ORIGINAL

Overexpression of osteoactivin protects skeletal muscle from severe degeneration caused by long-term denervation in mice

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Abstract : We have previously shown that osteoactivin, a type I membrane glycoprotein expressed in myofibers, upregulated expression of matrix metalloprotease (MMP)-3 and MMP-9 in fibroblasts infiltrated denervated skeletal muscle in mice. To address whether osteoactivin-mediated increase in MMPs in skeletal muscle is useful for regeneration of denervated skeletal muscle, we subjected osteoactivin-transgenic mice to long-term denervation for 70 or 90 days. Long-term denervation caused severe degeneration of myofibers and fibrosis in skeletal muscle of wild-type mice. However, overexpression of osteoactivin protected skeletal muscle from such changes. Infiltration of fibroblast-like cells and collagen deposition were sustained at low levels after long-term denervation in skeletal muscle of osteoactivin-transgenic mice. This cytoprotective effect of osteoactivin was supported by the expression of regeneration/degeneration-associated genes in the gastrocnemius muscle during denervation. Denervation significantly upregulated the expression of anti-fibrotic genes, such as glypican-1 and decorin-1, in the gastrocnemius muscle of osteoactivin-transgenic mice, compared with wild-type mice. In contrast, overexpression of osteoactivin caused a significant reduction in denervation-induced expression of elongation factor 1A-1, an indicator for the persistence of degenerated cells. Our results suggest that an osteoactivin-mediated increase in MMPs in skeletal muscle might be useful for protecting injured muscle from fibrosis, leading to full regeneration after denervation. J. Med. Invest. 54 : 248-254, August, 2007

Keywords **:** *denervation, fibrosis, matrix metalloproteases, osteoactivin-transgenic mice, skeletal muscle*

INTRODUCTION

Osteoactivin is a rat homolog of the Gpnmb family, which was originally reported to be highly expressed in human melanoma cells (1). Recently, we found that unloading conditions, such as denervation (sciatic neurectomy) and spaceflight, caused a remarkable increase in the expression of osteoactivin in skeletal muscle (2), and that osteoactivin upregulated expression of matrix metalloprotease (MMP)-

Abbreviations :
ANOVA, analysis of variance ; BDF1, C57 BL/6xDBA/2) F1; eEF1A-1, elongation factor 1A-1 ; FGF2, fibroblast growth factor 2 ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase ; HE, hematoxylin and eosin ; IGF-1, insulin-like growth factor-1 ; MMP, matrix metalloprotease ; MCP-1, monocyte chemoattractant protein-1 ; NGF, nerve growth factor ; RT-PCR, reverse transcription and polymerase chain reaction ; TGF-β, transforming growth factor-β.

Received for publication February 9, 2007 ; accepted March 28, 2007.

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3 and MMP-9 in fibroblasts infiltrated into denervated skeletal muscle in mice (3). On the basis of these findings, we suggested that an osteoactivin-mediated increase in MMPs in skeletal muscle might be useful for regeneration of denervated skeletal muscle, leading to compensation for the loss of muscle volume or protection of muscle fibers against injury after denervation. To address this issue, in the present study we subjected osteoactivin-transgenic mice to long-term denervation for 70 or 90 days and examined its effect on regeneration or degeneration in the denervated skeletal muscle.

MATERIALS AND METHODS

Generation of osteoactivin-transgenic mice

We established three strains of hemizygous osteoactivin-transgenic mice as described previously (3). Briefly, the *BamHI/PmeI* fragment of pcDNA 3.1/V5-His-tagged rat osteoactivin was subcloned into an expression vector containing the cytomegalovirus immediate early enhancer chicken β-globin hybrid promoter (4). This V5-His-tagged rat osteoactivin cDNA construct was injected into fertilized (C57BL/6xDBA/2) F1 (BDF1) eggs for the production of transgenic mice (Japan SLC, Inc., Shizuoka, Japan). The transgenics expressing the highest levels of osteoactivin were back-crossed onto BDF1 mice. The mice were kept under specific pathogen-free conditions in a room maintained at 23±2°C on a 12-h light/dark cycle and were allowed free access to food and water. Osteoactivin-transgenic mice without denervation were fertile and apparently normal.

All animal experiments in the present study were approved by The Committee for the Care and Use of Animals in The University of Tokushima Faculty of Medicine and were performed by the Institutional Animal Care and Oversight Committee according to established guideline principles.

Denervation

Adult male osteoactivin-transgenic and BDF1 (wild-type) mice (approximately 9 weeks old), weighing 18-22 g, were subjected to denervation at the same time, as described previously (2). For the denervation procedure, the dorsal skin of the right thigh was cut and the posterior muscles divided to reveal the sciatic nerve. A chronic denervation was obtained by removing a 5-mm-long section of the sciatic nerve. Gastrocnemius muscles isolated on Day 10, 20, 70 and 90 after denervation were immediately frozen in chilled isopentane and liquid nitrogen and stored at -80°C until analysis.

Histochemical analysis

Sections (5 μm) were fixed in ice-cold acetone for 10 min. After being rinsed with phosphate buffered saline (PBS) three times, the fixed sections were incubated with a 1 : 200 dilution of monoclonal anti-human vimentin antibody labeled with Cy3 (Sigma, St. Louis, MO) at 4°C for 18 hr. Sections were also subjected to hematoxylin and eosin (HE), or Van Gieson staining (5).

Semi-quantitative reverse transcription and polymerase chain reaction (RT-PCR)

To measure the mRNA level, semi-quantitative RT-PCR was performed as described previously (3). Following the synthesis of first strand cDNA from mRNA, second-strand synthesis and amplification of target genes were performed. In this case, the PCR buffer contained two sets of primers to amplify a target gene cDNA as well as the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA simultaneously. The sense and antisense primers used in this study are shown in Table 1. The PCR products were separated by PAGE on an 8% gel and detected with a highly-sensitive nucleic acid staining reagent (TaKaRa, Tokyo, Japan). The intensities of staining of the target bands, and those of internal standard gene cDNAs, were estimated using an image analyzer (FMBIO II, TaKaRa), and the intensity ratio of a target gene cDNA to the internal standard gene cDNA was calculated.

Statistical analysis

The experimental data are expressed as means ± SD for 4 each group of 4 replicates and were statistically evaluated by one-way analysis of variance (ANOVA) using the SPSS computer program (release 6.1 ; SPSS Japan, Tokyo). Individual differences between groups were assessed using Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

RESULTS

Histochemical changes in the gastrocnemius muscle of osteoactivin-transgenic mice after denervation

HE staining showed that 20-day denervation de-

creased the size of myofibers and caused the infiltration of mononucleated cells into the interstitial space of myofibers similarly in wild-type or osteoactivin-transgenic mice, compared with the respective controls (before denervation) (Fig. 1A). Because these cells stained positively with an anti-vimentin antibody (Fig. 1B), they were identified as fibroblast-like cells as described previously (3). In wild-type mice, long-term denervation for 70 or 90 days caused regeneration and degeneration of myofibers, as indicated by the large numbers of muscle fibers with central nuclei, shown by arrows in Fig. 1A. Furthermore, long-term denervation stimulated the infiltration of fibroblast-like cells into interstitial space of myofibers of wild-type mice (Fig. 1A) and caused collagen deposition in the intestinal space (Fig. 1C). In contrast, in osteoactivin-transgenic mice, little degeneration of myofibers was observed even after

Table 1. Primers for PCR

Target gene		Sequence	Length (bp)
MMP-3	S	5'-GGAAATCAGTTCTGGGCTATACGAGG-3'	301
	AS	5'-CCAACTGCGAAGATCCACTGAAGAAG-3'	
MCP-1	S	5'-CCCAATGAGTAGGCTGGAGA-3'	125
	AS	5'-TCTGGACCCATTCCTTCTTG-3'	
eEFlA-1	S	5'-AGTGAGCTCTTCCTGGGACA-3'	292
	AS	5'-TCGCCAGACTTCAGGAACTT-3'	
Glypican-1	S	5'-GGGACACTGTGCAGTGAGAA-3'	129
	AS	5'-AGGGTTGTTGATCTGGTTGG-3'	
Decorin-1	S	5'-TTTCTTGGAGCCAGCAGAAT-3'	162
	AS	5'-TGGGCTTTCTGTGTTTACCC-3'	
GAPDH	S	5'-ACCCAGAAGACTGTGGATGG-3'	125
	AS	5'-TTCAGCTCTGGGATGACCTT-3'	

AS, antisense primer ; S, sense primer ; eEFlA-1, elongation factor 1 A-1.

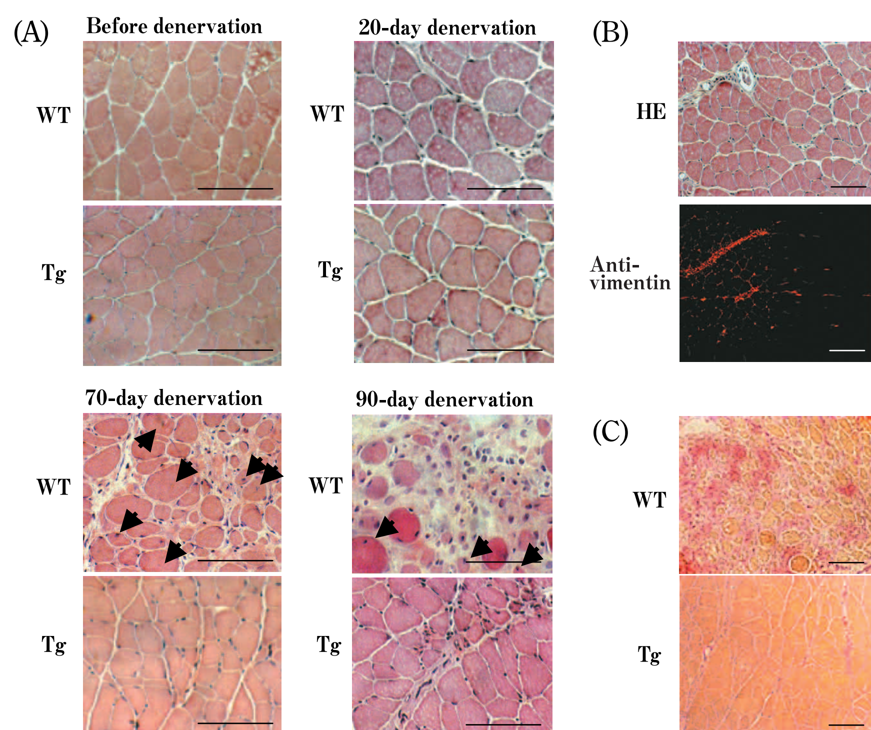

Fig. 1. Histochemical changes in the skeletal muscle of wild-type or osteoactivin-transgenic mice subjected to denervation (A) Wild-type (WT) or osteoactivin-transgenic (Tg) mice were subjected to denervation for the indicated periods. Sections (5 μm) from the gastrocnemius muscle of denervated mice were stained with hematoxylin and eosin (HE). Arrows indicate muscle fibers with central nuclei. (B) Serial sections (5 μm) from the gastrocnemius muscle of 20-day denervated wild-type mice were stained with hematoxylin and eosin (HE) and an antibody against vimentin, a fibroblast-specific protein. (C) Sections (5 μm) from the gastrocnemius muscle of 90-day denervated mice were subjected to Van Gieson staining to detect collagen deposition. Similar results were obtained in three separate experiments. Scale = 100 μm.

such long-term denervation (Fig. 1A). More interestingly, the infiltration of fibroblast-like cells and collagen deposition remained at low levels up to 70 and 90 days after denervation in the gastrocnemius muscle of osteoactivin-transgenic mice (Fig. 1A & C).

Expression of MMP-3 and regeneration/degeneration-associated genes in the skeletal muscle of osteoactivin-transgenic mice after denervation

We previously reported that 16-day denervation further enhanced expression of MMP-3 in the gastrocnemius muscle of osteoactivin-transgenic mice (3). Consistent with this previous finding, long-term denervation for more than 20 days stimulated expression of MMP-3 in the muscle, compared with wild-type mice (Fig. 2A and B). The amounts of MMP-3 in the gastrocnemius muscle of osteoactivin-transgenic mice were sustained at this high level even after 70-day denervation, while those of wild-type mice returned to the basal level (the pre-denervation value).

Since infiltration of macrophages into skeletal muscle is necessary to regenerate muscle fibers (6-8), we examined expression of a macrophage specific gene, monocyte chemoattractant protein-1 (MCP-1). Denervation for 10 or 20 days stimulated expression of MCP-1 in the gastrocnemius muscle of wild-type mice (Fig. 2A and C). This increased expression of MCP-1 was further enhanced in the skeletal muscle of osteoactivin-transgenic mice. However, MCP-1 expression in wild-type and osteoactivin-transgenic mice returned to the basal level by 70 days after denervation.

We also examined changes in expression of regeneration/degeneration-associated genes in the gastrocnemius muscle during long-term denervation. In the muscle of wild-type mice, denervation suppressed expression of the heparan sulfate proteoglycan glypican-1, a low affinity receptor for fibroblast growth factor 2 (FGF2) (9, 10), in a time-dependent manner, whereas the expression of glypican-1 in osteoactivin-transgenic mice was stimulated following denervation (Fig. 2A and D). Furthermore, expression of decorin-1, an anti-fibrotic agent (11, 12), in the gastrocnemius muscle was remarkably stimulated in denervated osteoactivin-transgenic mice, while denervation alone only tentatively induced its expression in wild-type mice (Fig. 2A and E). In contrast, overexpression of osteoactivin caused a significant reduction in denervation-induced expression of elongation factor 1A-1 (eEF 1A-1), an indicator for the persistence of degenerated cells (13), in the gastrocnemius muscle (Fig. 2A and F).

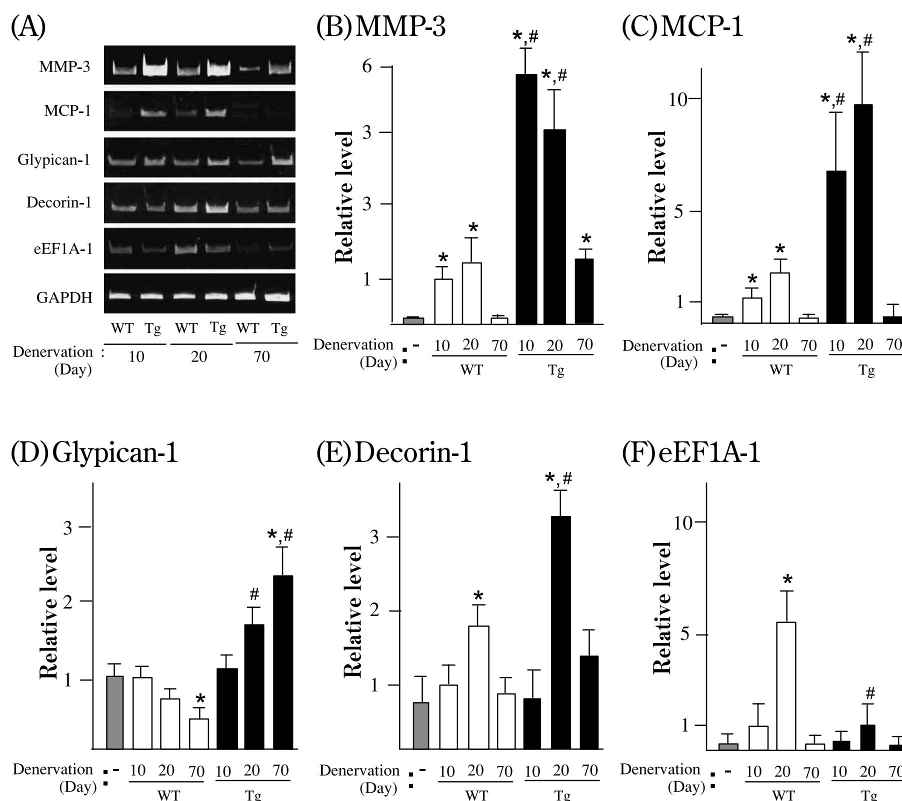

Fig. 2. Expression of MMP-3 and regeneration/degeneration-associated genes in the skeletal muscle of osteoactivin-transgenic mice after denervation
Wild-type (WT) or osteoactivin-transgenic (Tg) mice were subjected to denervation for the indicated periods. The amounts of MMP-3, MCP-1, glypican-1, decorin-1, eEF1A-1 or GAPDH in total RNA extracted from the gastrocnemius muscles were quantified using semi-quantitative RT-PCR, as described in MATERIALS AND METHODS. The intensity ratio of the cDNA of interest to GAPDH in each group was calculated and shown as a value relative to that in 10-day-denervated wild-type mice, which was set as one. The values of osteoactivin-transgenic and wild-type mice before denervation were similar. Values shown are means ± SD (n = 4). *$P < 0.05$, compared with the value of animals without denervation, $^{\#}P < 0.05$, compared with the value of wild-type mice.

DISCUSSION

A lot of investigations have shown that growth factors, including insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF2), and nerve growth factor (NGF), can improve muscle regeneration during the preliminary phase of healing (14-17). However, none of the growth factors that have been studied appear able to completely heal injured muscle, because the development of fibrosis hinders muscle regeneration and prevents full strength recovery in the injured skeletal muscle (18, 19). This process begins 2 weeks after muscle injury and continues over time (20, 21). Therefore, preventing fibrosis after muscle injury is one of the most important subjects affecting regeneration of myofibers.

We have previously reported that osteoactivin upregulates expression of MMP-3 and MMP-9 in fibroblasts infiltrated into denervated skeletal muscle in mice (3). MMP-3 has been reported to regulate growth and development of tissues by selective degradation of IGF-1/IGF-1-binding protein complexes (22). Secreted MMP-9 is also involved in the migration and myotube formation of myoblastic cells (23). Furthermore, targeted disruption of the MMP-3 gene in mice caused a delay in wound healing due to a failure in fibroblast contraction (24, 25). Synthetic MMP inhibitors tested in clinical trials had reversible musculoskeletal toxicity as the main side effect (26). On the basis of these findings, an osteoactivin-mediated increase in MMPs in skeletal muscle might be useful for protecting injured muscle from fibrosis, leading to full regeneration after denervation.

HE staining in the present study showed for the first time, to our knowledge, that overexpression of osteoactivin clearly prevents denervation-mediated fibrosis in skeletal muscle *in vivo*. This cytoprotective effect of osteoactivin is supported by the expression of regeneration/degeneration-associated genes in the gastrocnemius muscle during long-term denervation. Denervation significantly induces expression of anti-fibrotic agents, such as glypican-1 and decorin-1, in the gastrocnemius muscle of osteoactivin-transgenic mice, compared with wild-type mice. The overexpression of glypican-1 in turkey myogenic satellite cells increases their proliferation and responsiveness to FGF2, leading to regeneration of injured skeletal muscle (9, 10). In contrast, transforming growth factor-β(TGF-β), which is highly expressed in injured skeletal muscle (27), stimulates the deposition of collagens and overgrowth of the extracellular matrix, thereby leading to the formation of fibrosis (28, 29). Since decorin-1 has a potent anti-TGF-β action (11, 12), it is likely that its increased expression supports the reduction of fibrosis. In fact, the combined administration of decorin-1 and IGF-1 has been reported to enhance muscle regeneration and reduce fibrosis (30). At present, we cannot determine whether overexpression of osteoactivin induces expression of such anti-fibrotic agents directly or indirectly (by a macrophage-mediated mechanism). Further investigations are necessary to evaluate this hypothesis.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-aid "Initiative Education and Research for Graduate School University" promoted by the Ministry of Education, Culture, Sports, Science and Technology.

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