih-image/). The relative ratio of α1 chain band density to the number of RCC in each group was calculated. Standard collagen I, II and III were obtained from Sigma Chemical Co. (St. Louis, MO, USA)

**RESULTS**

**Cell morphology**

The most RCCs were initially fibroblastic (bipolar, spindle-shaped with a low cytoplasm: nucleus ratio) in shape (Fig. 1A). In some culture, it was possible to see clusters of polygonal cells when the culture developed, which were rounded than the cells normally seen (Fig. 1B).

---

**Fig. 1.** Phase contrast photomicrographs of living RCC in vitro. (A) Cells displaying typical bipolar morphology (original magnification ×20); (B) cluster of polygonal cells (original magnification ×20).
Growth curves

RCC cultured without bFGF reached confluence in 6 days (Fig. 2A). The proliferation of RCC was promoted by bFGF. Maximal responses were obtained at around 1 ng/ml for RCC. At a concentration of 1 ng/ml of bFGF, the cells grew more rapidly than without bFGF (control) group and reached confluence at 8 days. The most RCC at confluence were fibroblastic and the shape is more spindly than control. The cell number at confluence was almost 1.6 times compared with control group. At a concentration of 100 ng/ml, there is no difference of the cell number at confluence compared with control group (Figs. 2B–2E).

MTT assays

To determine whether bFGF had any effect on the proliferation or viability of RCC, MTT

Fig. 2. Growth curves for RCC. A: control; B: bFGF 0.1 ng/ml; C: bFGF 1 ng/ml; D: bFGF 10 ng/ml; E: bFGF 100 ng/ml. Results are the means and standard deviations of three replicate cultures.
assays were performed (Fig. 3). The proliferation of RCC was promoted by bFGF maximally around two-fold, in a dose-dependent manner. Maximal response was obtained at around 10 ng/ml.

Histology for Collagens

RCC cultures in chamber slides synthesized collagens as demonstrated by the histological staining (Lopez-De Leon and Rojkind 1985) (Fig. 4A). Immunohistochemical staining using specific antibodies demonstrated that RCC secreted both type I and III collagens (Figs. 4B and 4C), but we could not detect type II collagen (data not shown). Type I was the predominant collagen but significant amount of type III was present.

SDS-PAGE analysis of collagens

Fig. 5A shows the electrophoretic pattern of the peptides obtained from pepsin digestion of standard collagen type I, II and III. The -\( \gamma \) and -\( \alpha 1 \) chains of all three collagens can be identified. The -\( \beta 11 \) and -\( \beta 12 \) chains of type I and III collagen can be recognized and -\( \alpha 2 \) chain is unique to type I. The electrophoretic patterns of the samples after digestion by pepsin are shown in Fig. 5B. The -\( \gamma \), -\( \alpha 1 \), -\( \alpha 2 \), -\( \beta 11 \) and -\( \beta 12 \) chains were present. The administration of bFGF suppressed the secretion of collagens from RCC in a dose-dependent manner. All the chains can not be observed at a concentration of 10 and 100 ng/ml (Fig. 5B). The relative ratio of -\( \alpha 1 \) chain band intensity to the RCC number of each group was calculated (Fig. 5C). The relative ratio indicates the amount of -\( \alpha 1 \) chain secreted by one RCC. The amount of (1 chain at 1 ng/ml bFGF is less than 10% of that of control group.

DISCUSSION

The aims of this study were to determine the phenotype of cells derived from rotator cuff tendons and the effect of bFGF on these cells. The explant technique, in which cells are permitted to grow out from small fragments of tissue, was used to obtain rotator cuff tendon cells for this study. This is by far the commonest technique used in studies of tendon cells, although Riederer-Henderson et al and others have used other methods, most notably enzymic digestion (Riederer-Henderson 1983). However, such methods not only affect the cell membrane, but also destroy the cell-cell and cell-matrix relationship. Evans and Trail reported that this explant technique is best to determine the phenotype of the cells derived from tendons (Evans and Trail 1998).

We demonstrated that most RCC had fibroblast-like morphology and confluence occurred in 6 days. This result is similar to the previous study (Evans and Trail 1998). At the presence of 10% FBS, the proliferation of RCC was promoted by bFGF, although we did not find its obvious dose-dependency of bFGF. MTT assay under 0.1% FBS, however, showed that bFGF promoted the RCC proliferation in a
dose-dependent manner up to approximately 10 ng/ml. These results indicate that bFGF enhance the proliferation of RCC in a dose-dependent manner. At bFGF concentrations 10 and 100 ng/ml, cytotoxicity or some suppressive effect was observed. This inhibition was not as remarkable as previously reported effect of bFGF on proliferation of fibroblast and endothelial cells (Tanaka et al. 1996). Yoshida and Fujii (1999) reported the effect of bFGF on the anterior cruciate ligament (ACL) cells and the medial collateral ligament (MCL) cells. Their report showed that thymidine uptake by both human ACL and MCL cells was stimulated by human recombinant bFGF, maximally five-fold, in a dose-dependent manner, within a range of concentration of 0.1-10.0 ng/ml (Yoshida and Fujii 1999). Present study shows that the proliferation of RCC was promoted by bFGF maximally two-fold at around 10 ng/ml. These results suggest that RCC has lower response to bFGF compared with human ACL and MCL cells.

Histological staining demonstrated a great amount of collagen secretion by RCC. To determine the type of collagen, immunohistochemical staining using mono-clonal antibodies to type I, II and III collagen and SDS-PAGE analysis of the samples after digestion by pepsin were done. Immunohistological staining showed that type I and III collagen fibers were secreted by RCC. The ratio of -α1 to -α2 chains in control group was approximately 2 : 1, suggesting that type I was the predominant collagen, because it has two -α1 chains and one -α2 chain. Using NaCl fractionation of supraspinatus collagen, Fan et al. (1997) reported almost the same results that type I was the predominant collagen but significant amounts of type III and possibly some type II and V
Fig. 5. Standard collagen was subjected to acid-pepsin digestion as described in Materials and Methods and the resulting polypeptides were separated by SDS-PAGE on 7.5% gels. Collagen was solubilized from RCC by acid peptic digestion and electrophoresed on 7.5% gel as described in Materials and Methods. A: Lane 1, standard collagen type I (calf skin); lane 2, standard collagen type II (bovine tracheal cartilage); lane 3, standard collagen type III (human placenta). B: lane 1, control; lane 2, bFGF 0.1 ng/ml; lane 3, bFGF 1 ng/ml; lane 4, bFGF 10 ng/ml; lane 5, bFGF 100 ng/ml. C: The relative ratio of α1 chain band density to the number of RCC of each group.

were also present. Yoshida and Fujii (1999) reported that collagen synthesis of ACL and MCL cells was not stimulated by bFGF (10 ng/ml). In their experiments, ACL and MCL cells were exposed by bFGF for 24 hours. The present study demonstrated that 10-day administration of bFGF suppressed the secretion of collagens from RCC in a dose-dependent manner. Because of the difference of the exposure time, it is not possible to compare the nuisance value of bFGF to the collagen synthesis.

Finally, we showed that bFGF had a stimulatory effect on the proliferation of RCC, but suppressive effect on the collagen synthesis. Okumura et al. (1996) reported that bFGF restored injury-induced angiogenesis and granulation tissue formation and accelerated wound closure in healing-impaired animal. Another study showed that in vivo supplementation with bFGF affected the initial events of tendon healing such as cell proliferation and type III collagen expression (Chan et al. 2000). Based on the previous studies and the results of the present study, bFGF may have unique therapeutic potential in the early phase of rotator cuff tendon healing when cell proliferation mainly takes place and collagen (type I) synthesis has not been initiated. Further studies of the effect of brief exposure of bFGF and the combination with other growth factors would be useful to elucidate their contribution to the healing process of the rotator cuff.
tendon.

CONCLUSION

Our present study demonstrated that bFGF had a stimulatory effect on proliferation of RCC, however, the administration of bFGF suppressed the secretion of collagens from RCC in a dose-dependent manner.

References


