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III. 研究成果の刊行物・別刷

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REGULAR ARTICLE

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Identification of *Pip4k2β* as a mechanical stimulus responsive gene and its expression during musculoskeletal tissue healing

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Abstract To investigate the mechano-transduction system of cells, we identified genes responsive to a cyclic mechanical stimulus. MC3T3.E1 cells were cultured on a computer-controlled vacuum-pump-operated device designed to provide a cyclic mechanical stimulus. A maximum elongation of 15% of membrane at 10 cycles/min (3 s extension followed by 3 s relax per cycle) was repeated for 48 h. By means of a differential display, the gene expression pattern of cells exposed to the stimulus was compared with that of unexposed cells. As a result, a gene fragment that was exclusively expressed in mechanically stressed cells was identified. By using expressed sequence tag walking together with the oligo-capping method, this gene was identified as phosphatidylinositol 4-phosphate 5-kinase type II β (initially known as *Pip5k2β* but now reclassified as *Pip4k2β*). The specific up-regulation of *Pip4k2β* upon mechanical stimulus was also confirmed by using another apparatus, viz. a computer-controlled linearized-stepping motor system. To examine the involvement of the cyclic mechanical stimulus in the regulation of *Pip4k2β* expression in musculoskeletal tissue, we created an Achilles tendon transection model in rabbits. The temporal expression of *Pip4k2β* was assessed by means of a quantitative reverse-transcribed polymerase chain reaction. In the gastrocnemius muscle, expression of *Pip4k2β* rapidly decreased 1 week after transection but was restored to normal levels at 4 weeks. In the Achilles tendon, however, expression remained decreased until 4 weeks after transection. We suggest that the expression of *Pip4k2β* can be used as a marker for cells receiving a suitable mechanical stimulus.

Keywords Cyclic mechanical stimulus · Differential display · *Pip4k2β* · Mechanical stimulus-responsive genes · Musculoskeletal tissue healing · Rabbit (NZW)

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Introduction

Cells and tissues are constantly exposed to mechanical stimuli in many parts of the body. A number of attempts have recently been made to understand the biological and physiological roles that mechanical stimuli might play in many aspects of cellular functions (Benjamin and Hillen 2003; Daffara et al. 2004; Martinac 2004).

Investigators have reported the importance of mechanical stimuli not only during development (Elder et al. 2000; Robling et al. 2001; Wang and Mao 2002) and in the overall maintenance of physiological homeostasis (Rubin et al. 2001, 2002), but also in disease development (Park et al. 1998). Examination of the mechanisms by which the induction of gene expression is regulated by mechanical stimuli allows us to determine the way that cells respond to mechanical stimuli and the manner in which these stimuli are converted into intra-cellular signals. Moreover, accumulation of specific knowledge regarding the transcriptional regulation of MSR (mechanical stimulus-responsive) genes might improve our understanding of their roles in numerous cell regulatory contexts.

MSR genes are well known to play important roles in the signal transduction of endothelial cells, osteoblasts, fibroblasts, smooth muscle cells and cardiac muscle cells (Birukov et al. 1995; Danciu et al. 2003; Naruse et al. 1998; Sai et al. 1999); however, a number of known and/or unknown genes might not yet have been characterized as MSR genes. Furthermore, few reports have assessed the roles of MSR genes in vivo.

In musculoskeletal tissue healing, the tendon and muscle regeneration process begins immediately after their injury.

Once they are healed and gain continuity, they can transmit the physiological force to cells of the tendon and the muscle, thereby enabling them to receive mechanical stimuli once again. Based on this, we have hypothesized that the expression of MSR genes would be robust in intact tendon and muscle, attenuated when the tendon or muscle is transected and then increased as they heal. This could be clinically relevant to future treatments for musculoskeletal injury, because the expression levels of MSR genes would allow us to know to what extent injured tendon and muscle have healed and recovered their function.

With this goal in mind, we initially identified an MSR gene responsive to a cyclic mechanical stimulus by means of a differential display and then determined the full length cDNA sequence of the gene. Its responsiveness to a cyclic mechanical stimulus was tested by using two different in vitro systems and an in vivo model.

Materials and methods

Cells and cell culture

Five osteoblastic cell lines were cultured in a computer-controlled vacuum-pump-operated device (Flexercell, Flexcell, McKeesport, Pa.). MC3T3.E1 cells were provided courtesy of Dr. Kodama (Ohu University, Koriyama, Japan). Cells were cultured in RPMI-1640 (Gibco BRL, Gaithersburg, Md.) containing 10% fetal calf serum and grown under 5% $C0_2$ at 37°C.

Cell culture under a cyclic mechanical stimulus

To culture MC3T3.E1 cells under a cyclic mechanical stimulus, we used a computer-controlled vacuum-pump-operated device equipped with a specially designed microplate (FLEX-I, Flexcell) containing a deformable elastic silicone film lying at the bottom. Rigid-bottomed plates (FLEX-II, Flexcell) were used for control cultures.

Specifically, when the pump draws in air, the bottom of the experimental plate bulges and, when the valve shuts off, it flattens. Cells attached to the [illegible]

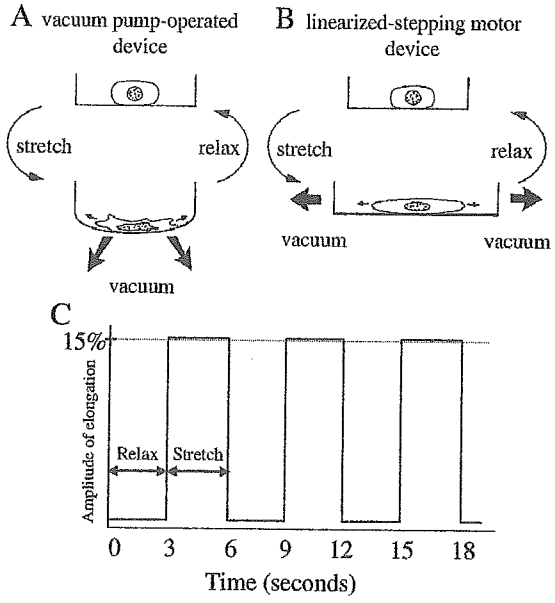


Fig. 1 Mechanical stimulus provided by two different pieces of apparatus. **A** The Flexercell computer-controlled vacuum-pump-operated device allows the cells to stretch and relax cyclically in response to the stimulus. **B** The NS-300 computer-controlled linearized-stepping motor device allows cells to stretch and relax in one direction by the computer-controlled linearized-stepping motor. **C** Mechanical stimulus parameters (*stretch*, *reflex*). A maximum elongation of 15% at 10 cycles/min (3 s extension followed by 3 s relax per cycle) was repeated over 48 h

relaxes cells cyclically. MC3T3.E1 cells were allowed to attach to deformable rectangular plates designed for this machine and were stretched and relaxed in one direction by a computer-controlled linearized-stepping motor system (Fig. 1b). Both ends of the chamber were firmly attached to a movable frame, which was connected to a motor-driven shaft. The amplitude and frequency of stretch was controlled by a programmable microcomputer. The silicon membrane was uniformly stretched over the whole membrane area and lateral thinning did not exceed 1% at 15% stretch. The stimulus parameters and other conditions used for this machine were the same as those used for the vacuum-pump-operated system. Following [illegible]

manufacturer's instructions and blotted onto a nylon membrane (HybondTM-N+, Amersham Pharmacia Biotech., Piscataway, N.J.). The expression levels of the candidate gene fragments were then compared.

Analysis of gene expression for *Pip4k2β* in an in vivo model

NZW rabbits (9–10 weeks old) were used to create an Achilles tendon transection model. In short, a 1.5-cm longitudinal skin incision was made on the distal end of the right hind limb of rabbits and the Achilles tendon was cut transversely at the calcaneus attachment portion. In this model, the tendon healed and started to become functional 4 weeks after transection (S. Sano et al., unpublished). Fifteen rabbits were used; five rabbits were sacrificed at 1 and 4 weeks and five rabbits were sham-operated and used as controls. When harvesting the Achilles tendon, special care was taken to dissect only the tendinous portion far from the newly formed granular tissue at the injured site. The gastrocnemius muscle sample was also taken from the centre of the muscle belly. These experimental procedures were performed under the approval of the Animal Care and Use Committee of Chiba University.

Total RNA was extracted from the tendon and the gastrocnemius muscle by using Trizol (Gibco BRL). Gene expression of *Pip4k2β* was examined by quantitative RT-PCR with primers (C)/(E) (see Fig. 3). Following standard Southern blotting, the radioactivity of hybridized bands was quantified by utilizing a bio-image analyzer (Image Gauge, Fujifilm, Tokyo, Japan) and the relative expression levels of *Pip4k2β* to β-actin were calculated. Statistical analysis was performed with Fisher's protected least significant differences at a level of significance of $P<0.05$.

Results

Mechanical stimulus responsiveness of cell line

MC3T3.E1 cells responded to cyclic stretching and relaxation with a morphological change. Specifically, after the stimulus, cells grew in concentric circles. We used MC3T3.E1 cells for further experiments.

Cloning of a gene fragment responsive to the cyclic mechanical stimulus and Northern blot analysis

More than 1000 bands were identified on a differential display gel. Among them, relative to control cells, five bands were expressed more highly in cells exposed to the mechanical stimulus. Subsequently, we investigated the expression of each fragment by Northern blot analysis. One of them showed exclusive expression in cells exposed to the stimulus and no expression in unexposed cells. This fragment was tentatively named 103A.2b. However, one fragment, named FG1a, was a false-positive (Fig. 2a).

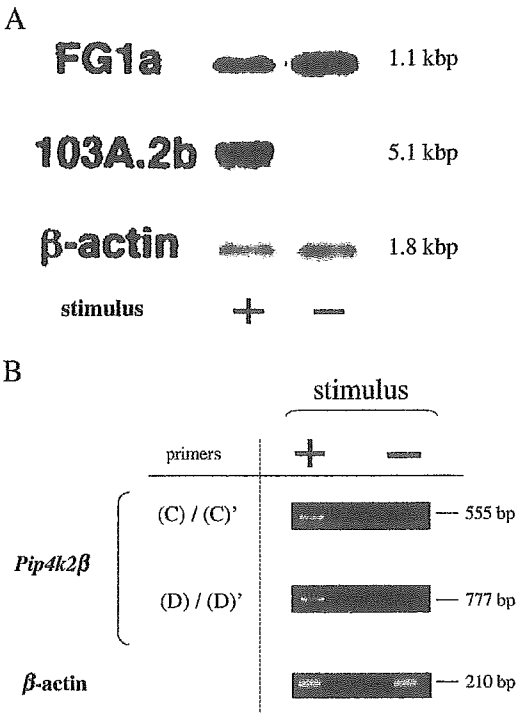


Fig. 2 **A** Exclusive expression of gene fragment 103.A2 in cells exposed to the cyclic mechanical stimulus. Total RNA was extracted from two sources (MC3T3.E1 cells cultured under a cyclic mechanical stimulus and those cultured quiescently) for Northern blot analysis. FG1a and 103A.2b were cloned gene fragments derived from the differential display and were used as probes. 103A.2b was exclusively expressed in cells exposed to a cyclic mechanical stimulus but FG1a was a false positive. Experiments were performed three times and representative data are shown. **B** Reproducibility of the mechanical stimulus responsiveness of *Pip4k2β* by using another type of stimulus. Up-regulation of *Pip4k2β* induced by a cyclic mechanical stimulus was verified by employing a linearized-stepping motor device (NS-300). Two sets of RT-PCR were performed with different primers (C)/(C') and (D)/(D') (Fig. 3). Intensive up-regulation of *Pip4k2β* was detected in cells exposed to the stimulus but not in cells which did not receive the stimulus. Experiments were performed three times and representative data are shown

Determination of the full-length cDNA sequence of the MSR gene

The 103A.2b fragment was 206 bp in length. No sequence similarity was found between 103A.2b and any known genes in the database. Therefore, expressed sequence tag (EST) walking was performed. An EST contig was extended to 3.6 kb until the 5'-end showed sequence similarity with *PIPKIIβ* (*Mus musculus* phosphatidylinositol phosphate kinase type II β, partial cds, Gen-Bank accession no. AB054987; between & and & in Fig. 3). Moreover, we found that the 5'-end of *PIPKIIβ* had sequence similarity with *Pip5k2β* (*Mus musculus* phosphatidylinositol-4-phosphate 5-kinase type II β, partial cds, Gen-Bank accession no. AY050219; between # and # in Fig. 3). The sequence continuity of 103A.2b, the EST contig, *PIPKIIβ* and *Pip5k2β* were confirmed by three sets of RT-PCR with primers (A)/(A'), (B)/(B') and (C)/(C')

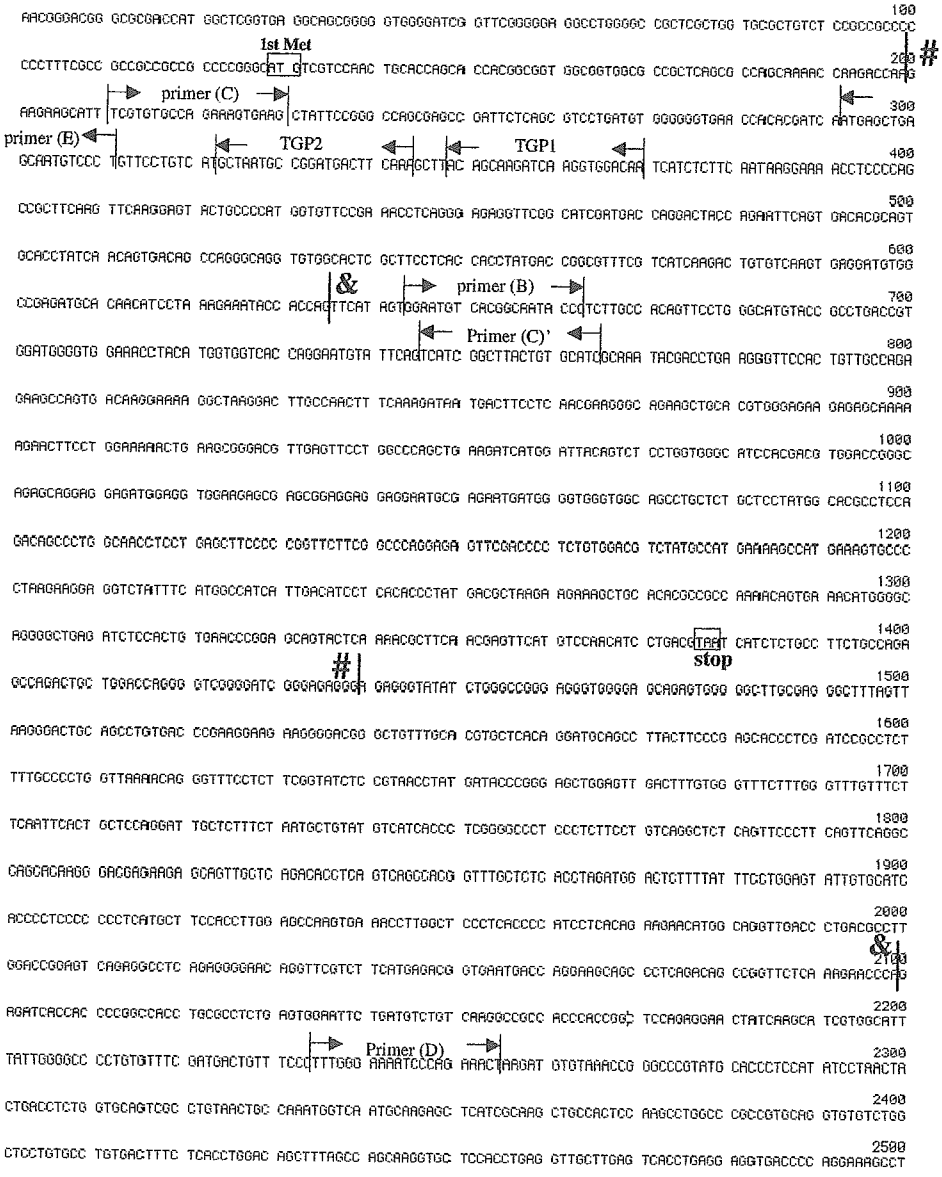


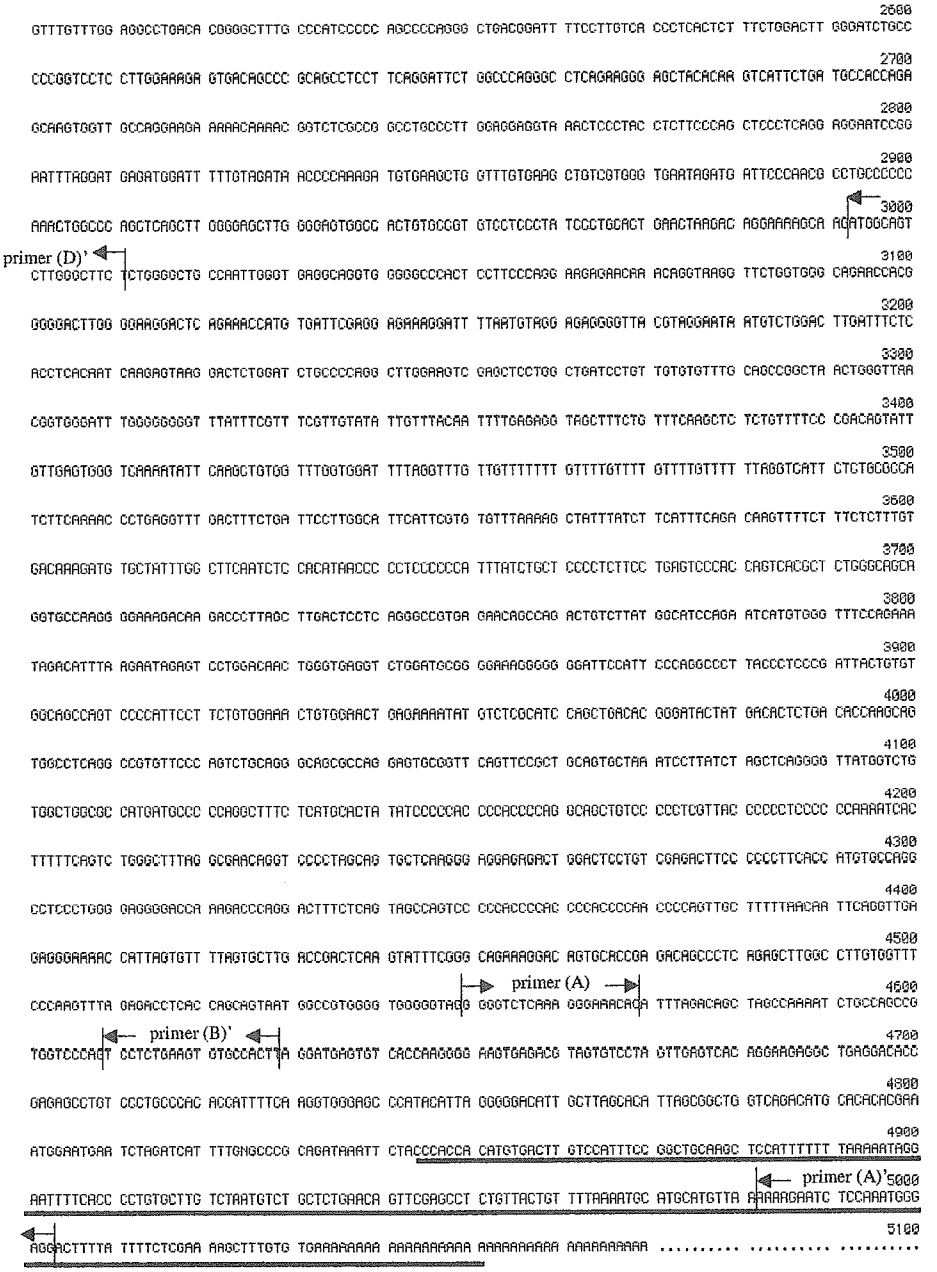
Fig. 3 Full-length 5043-bp cDNA sequence of the *Pip4k2β* gene. The 1st Met and the stop codon are each represented by a *rectangle*. 103A.2b, a 206-bp fragment identified by differential display, is *underlined*. Partial cDNA sequences registered as *PIPKIIβ* and *Pip5k2β* in the Gen-Bank correspond to nucleotide nos. 636-2099 (between & and &) and 200-1439 (between # and #), respectively. The sequences and directions for specific primers used in this study are also shown

(Fig. 3) with the mouse skeletal muscle being used as an RNA source. The transcription initiation site was determined by using the oligo-capping method (Maruyama and Sugano 1994) with Cap Site cDNA Mouse Skeletal Muscle (Nippon Gene, Tokyo, Japan) and the specific primers of TGP-1and-2 (Fig. 3). Thus, we determined that the full-length of the gene was 5043 bp and included a coding region of 1251 bp (nucleotide nos. 129-1379; Fig. 3). Since *Pip5k2β* is now reclassified as *Pip4k2β* (Rameh et al. 1997), we defined this gene as *Pip4k2β*. The strategy by which we determined the full-sequence is summarized in Fig. 4.

Reproducibility of the cyclic mechanical stimulus responsiveness of *Pip4k2β* by using another type of stimulus

Total RNA was extracted from cells cultured under the cyclic mechanical stimulus provided by the linearized-stepping motor device (NS-300) and those cultured quiescently. Two sets of RT-PCR was performed with different primers (C)/(C′) and (D)/(D′) (Fig. 3). Both of them showed intensive up-regulation of *Pip4k2β* in cells exposed to the stimulus; however, no expression was detected in cells that did not receive the stimulus (Fig. 2b).

Fig. 3 (continued)



Temporal expression of *Pip4k2β* during Achilles tendon healing

Before transection (i.e. in a control), expression of *Pip4k2β* was clearly detected both in the gastrocnemius muscle and in the Achilles tendon (Fig. 5a,b). After transection, the expression levels of *Pip4k2β* in the gastrocnemius muscle rapidly decreased to 40% of control at 1 week but were restored to normal at 4 weeks (Fig. 5a). Similarly, the expression levels in the Achilles tendon decreased to 25% of control at 1 week; however, it remained decreased until 4 weeks after transection (Fig. 5b).

Discussion

Recently, increasing attention has been paid to the relationship between mechanical stimuli and cellular responses. Previous studies designed to investigate cellular responses to mechanical stimuli in vitro have revealed that cells in a culture dish proliferate and rearrange themselves (Brighton et al. 1991; Buckley et al. 1988). Furthermore, the development of a special device to allow the culture of cells under mechanical stimuli has helped to increase our knowledge of their responses (Neidlinger-Wilke et al. 1994).

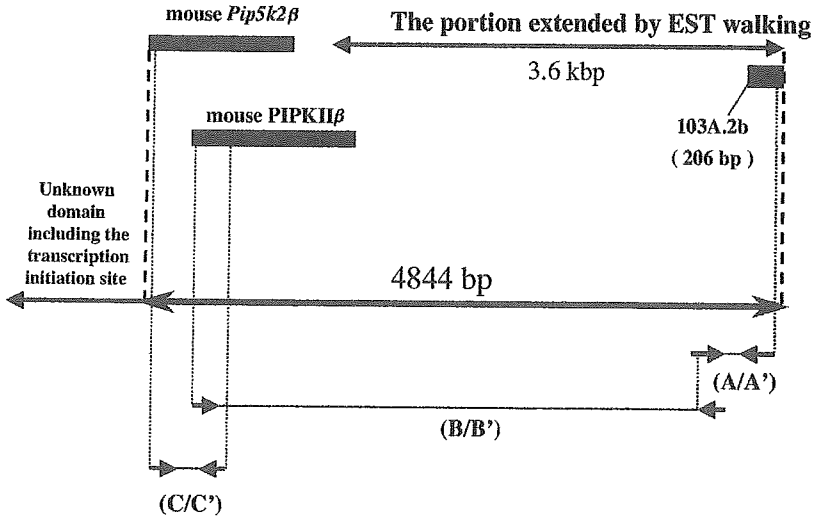


Fig. 4 Strategy for the determination of the full-length cDNA sequence of the *Pip4k2β* gene. Repeated BLAST homology searches of the EST database in an upstream direction was used to extend the 103A.2b fragment to 3.6 kb. The 5′-portion of this fragment was identical to mouse *PIPKIIβ*. Similarly, the 5′-portion of *PIPKIIβ* was identical to mouse *Pip5k2β*. Their sequence continuity was confirmed by three sets of RT-PCR with primers (A)/(A′), (B)/(B′) and (C)/(C′). A 200-bp sequence further upstream that included the transcription initiation site was determined by the oligo-capping method

As in previous studies, we used a computer-controlled vacuum-pump-operated device in the first part of our study. However, as has been reported, cells grown on this apparatus do not necessarily receive homogeneous stimuli in terms of direction and magnitude (Gilbert et al. 1994; Waters et al. 2001). Therefore, from our first experiment alone, we could not determine the magnitude of stretch that induced *Pip4k2β*. In addition, we could not refute the possibility of false-positives, even after having confirmed exclusive expression by Northern blot analysis. Thus, we further cultured cells on a different device in which they received a more homogeneous stimulus. This clearly verified that *Pip4k2β* was specifically induced in the presence of a cyclic mechanical stimulus.

Previous reports have shown that a transcription factor, nuclear factor kappa B (NFkB), is an important mediator for the induction of MSR genes (Chaqour et al. 1999; Inoh et al. 2002). Therefore, we examined whether NFkB-binding sites were present in the putative promoter region of the *Pip4k2β* gene. Indeed, we identified a candidate NFkB-binding site, a GGGGAGGCCT sequence in the putative promoter region (nucleotide nos. 56-65; Fig. 3), suggesting NFkB-mediated up-regulation of *Pip4k2β*. However, promoter analysis, such as the luciferase assay, is required to elucidate the involvement of NFkB in the regulation of *Pip4k2β* expression. *Pip4k2β* encodes type II phosphatidylinositol phosphate (PIP) kinase, which in turn produces phosphatidylinositol 4,5 bisphosphate (PI4,5P_2),

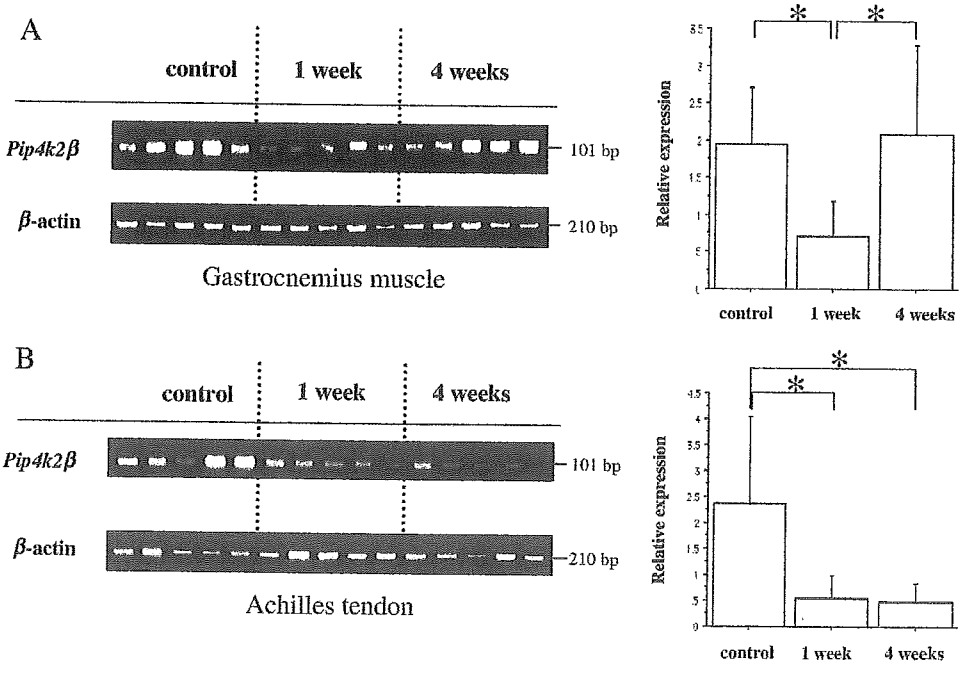


Fig. 5 Temporal expression of *Pip4k2β* during Achilles tendon healing. Expression of *Pip4k2β* in the gastrocnemius muscle (**a**) and Achilles tendon (**b**) was monitored by RT-PCR after transection. Five rabbits were sacrificed at different time-points. Relative expression levels of *Pip4k2β* to β-actin are also shown. **a** Expression of *Pip4k2β* in the gastrocnemius muscle rapidly decreased 1 week after transection and was restored to normal at 4 weeks. **b** In the Achilles tendon, expression also decreased at 1 week but it remained decreased until 4 weeks. Experiments were performed three times and representative data are shown. Values are expressed as a mean±SD. *Significantly different from control, $P<0.05$

which utilizes PI5P as a substrate. $PI4,5P_2$ is a crucial second messenger that regulates a myriad of cellular activities, including modulation of the actin cytoskeleton, vesicle trafficking, focal adhesion formation and nuclear events (Doughman et al. 2003). Although the functional role of Pip4k2β under physiological conditions has not been fully elucidated, Luoh et al. (2004) have demonstrated that overexpression of *Pip4k2β* in breast cancer cell lines confers proliferation advantage and promotes anchorage-independent growth and they imply that *Pip4k2β* plays important roles in the development and/or progression of breast cancer. In the present study, we have used MC3T3. E1 cells but the cells exposed to the cyclic mechanical stimulus show increased proliferation activity compared with those not exposed (data not shown). We therefore speculate that the specific up-regulation of *Pip4k2β* observed in the cells exposed to the cyclic mechanical stimulus might contribute to their proliferation.

To assess the involvement of the cyclic mechanical stimulus in the regulation of *Pip4k2β* in vivo, we first examined the tissue distribution of its expression under physiological normal conditions by Northern blot analysis. Expression was observed in the heart, brain, lung, liver, kidney and skeletal muscle but not in spleen and testis (data not shown). We speculate that, in brain, liver and kidney, which are abundantly vascular, the cyclic changes in blood pressure exerted on the endothelial cells induces the expression of *Pip4k2β*, although factors other than cyclic mechanical stimulus might also be involved in controlling the expression levels of this gene.

Next, we investigated whether the loss of mechanical stimulus could attenuate the expression of *Pip4k2β* by using the Achilles tendon transaction model. Loss of the mechanical stimulus indeed induced a rapid reduction of *Pip4k2β* expression both in the tendon and the muscle. Moreover, expression of *Pip4k2β* in the muscle was restored to normal 4 weeks after transection and appeared to be healed as evidenced by the functioning of the animals. These observations support the idea that *Pip4k2β* is synthesized under mechanically normal physiological conditions and that its production decreases concomitantly with the loss of a suitable mechanical stimulus. Although the expression of *Pip4k2β* might have reflected other factors than the mechanical stimulus, taken together with our in vitro results that *Pip4k2β* is intensely expressed in cells exposed to the cyclic mechanical stimulus, *Pip4k2β* seems to be, in part, involved in the recovery of mechanical stimulus responsiveness during Achilles tendon healing. Contrary to our expectations, expression in the tendon was not restored to normal at 4 weeks after transection. We speculate that an adhesive change, which occurred around the transected tendon, could have disturbed the transmission of the physiological force to the tendon cells. Another possibility is that a time-point of 4 weeks is too early for tendon cells to re-express the normal levels of *Pip4k2β*. Further evaluations of the expression levels of *Pip4k2β* at later stages of healing will therefore be needed.

In this study, we have reported, for the first time, that *Pip4k2β* is one of the MSR genes. Its specific up-regulation upon mechanical stimulus has been verified by using two different in vitro systems and an in vivo model. Further studies are required to elucidate the molecular mechanism by which a cyclic mechanical stimulus induces the expression of *Pip4k2β* and to establish its role under physiological conditions. Our results also indicate the possible usefulness of the *Pip4k2β* gene as a marker for monitoring functional musculoskeletal tissue healing.

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Effect of GDF-5 on Ligament Healing

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ABSTRACT: The effects of growth and differentiation factor-5 (GDF-5) on ligament healing were studied using a gap injury model of the medial collateral ligament in rat knee joints. The administration of GDF-5 once at the time of surgery significantly improved the mechanical properties of the femur–ligament–tibia complex. At 3 weeks after surgery, 30 μg of GDF-5 improved the ultimate tensile strength of the complex by 41%, and the stiffness by 60%, compared with the vehicle control ($p < 0.05$ for both; Fisher's PLSD test). The observation with a transmission electron microscopy revealed that GDF-5 increased the diameter of collagen fibrils in the repair tissue, which was considered to be a possible mechanism for the positive result in the biomechanical testing. Quantitative PCR and in situ hybridization revealed enhanced type I procollagen expression by GDF-5, and the PCR analysis also revealed that the GDF-5 treatment reduced the expression of type III procollagen relative to type I procollagen. The PCR analysis further showed that the expression of decorin and fibromodulin was relatively reduced against type I procollagen by the growth factor, which was considered to be responsible for the increase of collagen fibril diameter in the repair tissue. No adverse effects were observed, and the use of GDF-5 was considered a promising approach to facilitate ligament healing. © 2005 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:71–79, 2006

Keywords: ligament healing; growth and differentiation factor-5; biomechanical evaluation; transmission electron microscopy; mRNA expression

INTRODUCTION

Various attempts have been made to facilitate ligament healing to date. Since the discovery of growth factors, their use has become a focus of research because they are known to play pivotal roles in tissue healing. Several growth factors have been used for ligament healing, namely, platelet-derived growth factor-BB (PDGF-BB),[1,2] insulin-like growth factor-1 (IGF-1),[2] fibroblast growth factor-2 (FGF-2),[2,3] and transforming growth factor-beta1 and beta2 (TGF-β1 and β2),[1,4] alone or in combination. Promising results have been reported with some of them, but the best protocol to improve ligament healing has not yet been established.

Growth and differentiation factor-5 (GDF-5) in mice, or cartilage-derived morphogenetic protein-1 (CDMP-1) in humans, is a growth factor that belongs to the TGF-β superfamily.[5,6] During embryonal development, GDF-5 is expressed in cartilaginous tissue and plays important roles in the formation of joints and long bones.[5,6] Recent studies on GDF-5–deficient mice revealed that the factor is also involved in the development of tendons.[7,8] An earlier study showed that GDF-5 induces a tendon- or ligament-like tissue when ectopically administered in rat muscles,[6] suggesting that the factor may reintroduce the process of tendon development in mature individuals. Considering that the healing process is closely related to the developmental steps, it seems plausible that GDF-5 facilitates tendon or ligament healing. In

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fact, several investigators have applied GDF-5 on injured Achilles tendons and reported favorable results.[9–11] Based on these facts, we hypothesized that the addition of exogenous GDF-5 would facilitate the healing of an injured ligament, and examined the hypothesis using a gap injury model in a medial collateral ligament (MCL) in rat knee joints.

MATERIALS AND METHODS

Animal Model and Application of GDF-5

A total of 97 male Sprague–Dawley rats, 6 weeks old and weighing 215 ± 18 g (mean ± SD), were used for the study. All experiments were performed under the approval of the institutional review boards. Following anesthesia, a 1.5-cm skin incision was made in the medial side of the right knee, and an MCL was exposed. A 2-mm segment of the ligament centered on the joint line was sharply removed with a scalpel to create a full-width ligament gap, based on a reported model for delayed ligament healing.[12] This model had an advantage that the volume of the repair tissue was larger than that in a simple laceration model, which made the analysis easier. The rats were assigned to one of four groups. In Group I, the gap in the ligament was left untreated and served as a nontreatment control ($n = 36$). In Group II, the gap was filled with 10 μL of fibrin sealant (Beriplast® P, Aventis Pharma, Tokyo, Japan) mixed with 10 μL of 1 mM HCl solution to evaluate the effect of the carrier and vehicle on ligament healing ($n = 10$). In Groups III and IV, 10 μL of fibrin sealant was mixed with 10 μL of HCl solution containing 3 or 30 μg of recombinant mouse GDF-5 (R&D systems, Minneapolis, MN), respectively, and applied to the ligament gap ($n = 15$ and 36, respectively). Following the treatment, the fascia and skin were closed with 5-0 sutures. After surgery, the animal was housed in a cage with the operated limb free until the time of evaluation.

Histological Analysis

Eighteen rats in Groups I and IV were used for histological analysis. At 1, 3, and 6 weeks after surgery, three animals in each group were sacrificed, and medial collateral ligaments were obtained from the right knee joints. Coronal sections (4 μm thick) were made and stained with hematoxylin and eosin, and then the cellularity, cell morphology, and orientation of the tissue matrix were evaluated under a light microscope.

Biomechanical Testing

Biomechanical testing was performed using 10 animals in each of Groups I through IV. The animals were sacrificed at 3 weeks after surgery, and the right hind limbs were obtained by disarticulation at the hip and ankle joints. The limbs were wrapped in a plastic film and stored at −80°C until use. Before measurement, the specimen was thawed overnight at 4°C, and all soft tissues were carefully dissected free, sparing the cruciate ligaments, lateral collateral ligament, menisci, and MCL. The specimen was mounted in custom-built clamps with acrylic resin and attached to a uniaxial tensile testing machine (Tensilon® UTM 2.5T, Toyo Baldwin, Tokyo, Japan). Prior to the measurement, the positions of the clamps were adjusted so that the knee was flexed at 60 degrees, and the MCL was aligned in parallel with the load axis of the actuator. Then all knee ligaments except the MCL and the menisci were carefully removed. The femur–MCL–tibia complex was loaded at a rate of 20 mm/min until failure, and the load–deformation curve was recorded by an X-Y plotter (SS 207-EP, Toyo Baldwin). The site of failure was recorded for each specimen. The stiffness of the femur–MCL–tibia complex was obtained based on the linear slope of the load–deformation curve between 1 and 3 mm of elongation.

Transmission Electron Microscopy Analysis

Ultrastructural analysis of the healing tissue was performed on three animals in each of Groups I and IV at 6 weeks after surgery. For the evaluation, the animals were sacrificed and MCLs were obtained. The samples were then prepared according to a previously reported method[13] with some modifications. In brief, immediately after sacrifice, the operated MCL was drip-fixed by modified Karnovsky's fixative while the knee joint was held at 30 degrees flexion. An entire MCL was detached from bones and processed by 2% osmium tetroxide in the cacodylate buffer for 2 h at 4°C. After dehydration with a series of graded ethanol, the central part of the repair tissue was cut out in the form of a 2-mm cube. The tissue was then embedded in Epon 812 with careful attention to the orientation. Sections were cut at 100-nm thickness and stained with aqueous uranyl acetate and lead citrate. The examination was performed under a Hitachi H-7000 electron microscope. From each ligament, five photomicrographs of fibril cross sections were taken at the magnification of ×50,000. Locations of all photomicrographs were randomly selected, but those with artifacts and cellular components were carefully excluded. These images were scanned into a computer, and quantitative assessment was performed using an image analysis software (NIH Image version 1.61; National Institutes of Health, Bethesda, MD). To evaluate fibril diameters, the minimum fibril diameter was always measured to prevent errors due to obliquity in the sectioning. Fibrillar structures with a diameter less than 20 nm were excluded to avoid measurement of microfibrils or of noncollagen fibrillar components. Next, for each specimen, the total area occupied by the collagen fibrils and the total number of fibrils were counted on all five

photomicrographs, and the averaged percentage occupied by the fibrils and the averaged number of fibrils per 1 μm^2 were calculated.

In Situ Hybridization

Eighteen animals in Groups I and IV were used for in situ hybridization to evaluate the expression of pro α1(I) collagen mRNA. At 1, 3, and 6 weeks after surgery, three animals in each group were sacrificed and the ligaments were obtained. The method of in situ hybridization was described previously.[3] Briefly, after harvest, the ligaments were immediately fixed by immersion in 4% paraformaldehyde solution at 4°C overnight. The tissues were then embedded in OCT compound (Miles Laboratories, Elkhart, IN) and coronal sections of 10-μm thickness were prepared and mounted on glass slides coated with gelatin and poly-L-lysin. The sections were treated with proteinase K, then acetylated and prehybridized at 50°C for 3 h in the hybridization buffer. The hybridization was then allowed to proceed at 50°C for 24 h in the hybridization buffer containing the digoxigenin-labeled RNA probe. The probe was synthesized by T7 RNA polymerase using a pBluescript vector containing partial human pro α1(I) collagen cDNA as a template. In the transcription process, the probe was labeled with digoxigenin using DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Basel, Switzerland).

After hybridization, the sections were washed under an appropriate condition, and the hybridized probe was visualized by the color reaction for digoxigenin. The nuclei were stained with methyl green, and the sections were observed under a light microscope. The intensity of gene expression was assessed in a semiquantitative manner by a blinded observer (H.H.).

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis

Five animals in each of Groups I, III, and IV were sacrificed and served for quantitative PCR analysis at 1 week after surgery. After sacrifice, the ligament repair tissue was harvested, carefully avoiding contamination of the surrounding tissue. The tissue was then minced with a scalpel, and RNA was extracted using a commercially available kit (RNeasy® Micro; Qiagen, Valencia, CA). The synthesis of cDNA was performed with Sensiscript® reverse transcriptase (Qiagen). The cDNA was then used for real-time quantitative PCR on a LightCycler® (Roche Diagnostics) using gene specific primers and probes (Table 1). The expression of pro α2(I) collagen, pro α1(III) collagen, decorin, fibromodulin, and lumican was evaluated together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. For some genes, the amplification was monitored by the fluorescence of SYBRE® Green I dye that bound to the double-strand DNA. For the others, the probes carrying fluorescent dyes were used for monitoring. The initial amount of cDNA for the gene was determined with a standard curve based on the known amounts of the PCR product.

Table 1. Primer and Probe Sequences Used for Quantitative PCR

Gene	GenBank Accession Number	Primer/Probe Sequence	Amplicon Size (bp)
Col1A2[a]	AF121217	Forward: 5′- TACAACGCAGAAGGGGTGTC -3′	193
		Reverse: 5′- CCTCAGCAACAAGTTCGACG -3′	
		Probe Flu: 5′- GCTTCTCAGAACATCACCTACCACTGCA -3′	
		LC: 5′- GAACAGCATTGCGTACCTGGACGA -3′	
Col3A1	X70369	Forward: 5′- ATGGTGGCTTTCAGTTCAGC -3′	284
		Reverse: 5′- TGTCTTGCTCCATTCACCAG -3′	
Decorin[a]	Z12298	Forward: 5′- TGGACTGAACCGTATGATTG -3′	208
		Reverse: 5′- GCTGGCTGCATCAACTTT -3′	
		Probe Flu: 5′- GCAGGGAATGAAGGGTCTCGGATACA -3′	
		LC: 5′- CCGCATCTCAGACACCAACATAACTGC -3′	
Fibromodulin	NM080698	Forward: 5′- TGTCCGGCTGTCTCACAACA -3′	198
		Reverse: 5′- TCCACCACCGTGCAGAAACT -3′	
Lumican	BC061878	Forward: 5′- GGCGTGCCTGGAAACTCATT -3′	198
		Reverse: 5′- GCGCAAATGCTTGATCTTGG -3′	
GAPDH[a]	AF106860	Forward: 5′- TGAACGGGAAGCTCACTGG -3′	307
		Reverse: 5′- TCCACCACCCTGTTGCTGTA-3′	
		Probe Flu: 5′- CTGAGGACCAGGTTGTCTCCTGTGA -3′	
		LC: 5′- TTCAACTGCAACTCCCATTCTTCCACC -3′	

[a]Gene-specific probes were used for quantitative PCR. Flu: probe conjugated with Fluorescein; LC: probe conjugated with LC red 640.

Statistics

For the results of biomechanical measurement and quantitative PCR, the data were first analyzed by one-way factorial analysis of variance (one-way factorial ANOVA), and when a significant variance was observed, Fischer's PLSD test was performed as a post hoc test. For the result of ultrastructural analysis, unpaired t-test was performed to compare the data between the two experimental groups. A p-value of <0.05 was used for statistical significance.

RESULTS

All rats survived the surgery well and resumed normal cage activity within 1 or 2 days after surgery. At sacrifice, it was confirmed that the skin wound had healed normally and the operated knee had resumed a normal range of motion equal to the unoperated side. The average weight of the animals did not show a significant difference among the groups at any evaluation period.

Gross Appearance and Histological Evaluation

At 1 week after surgery, a larger volume of the repair tissue was formed at the ligament gap in Groups III and IV, compared with the control groups. The tissue in these GDF-5–treated groups tended to have a firmer consistency, although there was no obvious difference in their appearance. At 3 weeks, the difference in the volume became less obvious, but the repair tissue was still thicker in Group IV at 6 weeks.

On hematoxylin and eosin-stained sections, the effect of GDF-5 was most obvious at 1 week (Fig. 1A and B). In the animals treated with 30 μg of GDF-5, the repair tissue showed higher cellularity, and

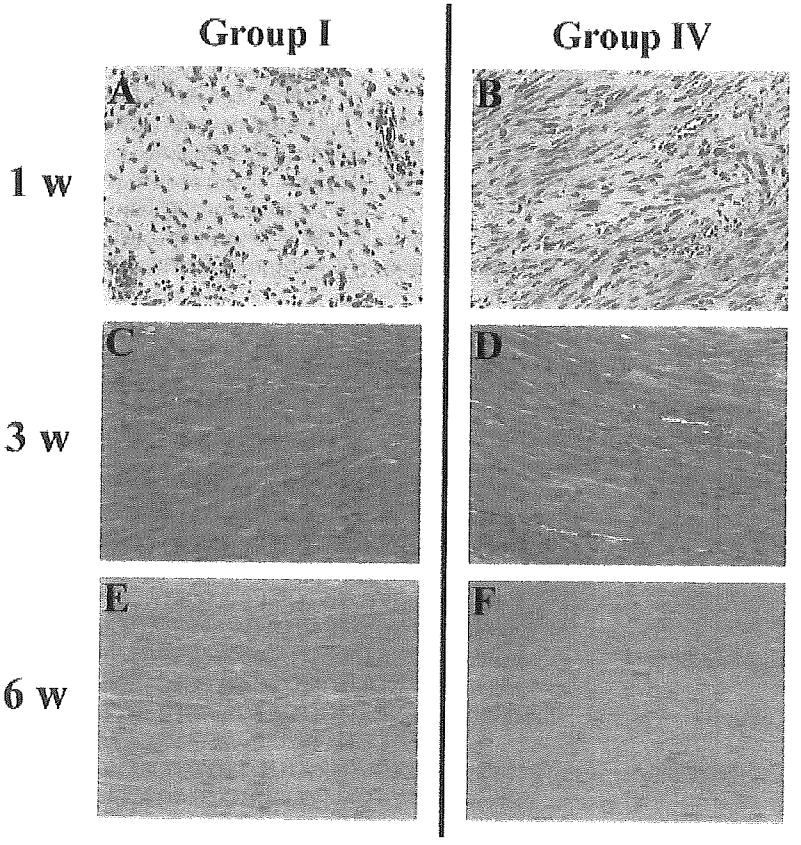

Figure 1. Histology of the repair tissue formed at the ligament gap 1, 3, and 6 weeks after surgery. The left and right columns show the results of the control animals (Group I; A, C, and E) and those treated with 30 μg of GDF-5 (Group IV; B, D, and F), respectively. Three animals in each group were evaluated at each time point, and representative photomicrographs are shown. Hematoxylin and eosin, original magnification ×20. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

the cells presented elongated forms while those in the control group were rounder and more spherical. These trends were observed at 3 weeks to a lesser extent, and the matrix in Group IV had better alignment and clearer orientation compared with the control animals (Fig. 1C and D). At 6 weeks after surgery, the difference between the treated and control groups was less obvious, and the repair tissues in both groups presented a similar histology (Fig. 1E and F). Unlike previous studies on tendon healing,[9–11] we did not find cartilaginous tissue formation by the GDF-5 treatment.

Biomechanical Testing

In all specimens, failure occurred at the ligament midsubstance. The ultimate tensile strength of the femur–MCL–tibia complex was 7.6 ± 3.3, 9.1 ± 3.0, 9.5 ± 2.9, and 12.9 ± 3.0 N for Groups I, II, III, and IV, respectively. The variation among the 4 groups was significant ($p < 0.01$; one-way factorial ANOVA), and the strength of Group IV was significantly greater than that of Group I or II ($p < 0.05$ for either group; Fischer's PLSD test) (Fig. 2A). The stiffness of Group I, II, III, and IV was 3.6 ± 1.8, 3.8 ± 1.7, 5.4 ± 1.7, and 6.2 ± 1.9 N/mm, respectively, and their variation was statistically significant ($p < 0.05$; one-way factorial ANOVA). Again, the stiffness of Group IV was significantly higher than that of Group I or II ($p < 0.05$ for either group; Fischer's PLSD test) (Fig. 2B).

Transmission Electron Microscopic Study

In Group I, the repair tissue contained a rather uniform population of collagen fibrils with small diameters (Fig. 3A and B), while the tissue in Group IV contained some thicker fibrils (Fig. 3C and D). The mean fibril diameters for Groups I and IV were 36.5 ± 8.0 and 45.9 ± 15.7, respectively, and the difference between the two groups was statistically significant ($p < 0.01$; unpaired t-test) (Fig. 3E). The numbers of fibrils per 1 μm^2 were 171 ± 21 and 224 ± 43 for Groups I and IV, respectively, and the repair tissue in Group IV contained significantly higher number of fibrils than Group I ($p < 0.01$; unpaired t-test) (Fig. 3F). Consequently, the percentages of the area occupied by collagen fibrils in Groups I and IV were 26.3 ± 4.9 and 49.1 ± 4.0, respectively, and the value in Group IV was significantly higher than that in Group I ($p < 0.01$; unpaired t-test) (Fig. 3G).

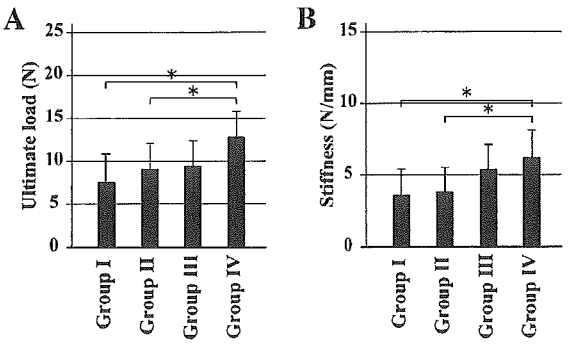

Figure 2. Ultimate tensile strength (A) and stiffness (B) of the femur–MCL–tibia complex 3 weeks after surgery. Each bar represents the average of 10 specimens. Values are expressed by means + SD. *$p < 0.05$.

In Situ Hybridization Study

In an attempt to elucidate the mechanisms for the advantageous effect of GDF-5, the expression of pro α1(I) procollagen mRNA was evaluated by in situ hybridization at 1, 3, and 6 weeks after surgery. At 1 week, abundant gene expression was observed in the repair tissue in both groups, but the expression in Group IV was more enhanced than in Group I (Fig. 4). The gene expression in the remnants of original MCL was barely detectable in both groups. At 3 weeks after surgery, the mRNA expression in the repair tissue was reduced in both groups, but the expression in Groups IV was still stronger than that in Group I. The expression in the repair tissue was hardly detectable at 6 weeks for both groups.

Quantitative PCR Study

To clarify the difference in the repair process, the levels of mRNA expression were studied on two dominant procollagens together with three small leucine-rich proteoglycans (SLRPs) that are known to affect collagen fibrillogenesis. Compared with Group I, the expression of pro α2(I) collagen was significantly higher in Groups III and IV ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5A), while the expression of pro α1(III) collagen was similar for all three groups (Fig. 5B). Thus, the expression of type III collagen relative to type I was significantly reduced in both GDF-5 treated groups ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5C). Among the three SLRPs studied, the expression of decorin was

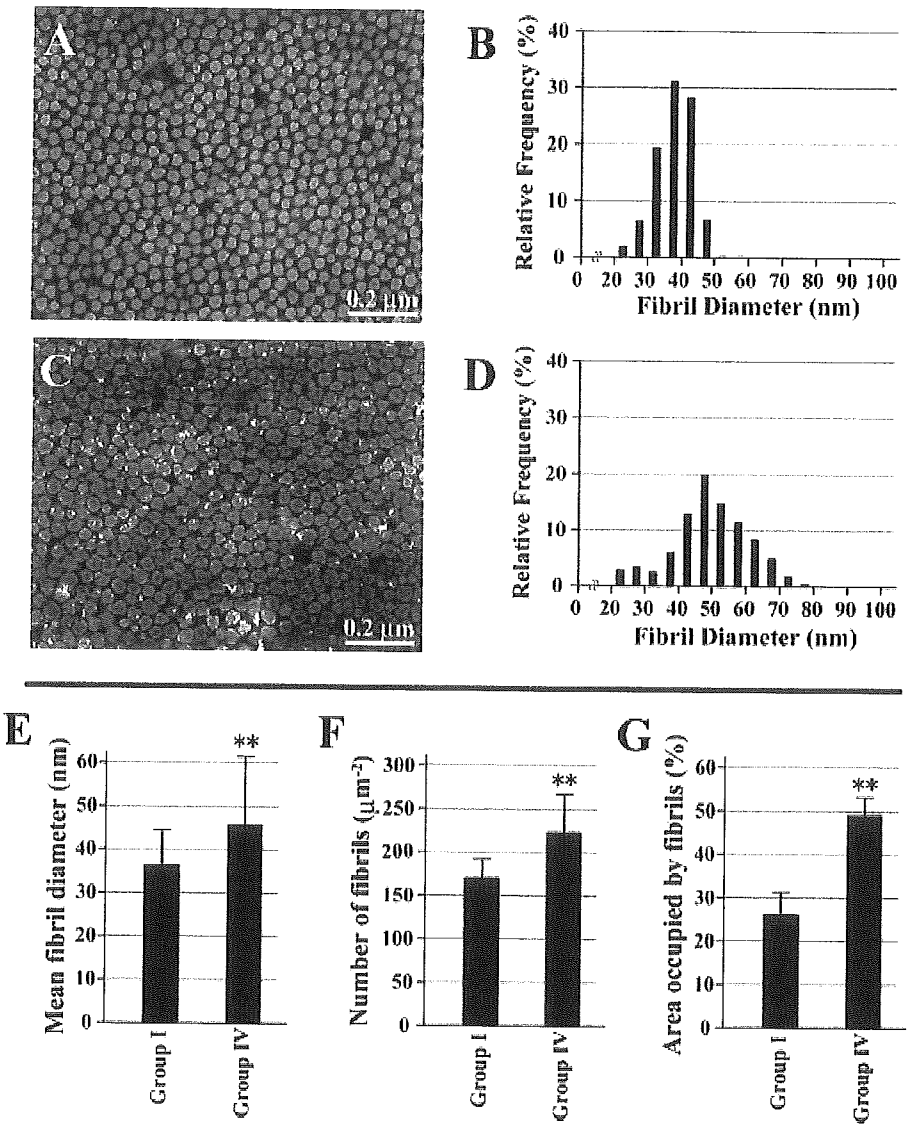

Figure 3. In the upper panel, photomicrographs of the repair tissue and the histograms for the distribution of fibril diameter in Group I (A and B) and Group IV (C and D) are shown. Fibril diameter (E), number of fibrils in a 1 μm square (F), and percentage of the area occupied by collagen fibril cross sections (G) of both groups are shown in the lower panel. The evaluation was performed on three animals in each group. Values are expressed by means + SD. $^{**}p < 0.01$ against Group I.

significantly reduced in Group IV ($p < 0.01$; Fischer's PLSD test) (Fig. 5D), and the expression of fibromodulin was remarkably suppressed in both GDF-5–treated groups ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5E). However, GDF-5 strongly stimulated the expression of lumican ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5F). When the expression of SLRPs was considered relative to type I collagen expression, the GDF-5 treatment strongly suppressed the expression of decorin and fibromodulin ($p < 0.01$ for both groups for both genes; Fischer's PLSD test) (Fig. 5G and H, respectively), while lumican expression was almost unaffected (Fig. 5I).

DISCUSSION

In this study, the results of biomechanical testing have shown that the process of ligament healing

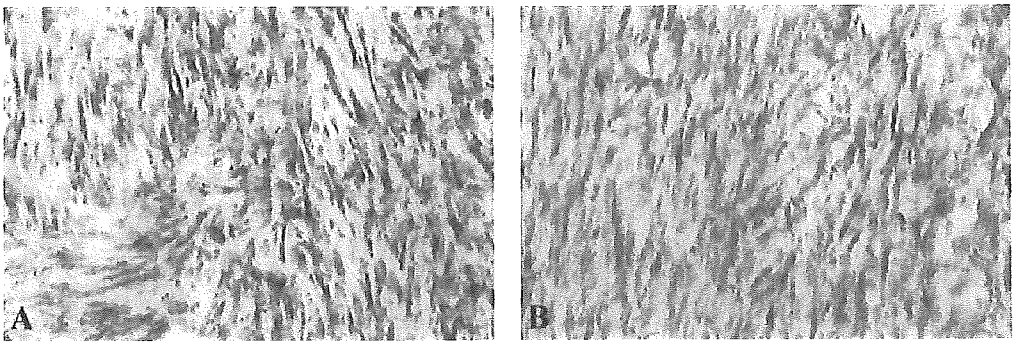

Figure 4. Results of in situ hybridization for pro α1(I) collagen mRNA in the repair tissues in Group I (A) and Group IV (B). Three animals in each group were evaluated at each time point, and representative photomicrographs of 1-week specimens are shown. Nuclei were stained with methyl green. Original magnification ×100. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

was in fact facilitated by GDF-5. Although a slight improvement was observed with the fibrin sealant alone, the data showed that the mechanical properties of the femur–MCL–tibia complex were significantly improved by 30 μg of GDF-5; the ultimate tensile strength of the complex was over 40% greater than that of the vehicle controls, and the stiffness was improved by 60%. The changes were both above the level of statistical significance. In all specimens, the failure occurred through the ligament midsubstance, and the result was attributed to the improved mechanical property of the healing tissue by GDF-5. A possible explanation for the positive effect was given by the results of in situ hybridization and quantitative PCR. These experiments revealed that the expression of type I procollagen genes was promoted in the healing tissue treated with GDF-5. Because type I collagen is the primary component of the repair tissue,[14] it is likely that the elevated type I collagen production led to an increase of repair tissue volume, giving higher mechanical strength to the tissue. Interestingly, the growth factor did not change the expression level of type III collagen, and thus, the expression of type III collagen relative to type I declined significantly. It is known that the ligament repair tissue contains an increased amount of type III collagen compared with a normal ligament,[14,15] which might be responsible for the poor mechanical property of the tissue.[16,17] Therefore, in this study, the relative decrease in type III collagen content might be related to the improvement of biomechanical results by altering the type I and type III collagen ratio toward normal.

The result of electron microscopic observation has shown another possible mechanism for the improved healing by GDF-5. In comparison to the controls, the repair tissue in the GDF-5–treated animals tended to contain thicker collagen fibrils at a higher density. Considering that the collagen fibril diameter is known to be a major determinant of the mechanical property of the ligaments,[18,19] the positive result might be the consequence of the increased size and density of collagen fibrils in the repair tissue.

The results of ultrastructural observation led us to examine the expression of small leucine-rich proteoglycans because they have been reported to affect collagen fibril formation in vivo.[20–22] In the present study, the expression of these genes was evaluated 1 week after surgery, the time point when the influence of GDF-5 on the healing process was most obvious by histology and in situ hybridization. The quantitative PCR analysis revealed that the effect of the growth factor varies on the genes; the expression of fibromodulin was strongly suppressed, lumican was enhanced, and decorin was not altered significantly. Because fibromodulin and lumican share similar effects on collagen fibrillogenesis,[21] the result did not seem to support the increase in the fibril size by GDF-5. Then, considering that these molecules modulate fibrillogenesis through the direct binding to collagen fibrils,[22] we obtained the expression ratios between the proteoglycans and type I procollagen. The results showed that the expression of decorin and fibromodulin were relatively suppressed, while the expression of lumican was nearly unaffected. Considering that these proteoglycans could inhibit collagen fibril assembly in the early phase of fibrillogenesis,[21,23] we currently presume that the relative suppression of decorin and fibromodulin could be related to the

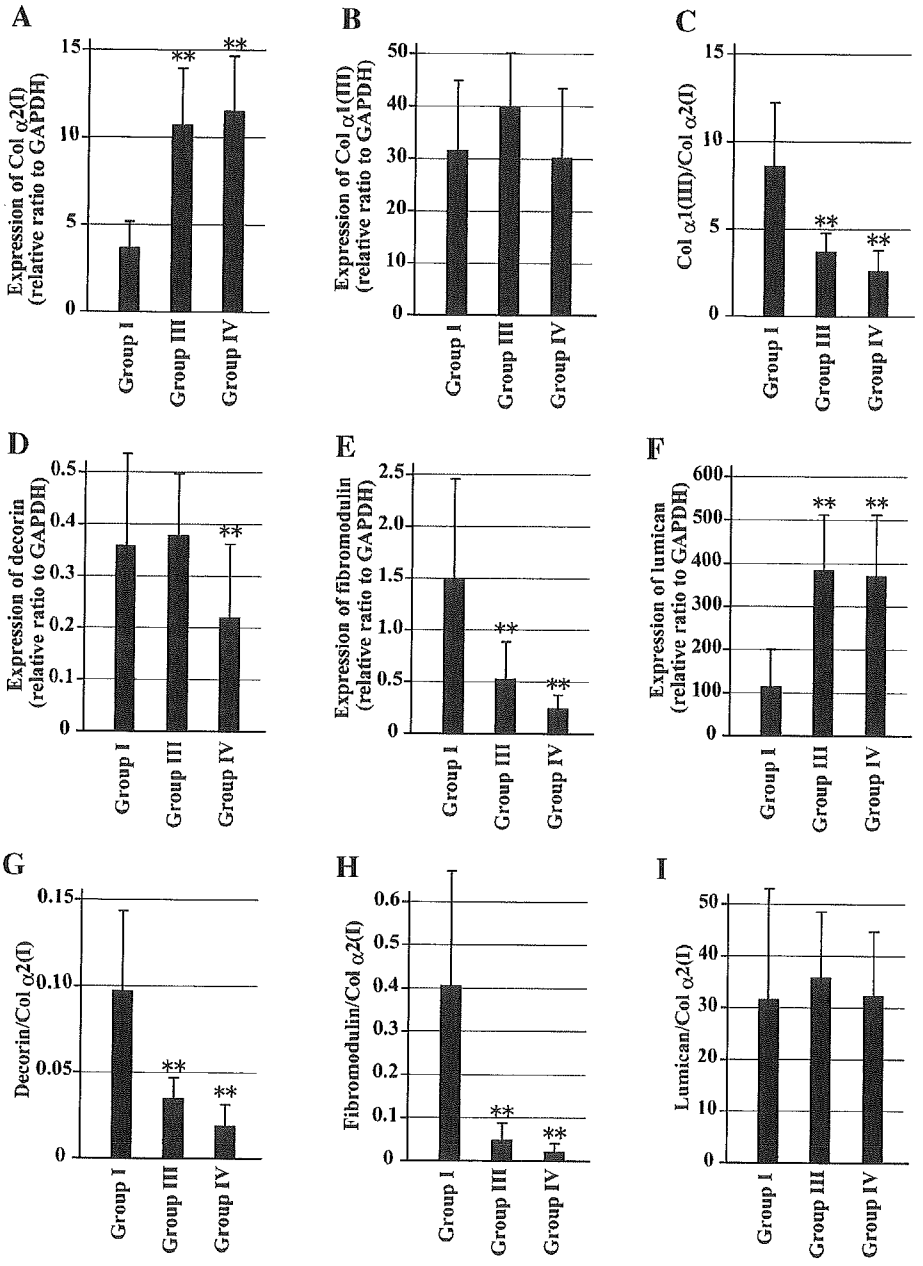

Figure 5. Result of quantitative PCR. Top panel shows the expression of pro α2(I) collagen (A), pro α1(III) collagen (B), and their expression ratios (C). Middle panel shows the expression of decorin (D), fibromodulin (E), and lumican (F) relative to GAPDH. Their expression ratios against pro α2(I) collagen are shown at the bottom panel (G–I, respectively). Each bar represents the average of five specimens. Values are expressed by means + SD. $^{*}p < 0.05$, and $^{**}p < 0.01$ against Group I.

appearance of thicker collagen fibrils by GDF-5. Similar data have been shown by a recent study that BMP-12, another member of the GDF family, suppressed decorin expression and elevated collagen expression in cultured human tendon fibroblasts.[24]

Collectively, exogenously added GDF-5 enhanced healing of an injured ligament histologically and biomechanically. No adverse effect was observed, and the use of GDF-5 was considered to be a promising approach to facilitate ligament healing.

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