The Journal of Veterinary Medical Science

NOTE

Anatomy

Neutralization of transforming growth factor (TGF)-β1 activity reduced fibrosis and enhanced regeneration of glycerol-injured rat muscle

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J. Vet. Med. Sci.
82(2): 168–171, 2020
doi: 10.1292/jvms.19-0446

Received: 17 September 2019
Accepted: 6 December 2019
Advanced Epub:
25 December 2019

ABSTRACT. Recently, we have shown that glycerol induces early fibrosis in rat muscles which persists up to two weeks after injury. The current study aims to determine the possible factor associated with fibrosis of rat muscle following glycerol injury. Eight-week-old male Wistar rats received either glycerol only (as a control) or a co-treatment of neutralizing antibody to transforming growth factor (TGF)-β1 (5 and 12.5 μg). Both antibody doses significantly decreased fibrosis and improved muscle regeneration suggesting that anti-TGF-β1 antibody has both anti-fibrotic and myogenic effects. In conclusion, fibrosis developed in glycerol-injured rat muscles, might be mediated, in part, by the upregulation of TGF-β1 expression. Targeting TGF-β1 could be a promising approach for inhibiting fibrosis and enhancing muscle regeneration.

KEY WORDS: anti-transforming growth factor-β1, fibrosis, glycerol injury, muscle regeneration, rat

Skeletal muscles are exposed daily to several types of injuries and strains especially in sports [8]. As a result, injured muscle undergoes degenerative and regenerative changes. Regardless of the high regeneration capability of skeletal muscle, excessive accumulation of extracellular matrix (ECM) components compromises muscle regeneration and results in fibrosis [5]. Muscle fibrosis is a common feature in muscular dystrophies, aged muscles, and following severe injuries. It impairs muscle function and regeneration after injury and renders the muscle to be more susceptible to re-injury [16]. Furthermore, replacing myofibers with ECM hinders cell and gene therapies through reduction of the amount of targeted muscle available for repair [9].

Recently, we have shown that intramuscular injection of glycerol induces significant fibrosis in rat muscles at early stages of regeneration with inflammatory cellular infiltration that persists up to 2 weeks after injury [19]. However, the possible factors responsible for this fibrosis are still unknown. The pro-fibrotic factor, transforming growth factor (TGF)-β has been reported as a key mediator of fibrosis in different organs including muscles [16, 22, 24]. A recent study showed that fibrosis induced by irradiation of rat muscles is associated with the up-regulation of TGF-β1 [29]. Moreover, TGF-β1 level increases following strain-induced injury [23] and acute kidney injury induced by intramuscular injection of glycerol in rats [11]. Therefore, we hypothesized that increased fibrosis in glycerol-injured rat muscles might be due to TGF-β1. To test our hypothesis, we treated glycerol-injured rat muscles with a neutralizing antibody to TGF-β1. Treatment with a neutralizing TGF-β1 antibody significantly reduced fibrosis and enhanced muscle regeneration, which suggests an active role of TGF-β1 in fibrous tissue accumulation and impaired regeneration in glycerol-injured rat muscles.

The animal experiments were approved by the Animal Research Committee of Tottori University, Japan (approval number 15-T-24). Adult male Wistar rats (CLEA Japan, Tokyo, Japan), 8-weeks of age and weighting 200–220 g were anesthetized by intraperitoneal injection of sodium pentobarbital (0.02 mg/g body weight). Animals were randomly divided into 3 groups ($n=5$). Two groups received 500 μl of 50% glycerol (Wako, Osaka, Japan) containing either 5 μg (lower dose) or 12.5 μg (higher dose) of chicken polyclonal anti-TGF-β1 IgY (AF-101-NA; R&D Systems, Minneapolis, MN, USA), respectively [20], while the third group received glycerol only and was used as a negative control. Injection was performed into the left tibialis anterior (TA) muscle. The skin of the left hind limb was shaved using a razor and disinfected with iodine. Then injection was performed along the TA muscle during withdrawing the needle [17]. Animals were killed by inhalation of an overdose of isoflurane (Intervet,

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Osaka, Japan). Muscle samples were harvested on day 7 after injection and routinely processed. Hematoxylin and eosin (HE)-stained paraffin sections were examined on an Olympus inverted microscope (IX71, Olympus, Tokyo, Japan). Digital images were obtained and used for evaluation of muscle morphology, as well as, performing morphometric measurements. To assess fibrosis, muscle sections were stained with picrosirius red solution (Polysciences, Warrington, PA, USA) for 1 hr then washed with 0.5% acetic acid solution in water, dehydrated in ascending series of ethanol, cleared in xylene, and mounted using Eukitt mounting medium (O. Kindler GmbH, Freiburg, Germany). Three non-overlapping fields at X10 objective lens were examined per section and three sections for each animal were selected. Fibrosis index was assessed by calculating the Sirius red-positive area, using the Image-J software (National Institutes of Health, Bethesda, MD, USA), in relation to the total myofiber area [19]. To evaluate muscle regeneration, the smallest diameters (minor axis diameters) of about 150 newly-formed myotubes (with central nuclei) in each injured muscle were measured using the Image-J software [19]. To compare the data between groups, data were analyzed using SPSS software, version 21 (IBM SPSS, Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Data were shown as mean ± standard deviation (SD), and significant difference was indicated when $P<0.05$.

To investigate whether TGF-β1 mediates muscle fibrosis after glycerol-induced injury, glycerol-injured muscles were treated with a neutralizing antibody to TGF-β1. Two different doses of the antibody were co-injected with glycerol, and muscle regeneration was assessed on day 7 after injection. Sirius red staining revealed that treatment with the neutralizing antibody to TGF-β1 decreased the fibrosis index by about 13% and 27% ($P<0.05$) in TA muscle that receive lower dose and higher dose of neutralizing antibody, respectively, compared with that in the control rat muscle (Fig. 1A). On the other hand, neutralizing antibody

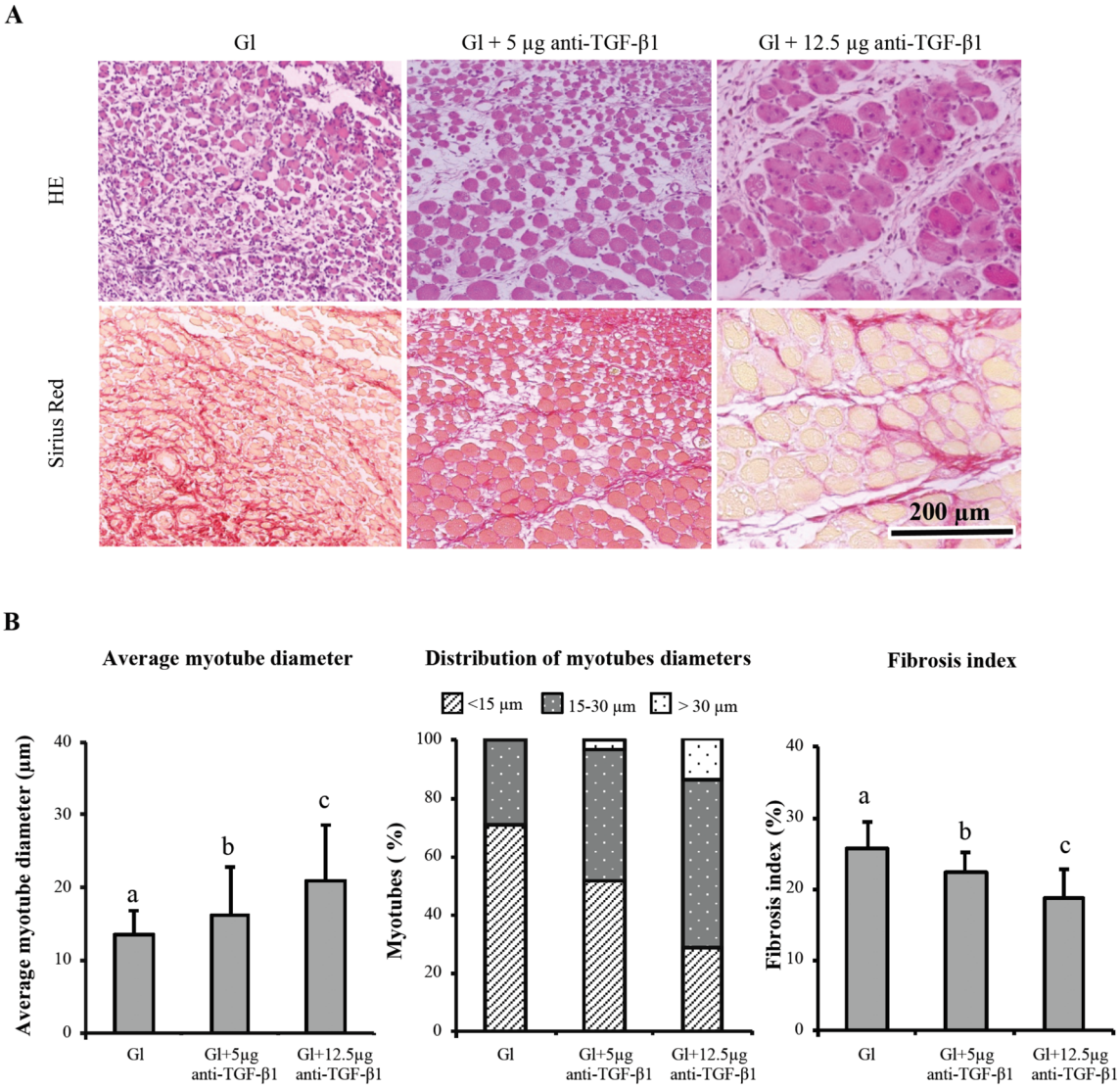

Fig. 1. Treatment with a neutralizing antibody to transforming growth factor (TGF)-β1 enhanced muscle regeneration and reduced fibrosis. (A) Sections of the tibialis anterior (TA) muscle injected with glycerol (Gl) as a control, Gl + 5 μg anti-TGF-β1, and Gl + 12.5 μg anti-TGF-β1 stained with hematoxylin and eosin (HE) and Sirius red. (B) Treatment with neutralizing antibody to TGF-β1 activity decreased the fibrosis index, increased the average myotube diameter, and shifted the distribution of myotube diameters toward larger values. Different letters indicate significant difference. Data are expressed as mean ± standard deviation (SD), and significant difference is indicated ($P<0.05$).

treatment markedly enhanced the regeneration of TA muscle on day 7, as evidenced by improved muscle architecture (Fig. 1A). Moreover, the average myotube diameter was approximately 1.2-fold and 1.5-fold ($P<0.05$) higher with the lower and higher dose of the neutralizing TGF-β1 antibody, respectively, than that in the control group. There was a shift in myotube size distribution towards larger diameters, compared to those in the control group. The number of myotubes with small diameters (less than 15 μm) decreased by about 27% and 60% with lower and higher dose of the neutralizing antibody, respectively, compared with that in the control group (Fig. 1B).

Our recent study revealed that glycerol induces significant fibrosis in rat muscles at early regenerative stage, at day 7, together with persistent inflammatory cellular infiltration up to 2 weeks after injury [19]. It is suggested that persistent inflammatory response induces the secretion of various inflammatory cytokines and alteration of ECM environment leading to muscle fibrosis [6]. A recent study showed that fibrosis induced by irradiation of rat muscles is associated with the up-regulation of the pro-fibrotic factor, TGF-β1 [29]. Moreover, TGF-β1 level increases following strain-induced injury [23] and acute kidney injury induced by intramuscular injection of glycerol in rats [11]. In addition, overexpression of TGF-β1 induces extensive fibrosis in the glycerol-injured muscle in mice [18]. Therefore, we hypothesized that TGF-β1 might be responsible for the extensive collagen deposition in the glycerol-injured rat muscles. To test this hypothesis, glycerol-injured rat muscles were treated with a neutralizing antibody to TGF-β1 at different doses and regeneration was evaluated at day 7 after injury.

Treatment with a neutralizing antibody to TGF-β1 significantly decreased fibrous tissue accumulation, as indicated by a decreased fibrosis index compared with that in the glycerol-injured muscle. This result is consistent with previous findings showing that specific inhibition of TGF-β1 activity reduces fibrosis and restores regeneration and vascularization in the dystrophic muscle [21]. Both doses of anti-TGF-β1 significantly decreased fibrosis index compared with that in the control muscle. Van Linthout *et al.* [25] reported that TGF-β1 stimulates fibroblast proliferation to produce ECM proteins. Decreased fibroblast migration and diminished ECM production at the injury site reduces fibrosis and enhances muscle repair [3]. Taken together, our results and those of the previous studies suggest that blockage of TGF-β1 activity by a neutralizing antibody reduces muscle fibrosis.

We also revealed that treatment with anti-TGF-β1 antibody enhanced muscle regeneration, as indicated by improved muscle architecture and increased average myotube diameter. Our results are consistent with those of Zimowska *et al.* [30], who reported enhanced muscle regeneration *in vivo,* as well as increased muscle differentiation *in vitro,* following neutralization of TGF-β1 activity. TGF-β1 negatively affects the regeneration of skeletal muscle by inhibiting the proliferation and differentiation of satellite cells [2]. Moreover, TGF-β1 inhibits the fusion of myoblasts and formation of myotubes in mouse C_2C_{12} myoblasts [27]. Li *et al.* [14] concluded that blockage of intrinsic TGF-β1 activity in rats after CTX injury is beneficial for muscle regeneration. In addition, inhibition of TGF-β1 activity improves skeletal muscle architecture in several genetic myopathies [10]. Krueger and Hoffmann [12] showed that TGF-β1 suppresses myoblast differentiation in a dose-dependent manner. In addition, it was found that retinoic acid attenuates the anti-myogenic effect of TGF-β1 on C_2C_{12} myoblasts in a dose-dependent manner [13]. These results suggest that treatment with a neutralizing TGF-β1 antibody reverses the anti-myogenic effect of TGF-β1.

Several growth factors have been reported to enhance muscle fibrosis, such as myostatin, the member of the TGF-β protein family which induces fibroblast proliferation and ECM proteins synthesis [15], interleukin (IL)-6 which is a pro-inflammatory factor with pro-fibrotic actions [4], and the profibrotic cytokine, connective tissue growth factor (CTGF) which is expressed in response to TGF-β1 and increases the expression of collagen I α2 chain, fibronectin and integrins [26]. In addition, Wnt/β-catenin signaling and vascular endothelial growth factor (VEGF) induce the transformation of fibroblasts into myofibroblasts [1, 7]. Furthermore, fibroblast growth factor (FGF), as well as, epidermal growth factor (EGF) treatment induce fibroblast proliferation *in vitro* [28].

In conclusion, treatment with a neutralizing antibody to TGF-β1 reduced fibrosis and enhanced muscle regeneration in glycerol-injured rat muscles. Our data showed that extensive fibrosis in rat muscles may be mediated in part by the upregulation of TGF-β1 protein expression. Targeting TGF-β1 activity appears to be a promising therapeutic approach for the inhibition of fibrosis and enhancement of muscle regeneration following muscular injury.

ACKNOWLEDGMENTS. This work was supported by JSPS KAKENHI Grant Number 16H02585. The first author acknowledges the Egyptian Government for financial support.

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