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Muscle injury-induced thymosin β4 acts as a chemoattractant for myoblasts

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Thymosin β4 (Tβ4) is a major intracellular G-actin-sequestering peptide. There is increasing evidence to support important extracellular functions of Tβ4 related to angiogenesis, wound healing and cardiovascular regeneration. We investigated the expression of 'Tβ4' and 'thymosin β10', a closely related peptide, during skeletal muscle regeneration in mice and chemotactic responses of myoblasts to these peptides. The mRNA levels of 'Tβ4' and 'thymosin β10' were up-regulated in the early stage of regenerating muscle fibres and inflammatory haematopoietic cells in the injured skeletal muscles of mice. We found that both Tβ4 and its sulphoxized form significantly accelerated wound closure and increased the chemotaxis of C2C12 myoblastic cells. Furthermore, we showed that primary myoblasts and myocytes derived from muscle satellite cells of adult mice were chemoattracted to sulphoxized form of Tβ4. These data indicate that muscle injury enhances the local production of Tβ4, thereby promoting the migration of myoblasts to facilitate skeletal muscle regeneration.

Keywords: chemotaxis/myoblast/regeneration/skeletal muscle/thymosin beta4.

Abbreviations: bFGF, basic fibroblast growth factor; CT, cardiotoxin; DIG, Digoxigenin; DMEM, Dulbecco's modified Eagle's medium; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; ILK, integrin-linked kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; TA, tibialis anterior; Tβ4, thymosin β4; Tβ4so, thymosin β4 sulphoxized; Tβ10, thymosin β10.

Skeletal muscles are damaged and repaired daily, and support the locomotor functions of life. During muscle regeneration, various cytokines and secretary peptides are transiently produced by inflammatory immune cells and regenerating muscle fibres. These factors play an important role in the cell cycle entry of satellite cells (muscle stem cells), the differentiation of myoblasts and in myotube formation (*1*). Since some factors, such as insulin-like growth factor-1 (*2*) and vascular endothelial growth factor (*3*), have been reported to ameliorate the damaged skeletal muscles in mouse models of muscular dystrophy, it is important to extend our knowledge of these muscle injury-induced secretory molecules.

We and others reported previously that the expression of 'thymosin β4 (Tβ4)' mRNA is up-regulated in the skeletal muscles of dystrophin-deficient *mdx* mice (*4*, *5*) and in a *mdx*-derived myoblastic cell line (*6*) compared with their respective wild-type controls. Tβ4 is an N-terminally acetylated polypeptide of 4.9 kDa and contains 43 amino acid residues (*7*). Tβ4 sequesters intracellular monomeric G-actin and inhibits the assembly of actin fibres within cells (*8*). However, a substantial amount of Tβ4 is released from cells, where it serves as a paracrine factor to support angiogenesis, wound healing, hair growth and cardiac regeneration (*9*–*14*). It has been reported that Tβ4 is chemotactic for endothelial cells (*9*), keratinocytes (*12*) and cardiomyocytes (*14*). In contrast, Tβ4 is inhibitory for the chemotaxis of neutrophils (*15*). Notably, Tβ4 sulphoxide (Tβ4so), in which the sixth methionine residue is oxidized, has an even greater ability to inhibit the migration of neutrophils (*15*). Moreover, a closely related peptide, thymosin β10 (Tβ10) (*16*, *17*) may have similar functions to Tβ4.

Recently, two groups reported a critical role for Tβ4 and its cleavage product in cardiac regeneration (*13*, *14*). Tβ4 was shown to be physically associated with the LIM (Lin-1, Isl-1, and Mec-3) domain protein, PINCH (particularly interesting new Cys-His protein) and with integrin-linked kinase (ILK) to activate Akt kinase in cells (*13*). Tβ4 promoted the survival and repair of cardiomyocytes after cardiac injury through this signalling pathway, in addition to stimulating the migration of cardiomyocytes and endothelial cells.

The expression level of Tβ4 is reported to be high in wound fluid and in regenerating tissues (*18*–*20*). Based on the fact that 'Tβ4' expression is up-regulated in the skeletal muscles of *mdx* mice (*4*, *5*) and injured porcine muscles (*20*), we hypothesized that it may play an important role in skeletal muscle regeneration. In this study, we show that 'Tβ4' and 'Tβ10' mRNAs are induced in regenerating muscles and inflammatory haematopoietic cells. More importantly, we

demonstrate that Tβ4 serves as a chemoattractant for myoblasts.

Materials and Methods

Muscle injury models

First experimental model was set up as described earlier (*21*). Briefly, 100 μl of cardiotoxin (CT, 10 μM in 0.9% NaCl; Wako, Osaka, Japan) was injected into the tibialis anterior (TA) muscles of 6-week-old male C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) using 27G needles. The CT-treated TA muscles were then harvested at various time points (from 6 h to 14 days), fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4, frozen, sectioned and stained with haematoxylin and eosin.

Alternatively, TA muscles were quickly frozen in liquid nitrogen for RNA extraction. A second muscle crush-injury model was established by puncturing the gastrocnemius muscles of 8-week-old male C57BL/10 mice (Nihon SLC) with 23G needles. At different time points (from 5 h to 14 days) after injury, the gastrocnemius muscles were isolated, frozen in liquid nitrogen and subjected to RNA extraction.

Mice were maintained under a 12-h light/dark cycle in a pathogen-free animal facility. All experimental procedures involving the mice were pre-approved by the ethical committee of the institute.

Reverse transcription–polymerase chain reaction (PCR)

Total RNAs were prepared from skeletal muscle tissues using Trizol (Invitrogen, Carlsbad, CA, USA). Five micrograms of RNA from each sample was reverse-transcribed using the SuperScript II pre-amplification system for first strand cDNA synthesis with oligo(dT) primers (Invitrogen). Part of the cDNA mixture (1/125) was used in a PCR reaction with an annealing temperature of 56°C, ExTaq DNA polymerase (Takara, Otsu, Japan), and the following primer sets: 5′-TCTGACAAACCCGATATGGCT-3′ and 5′-CGATTCGCCAGCTTGCTTCTCT-3′ for detection of 'Tβ4' (PCR product: 129 bp), 5′-GCAGACAAGCCGGACATGGGG-3′ and 5′-GGAGATTTCACTCCTCTTTTCC-3′ for 'Tβ10' (PCR product: 129 bp) and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for 'glyceraldehyde 3-phosphate dehydrogenase' ('Gapdh'; PCR product: 452 bp), respectively. 'Gapdh' was used as a template control. Real-time PCR was performed with SYBR® premix ExTaq II (Takara). The $\Delta\Delta C_t$ method was used to determine relative mRNA expression levels.

In situ *hybridization*

The PCR-amplified 3′-untranslated regions of the 'Tβ4' and 'Tβ10' cDNAs were cloned into the PCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA, USA). Digoxigenin (DIG)-labelled RNA probes were prepared by using the DIG RNA labelling kit (Roche Diagnostics, Indianapolis, IN, USA). The CT-treated TA muscles from C57BL/6 mice were dissected on the fifth day after injection and frozen in isopentane pre-cooled in liquid nitrogen. Cryostat cross sections (10 μm) were prepared, fixed in 4% paraformaldehyde in PBS, and treated with 1 μg/ml proteinase K (Wako) in PBS at room temperature for 7 min. After acetylation with acetic anhydride in triethanolamine (Wako), the sections were hybridized with a DIG-labelled anti-sense or sense RNA probe at 65°C for 18 h and the signals were detected colorimetrically (*22*).

Cells and reagents

Mouse myoblast-derived C2C12 cells (American Type Culture Collection, CRL-1772) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) containing 10% fetal calf serum (Invitrogen) and 0.5% penicillin-streptomycin (Sigma). Myotube differentiation of C2C12 cells was carried out as previously described (*23*). Muscle satellite cells were separated from 8-week-old female C57BL/6 mice by using SM/C-2.6 antibody as described earlier (*24*) and sub-cultured for 7 days in 20% fetal calf serum, human basic fibroblast growth factor (bFGF, 5 ng/ml; PeproTech, Rocky Hill, NJ, USA) and 0.5% penicillin-streptomycin on matrigel (BD Biosciences, San Jose, CA, USA)-coated plates. SM/C-2.6$^+$ cells were sorted on a FACSAria (BD Biosciences). Approximately 4×10^5 cells of SM/C-2.6$^+$ fraction were obtained from skeletal muscle of two adult mice.

Tβ4 was chemically synthesized by the Peptide Institute Inc. (Osaka, Japan) and oxidized in the presence of 30% H_2O_2. Unoxidized Tβ4 was separated from Tβ4so by high-performance liquid chromatography.

Scratch wound closure assay

Confluent monolayers of C2C12 cells in 6-well plates were scratched with a blue tip to generate a gap (~5 × 1 mm). Cells were incubated with DMEM containing 10 mM thymidine (Sigma) at 37°C for 8 h in the presence or absence of various concentrations of Tβ4 or Tβ4so. The medium was replaced after 4 h of incubation. The scratched areas were photographed under a microscope both before and after culture, and the width of each gap measured.

Chemotaxis assay

C2C12 cells or muscle satellite cells-derived myoblasts/myocytes were washed and resuspended at a concentration of 10^6 cells/ml (C2C12) or 3.6×10^5 cells/ml (primary myoblasts/myocytes) in DMEM containing 0.1% fatty acid-free bovine serum albumin (Sigma) and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid pH 7.3 (Invitrogen). The wells of a 24-well culture plate were filled with 550 μl of Tβ4, Tβ4so or bFGF at various concentrations. Chemotaxicell chambers (8 μm pore size; Kurabo, Osaka, Japan) were pre-coated with bovine fibronectin (100 μg/ml; Wako) for 2 h at room temperature and placed in each well. Cells (200 μl) were then added to the upper chamber. The plate was then incubated for 6 h at 37°C, after which the cells remaining in the upper chamber were scraped off. The cells that had migrated to the bottom surface of the membrane were stained with Diff-Quik (Kokusai Shiyaku, Kobe, Japan) and counted using a microscope.

Statistical analyses

All statistical analyses were performed using ANOVA (analysis of variance) repeated measures analysis (Statview J5.0, Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was considered significant for the unpaired Student's *t*-test.

Results

Expression of Tβ4 and Tβ10 mRNAs is enhanced in skeletal muscles after injury

First, we investigated the expression patterns of 'Tβ4' and 'Tβ10' mRNAs during the recovery of injured skeletal muscles. As previously demonstrated (*21*), injection of CT into TA muscles caused the extensive degeneration of muscle fibres within 6 h (Fig. 1A). On Days 3–5, many inflammatory haematopoietic cells were recruited into the interstitial spaces. From Day 7 after CT injection, regenerating muscle fibers with central nuclei were gradually formed and the entire TA muscles were eventually repaired by Day 14 (Fig. 1A). Expression of 'Tβ4' mRNA was increased within 1 day, reaching its highest level on Day 3, and decreasing to base line levels by Day 14 after CT injection (Fig. 1B). Although the relative amount of 'Tβ10' mRNA was smaller than that of 'Tβ4', its expression was also maximal around Day 3 (Fig. 1B). In the crush injury model, 'Tβ4' mRNA was similarly up-regulated until Day 4 (when inflammatory reactions are initiated) and then gradually decreased by Day 14 (Fig. 1C).

Localization of Tβ4 and Tβ10 mRNAs in the regenerating skeletal muscles

We next determined the type of cells that produce 'Tβ4' and 'Tβ10' during the regeneration of TA muscles by *in situ* hybridization. 'Myosin light chain' was utilized as a marker for the regenerating muscle fibres. As shown in Fig. 2, 'Tβ4' and 'Tβ10' mRNAs were

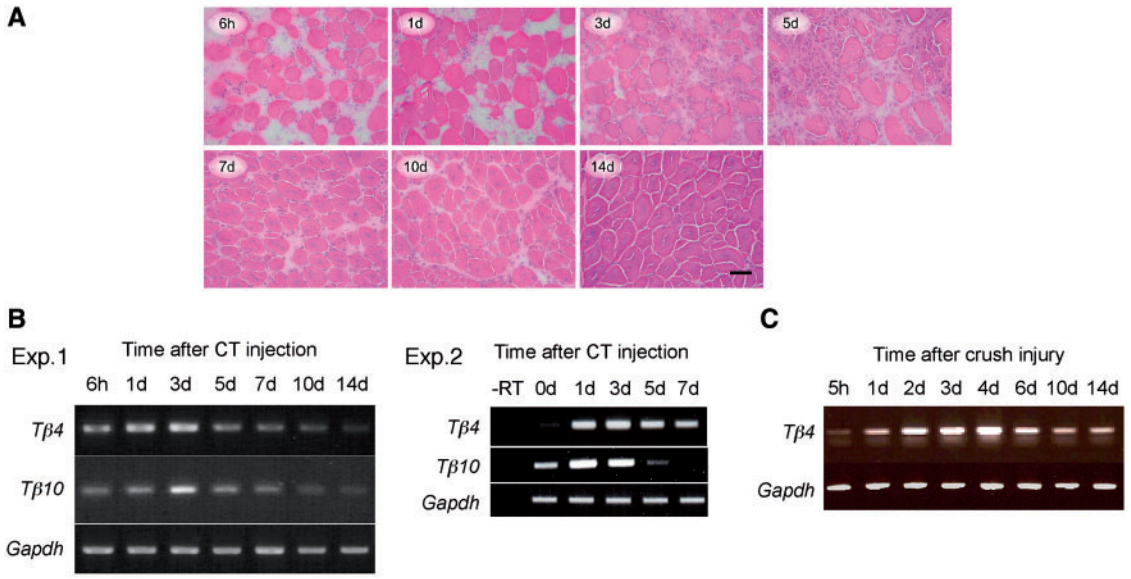

Fig. 1 Up-regulation of 'Tβ4' and 'Tβ10' mRNAs during skeletal muscle regeneration. (A) Histological appearance of skeletal muscle during the course of regeneration after CT injection. TA muscles were recovered from CT-injected mice at indicated time points (6 h to 14 days). Their frozen sections were stained with haematoxylin and eosin. Scale bar, 50 μm. (B and C) The mRNA levels of the indicated genes were analysed by reverse transcription–PCR at various time points (5 h to 14 days) after CT injection (B) or crush injury (C). Base line expression levels (Day 0) of 'Tβ4' and 'Tβ10' mRNAs were shown in the second CT injection experiment. DNA was visualized by ethidium bromide staining. 'Gapdh' was used as a template control.

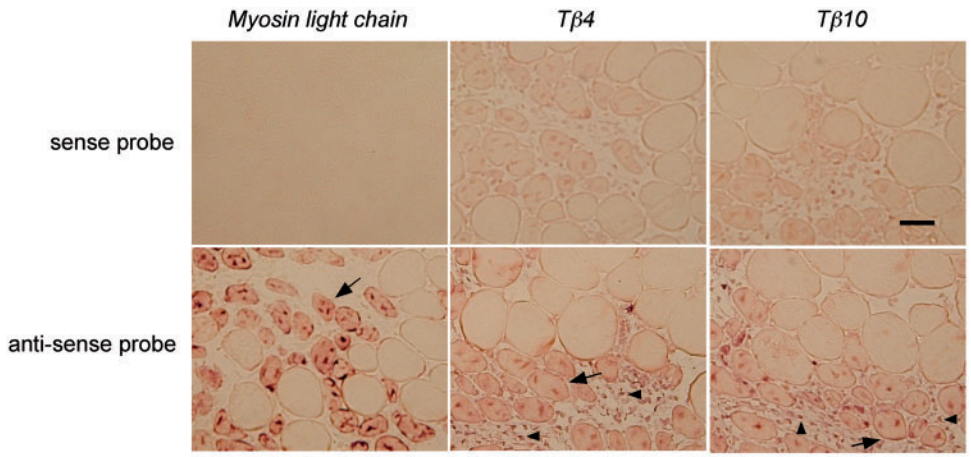

Fig. 2 Location of 'Tβ4' and 'Tβ10' mRNAs in the regenerating skeletal muscle. Transverse cryosections of TA muscles were harvested 5 days after CT injection and were hybridized with DIG-labelled sense (upper panels) or anti-sense (lower panels) cRNA probes for 'Myosin light chain', 'Tβ4' and 'Tβ10', respectively. Gene-specific signals in cytoplasm were visualized as light brown paints in the centrally nucleated muscle fibres (arrows) and interstitial haematopoietic cells (arrowheads). Scale bar, 50 μm.

detectable in centrally nucleated regenerating muscle fibres, as well as in the haematopoietic cells present in the interstitial spaces (Fig. 2). These staining patterns were not observed with the sense probes, confirming the specificity of signals.

Migratory responses of C2C12 cells to Tβ4 and Tβ4so

As Tβ4 is known to promote the migration of endothelial cells and cardiac myocytes (*9*, *14*), we examined the migratory responses of myoblasts to both Tβ4 and its oxidized form. As shown in Fig. 3A, both forms significantly enhanced the wound closing capacity of C2C12 cells in a dose-dependent manner. Maximum activity was observed at 100 pg/ml for Tβ4so, whereas Tβ4 exhibited a constantly high activity at concentrations >100 pg/ml. In contrast, the chemotactic responses of C2C12 cells to both Tβ4 and Tβ4so were very similar in terms of dose dependency (Fig. 3B). The total number of cells migrated in response to Tβ4 and Tβ4so was comparable to that seen with bFGF, a known chemotactic factor for C2C12 cells (*25*). When Tβ4 or Tβ4so was included in the upper chamber, or both the upper and lower chambers, of Chemotaxicell, enhancement of cell migration was cancelled (Fig. 3C). Therefore, Tβ4 and Tβ4so induce chemotaxis, but not chemokinesis to C2C12 cells.

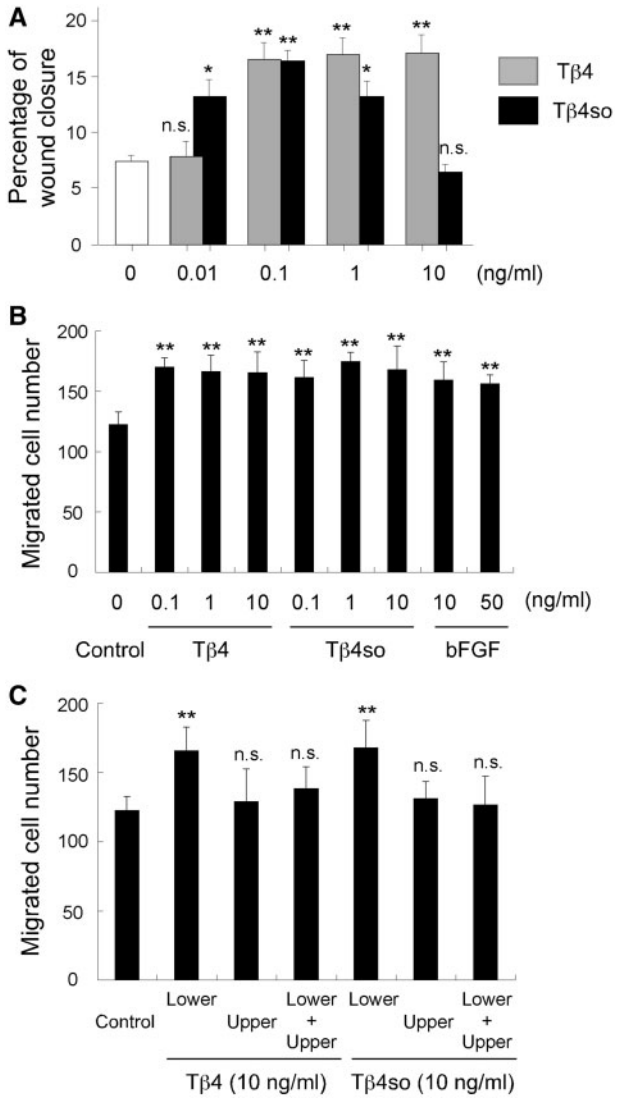

Fig. 3 Wound closure and chemotactic responses of C2C12 cells by exogenous Tβ4 and Tβ4so. (A) Recovery ratio of scratched wounded areas of C2C12 monolayer was measured in the presence or absence of indicated concentrations of Tβ4 or Tβ4so. (B) Chemotactic responses of C2C12 cells to various concentrations of Tβ4 and Tβ4so. bFGF was used as a positive control. (C) Evaluation of chemokinetic responses of C2C12 cells to Tβ4 and Tβ4so. In (B) and (C), assays were performed using Chemotaxicell (8 μm pore size) with indicated reagents added in the upper and/or lower chambers. After 6 h of incubation, cells that had migrated to the bottom surface of the membrane were stained and subjected to microscopic observation and cell counting. Each value represents the mean ± S.D. ($n = 8$–9). $^{*}P < 0.05$; $^{**}P < 0.01$. n.s., not significant compared to the value of medium control.

Chemotactic responses of satellite cells-derived primary myoblasts/myocytes

To confirm that Tβ4-responsiveness is a general property of myoblasts and not a C2C12-specific phenomenon, we prepared primary myoblasts and used them for the chemotaxis assay. For this purpose, we isolated SM/C2.6^{+} muscle satellite cells from adult mice and cultured them for *in vitro* differentiation. As previously demonstrated (*24*), they vigorously proliferated and gave rise to myoblasts, myocytes and myotube-like structures (Fig. 4A). When we applied these cells to

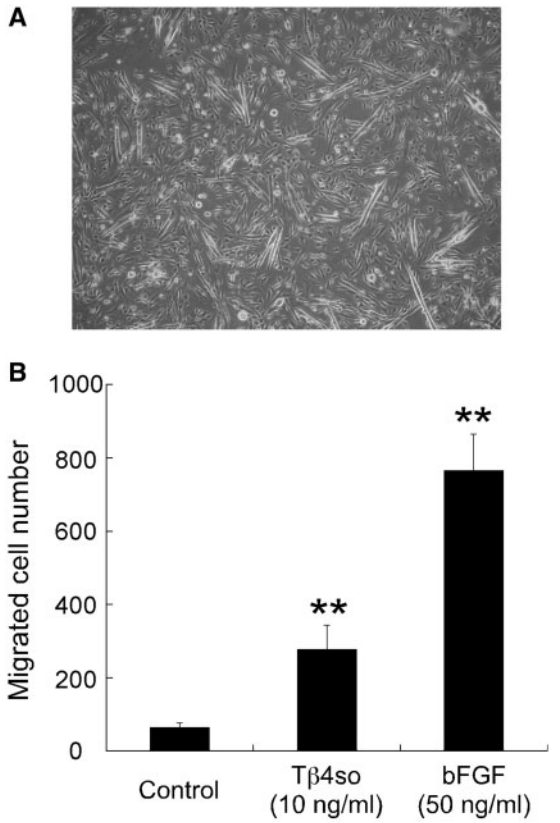

Fig. 4 Chemotaxis of satellite cells-derived myoblasts/myocytes to exogenous Tβ4so. (A) Morphological appearance of primary myoblasts and myocytes derived from muscle satellite cells of adult mice. (B) Chemotactic responses of primary myoblasts/myocytes to Tβ4so and bFGF. Cells and indicated reagents were added in upper and lower chambers of Chemotaxicell (8 μm pore size), respectively. After 6 h of incubation, cells that had migrated to the bottom surface of the membrane were stained and counted. Each value represents the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared to the value of medium control.

Chemotaxicell, significant number of cells migrated across the membrane in response to Tβ4so (Fig. 4B). These data demonstrated the responsiveness of primary myoblasts/myocytes to Tβ4so. It is noteworthy that satellite cells-derived primary cells were more strongly chemoattracted to bFGF than to Tβ4so (Fig. 4B) when compared to C2C12 cells (Fig. 3B). This is probably related to a higher frequency of myocytes in the primary cell population.

Up-regulation of Tβ4 and Tβ10 mRNAs during myotube differentiation

To understand whether expression of 'Tβ4' and 'Tβ10' mRNAs is changed between myoblasts and myofibers, we compared their expressions in C2C12 cells before and after myotube differentiation *in vitro*. As shown in Fig. 5, both 'Tβ4' and 'Tβ10' mRNAs were significantly increased by the myotube differentiation.

Discussion

Previous reports from several laboratories noted that the expression levels of 'Tβ4' in the skeletal muscle of *mdx* mice are significantly higher than

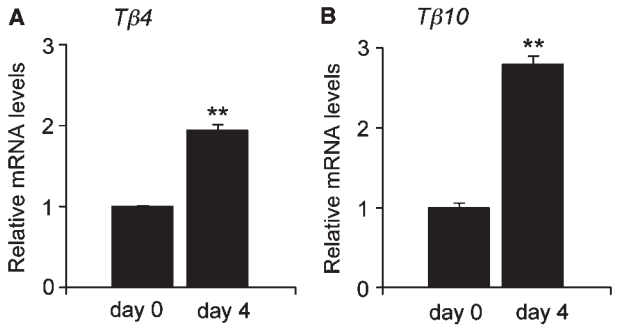

Fig. 5 Increase of 'Tβ4' and 'Tβ10' mRNAs during myotube differentiation of C2C12 cells. (A) C2C12 cells were cultured in the differentiation media to induce myotube-like structures. Expression levels of 'Tβ4' (A) and 'Tβ10' (B) mRNAs before (Day 0) and after (Day 4) the myotube differentiation were determined by real-time RT–PCR. 'Gapdh' was used to normalize the relative abundance of 'Tβ4' or 'Tβ10' mRNA. In each panel, value of Day 4 was expressed as a relative amount to that of Day 0. Each value represents the mean ± S.E. ($n = 3$). **, $P < 0.01$ compared to the value of Day 0.

those of wild-type mice (*4*, *5*). We also found that larger amounts of 'Tβ4' mRNA are produced by the *mdx*-derived myoblastic cell line (*6*) and the skeletal muscles of *mdx* mice (Nakayama, Y. and Hara, T., unpublished data). *In situ* hybridization data show that both 'Tβ4' and 'Tβ10' mRNAs are present in regenerating muscle fibres, but are absent from mature skeletal muscles. The specific localization of Tβ4 protein in regenerating muscles was shown in a very recent report using *mdx* mice (*26*). In addition to immature muscles, we found that haematopoietic cells in the interstitial spaces also produced 'Tβ4' and 'Tβ10' mRNAs. In the early stages (3–5 days) of muscle regeneration after CT injection, large amounts of inflammatory blood cells, including macrophages and T cells, infiltrate into the degenerated areas of skeletal muscle. These cells play an important role in tissue repair by secreting a number of cytokines. Accordingly, the levels of 'Tβ4' and 'Tβ10' mRNAs in the TA muscles were at their highest on Day 3 after CT injection.

Tβ4 is known to promote wound healing in skin (*11*), hair growth (*12*) and cardiac repair (*13*, *14*). However, the physiological relevance of this up-regulation of 'Tβ4' and 'Tβ10' in *mdx* muscles and injured skeletal muscles remains to be clarified. In this study, we demonstrated that Tβ4 and its oxidized form, Tβ4so, are capable of stimulating the migration of both C2C12 cells and skeletal muscle-derived primary myoblasts/myocytes. Tβ4so was more potent in promoting the migration of C2C12 cells in the scratch wound assay than Tβ4, as was shown for human umbilical vein endothelial cells (*15*). As the chemotaxis-inducing activity of Tβ4 and Tβ4so was very similar, these two assays might detect a distinct biological activity of Tβ4so. In this regard, it is interesting that Tβ4so, but not Tβ4, is a potent inducer of neutrophil locomotion, and suppresses footpad swelling (*15*). However, we cannot rule out the possibility that an action range of the chemotaxis assay is broader than that of wound closure assay.

As mentioned above, 'Tβ4' mRNA is expressed in myoblastic cell lines and its expression level is higher in *mdx*-derived myoblastic cells than that in C57BL/10 mice-derived myoblasts (*6*). If Tβ4 acts as a chemoattractant for myoblasts, how Tβ4 gradient is generated in the regenerating skeletal muscles? We demonstrated that both Tβ4 and 'Tβ10' mRNAs were markedly up-regulated during myotube differentiation of C2C12 cells. Therefore, newly formed myofibers as well as inflammatory haematopoietic cells would produce larger amounts of Tβ4 and Tβ10 locally, thereby facilitating mobilization of myoblasts into the regenerating skeletal muscle regions.

Given the fact that Tβ4 and Tβ4so mobilize myoblasts to injured muscles, it may also facilitate the regeneration of injured skeletal muscles. In fact, a very recent report by Spurney *et al*. (*26*) shows that Tβ4-treated *mdx* mice have significantly increased numbers of regenerating muscle fibres compared with control *mdx* mice. Considering the role of Tβ4 in cardiac regeneration, it is likely that Tβ4 mediates the recruitment and survival of myoblasts via the PINCH-ILK-Akt pathway. This is supported by the fact that ILK and Akt are activated in C2C12 cells in response to Tβ4 (*14*). Alternatively, a reduction of NF-κB activity induced by Tβ4 might play a role in the anti-apoptotic effects seen in skeletal muscles (*27*).

Unfortunately, the impaired skeletal muscle strength and fibrosis seen in *mdx* mice were not ameliorated after chronic administration of Tβ4 in the study of Spurney *et al*. (*26*). In transgenic mouse lines overexpressing Tβ4 in their skeletal muscles, we failed to observe significant differences in the kinetics of muscle regeneration after CT injection (Nakayama, Y. and Hara, T., unpublished data). In this case, it is possible that the concentrations of endogenous Tβ4 and Tβ10 were sufficiently high to mobilize myoblasts in the injured muscles of these transgenic mice. Regarding the clinical application of Tβ4 for the treatment of muscular dystrophies, the combination of Tβ4 with other regeneration-promoting cytokines would be beneficial.

Only endothelial cells (*9*), keratinocytes (*12*), cardiomyocytes (*14*) and skeletal myoblasts (this study) are chemoattracted to Tβ4. Fibroblasts, smooth muscle cells, monocytes and neutrophils do not respond to Tβ4 (*9*, *15*). Interestingly, both Tβ4 and Tβ4so are rather inhibitory for neutrophil chemotaxis elicited by N-formyl-methion-leucyl-phenylalanine (*15*). Therefore, Tβ4 modulates cell migration in a cell-type specific fashion to either promote tissue regeneration, or attenuate inflammatory responses. Future investigation of putative Tβ4 receptors would provide critical information for understanding how extracellular Tβ4 exerts its biological activities in cells.

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Conflict of interest
None declared.

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