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

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ABSTRACT. 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, Received: 17 September 2019
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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

![Figure 1](https://example.com/f1.png)

**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 ECM and enhances muscle regeneration following muscular injury.

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 C2C12 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 C2C12 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.

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REFERENCES

1. Cisternas, P., Henriquez, J. P., Brandan, E. and Inestrosa, N. C. 2014. Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis. Mol. Neurobiol. 49: 574–589. [Medline] [CrossRef]
2. Cohn, R. D., van Erp, C., Habashi, J. P., Soleimani, A. A., Klein, E. C., Lisi, M. T., Gamradt, M., ap Rhys, C. M., Holm, T. M., Loeys, B. L., Ramirez, F., Judge, D. P., Ward, C. W. and Dietz, H. C. 2007. Angiotensin II type 1 receptor blockade attenuates TGF-β-induced failure of muscle synapse and fibrosis. Mol. Neurobiol. 36: 574–589. [Medline] [CrossRef]
3. Delaney, K., Kasprzycka, P., Ciernyych, M. A. and Zimowska, M. 2017. The role of TGF-β1 during skeletal muscle regeneration. Cell Biol. Int. 41: 706–715. [Medline] [CrossRef]
4. Forcina, L., Miano, C., Sicchitano, B. M. and Musarò, A. 2019. Signals from the Niche: Insights into the Role of IGF-1 and IL-6 in Modulating Skeletal Muscle Fibrosis. Cells 8: 232. [Medline] [CrossRef]
5. Gillies, A. R. and Lieber, R. L. 2011. Structure and function of the skeletal muscle extracellular matrix. Muscle Nerve 44: 318–331. [Medline]
6. Gosselin, L. E. and McCormick, K. M. 2004. Targeting the immune system to improve ventilatory function in muscular dystrophy. Med. Sci. Sports Exerc. 36: 44–51. [Medline] [CrossRef]
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7. Gutpelt, K. M. and Hoffman, L. M. 2015. VEGF induces stress fiber formation in fibroblasts isolated from dystrophic muscle. J. Cell Commun. Signal. 9: 353–360. [Medline]  [CrossRef]

8. Järvinen, T. A. H., Järvinen, M. and Kalimo, H. 2014. Regeneration of injured skeletal muscle after the injury. Muscles Ligaments Tendons J. 3: 337–345. [Medline]  [CrossRef]

9. Kharraz, Y., Guerrera, P., Pessina, P., Serrano, A. L. and Muñoz-Cánoves, P. 2014. Understanding the process of fibrosis in Duchenne muscular dystrophy. BioMed Res. Int. 2014: 965631. [Medline]  [CrossRef]

10. Kollias, H. D. and McDermott, J. C. 2008. Transforming growth factor-beta and myostatin signaling in skeletal muscle. J. Appl. Physiol. 104: 579–587. [Medline]  [CrossRef]

11. Korrapati, M. C., Shaner, B. E. and Schnellmann, R. G. 2012. Recovery from glycerol-induced acute kidney injury is accelerated by suramin. J. Pharmacol. Exp. Ther. 341: 126–136. [Medline]  [CrossRef]

12. Krueger, C. and Hoffmann, F. M. 2010. Identification of retinoic acid in a high content screen for agents that overcome the anti-myogenic effect of TGF-beta1. PLoS One 5: e15511. [Medline]  [CrossRef]

13. Lamarche, É., Lala-Tabbert, N., Gunanayagam, A., St-Louis, C. and Wiper-Bergeron, N. 2015. Retinoic acid promotes myogenesis in myoblasts by antagonizing transforming growth factor-beta signaling via C/EBPβ. Skelet. Muscle 5: 8. [Medline]  [CrossRef]

14. Li, H., Hicks, J. J., Wang, L., Oyster, N., Philippou, M. J., Harwitz, S., Hogan, M. V. and Huard, J. 2016. Customized platelet-rich plasma with transforming growth factor β1 neutralization antibody to reduce fibrosis in skeletal muscle. Biomaterials 87: 147–156. [Medline]  [CrossRef]

15. Li, Z. B., Kollias, H. D. and Wagner, K. R. 2008. Myostatin directly regulates skeletal muscle fibrosis. J. Biol. Chem. 283: 19371–19378. [Medline]  [CrossRef]

16. Mahdy, M. A. A. 2019. Skeletal muscle fibrosis: an overview. Cell Tissue Res. 375: 575–588. [Medline]  [CrossRef]

17. Mahdy, M. A., Warita, K. and Hosaka, Y. Z. 2016. Early ultrastructural events of skeletal muscle damage following cardiotoxin-induced injury and glycerol-induced injury. Microsc 91: 29–40. [Medline]  [CrossRef]

18. Mahdy, M. A. A., Warita, K. and Hosaka, Y. Z. 2017. Effects of transforming growth factor-β1 treatment on muscle regeneration and adipogenesis in glycerol-injured muscle. Anim. Sci. J. 88: 1811–1819. [Medline]  [CrossRef]

19. Mahdy, M. A. A., Warita, K. and Hosaka, Y. Z. 2018. Glycerol induces early fibrosis in regenerating rat skeletal muscle. J. Vet. Med. Sci. 80: 1646–1649. [Medline]  [CrossRef]

20. Noirez, P., Torres, S., Cebrian, J., Agbulut, O., Peltzer, J., Butler-Browne, G., Daegelen, D., Martelly, I., Keller, A. and Ferry, A. 2006. TGF-beta1 favors the development of fast type identity during soleus muscle regeneration. J. Muscle Res. Cell Motil. 27: 1–8. [Medline]  [CrossRef]

21. Pessina, P., Kharraz, Y., Jardi, M., Fukada, S., Serrano, A. L., Perdiguero, E. and Muñoz-Cánoves, P. 2015. Fibrogenic cell plasticity blunts tissue regeneration and aggravates muscular dystrophy. Stem Cell Reports 4: 1046–1060. [Medline]  [CrossRef]

22. Pohlers, D., Brenmoehl, J., Löfler, I., Müller, C. K., Leipner, C., Schultz-Mosgau, S., Stallmach, A., Kinne, R. W. and Wolf, G. 2009. TGF-beta promotes the development of fast type identity during soleus muscle regeneration. J. Muscle Res. Cell Motil. 27: 1–8. [Medline]  [CrossRef]

23. Smith, C. A., Stauber, F., Waters, C., Alway, S. E. and Stauber, W. T. 2007. Regulation of fibrosis following skeletal muscle strain injury in rats. J. Appl. Physiol. 102: 755–761. [Medline]  [CrossRef]

24. Smith, L. R. and Barton, E. R. 2018. Regulation of fibrosis in muscular dystrophy. Matrés Biol. 68-69: 602–615. [Medline]  [CrossRef]

25. Van Linthout, S., Miteva, K. and Tschöpe, C. 2014. Crosstalk between fibroblasts and inflammatory cells. Cardiovasc. Res. 102: 258–269. [Medline]  [CrossRef]

26. Vial, C., Zühiga, L. M., Cabello-Verrugio, C., Cañón, P., Fadic, R. and Brandan, E. 2008. Skeletal muscle cells express the profibrotic cytokine connective tissue growth factor (CTGF/CCN2), which induces their dedifferentiation. J. Cell. Physiol. 215: 410–421. [Medline]  [CrossRef]

27. Wick, Z., Sadkowski, T., Jank, M. and Motyl, P. 2010. Transcriptional pattern of TGF-beta1 inhibitory effect on mouse C2C12 myoblasts differentiation. Pol. J. Vet. Sci. 13: 629–638. [Medline]  [CrossRef]

28. Yu, A., Matsuda, Y., Takeda, A., Uchinuma, E. and Kuroyanagi, Y. 2012. Effect of EGF and bFGF on fibroblast proliferation and angiogenic cytokine production from cultured dermal substitutes. J. Biomater. Sci. Polym. Ed. 23: 1315–1324. [Medline]  [CrossRef]

29. Zhou, Y., Sheng, X., Deng, F., Wang, H., Shen, L., Zeng, Y., Ni, Q., Zhan, S. and Zhou, X. 2018. Radiation-induced muscle fibrosis rat model: establishment and validation. Radiat. Oncol. 13: 160. [Medline]  [CrossRef]

30. Zimowska, M., Duchesnay, A., Dragun, P., Oberbek, A., Moraczewski, J. and Martelly, I. 2009. Immune neutralization of TGF-β1 improves skeletal muscle regeneration: effects on myoblast differentiation and glycosaminoglycan content. Int. J. Cell Biol. 2009: 659372. [Medline]  [CrossRef]