Model for Muscle Regeneration around Fibrotic Lesions in Recurrent Strain Injuries

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ABSTRACT

GREFTE, S., A. M. KUIJPERS-JAGTMAN, R. TORENSMA, and J. W. VON DEN HOFF. Model for Muscle Regeneration around Fibrotic Lesions in Recurrent Strain Injuries. Med. Sci. Sports Exerc., Vol. 42, No. 4, pp. 813–819, 2010. Purpose: The purpose of this study was to establish an in vivo model for muscle regeneration after strain injury in the presence of a fibrotic discontinuity. Methods: The musculus soleus of 5-wk-old male rats was exposed, completely lacerated, and sutured together with or without a collagen scaffold in between the muscle ends. The scaffold represents a fibrotic discontinuity in the muscle. Muscle healing was evaluated after 14 d by general histology and staining for myofibroblasts, satellite cells (activated), and inflammatory cells. Results: Around all wounds, satellite cells were activated. Inside the collagen scaffolds, satellite cells were absent, indicating that muscle regeneration was impaired. In the wounds without a collagen scaffold, the lacerated and the sutured myofibers contacted and had already started to regenerate, whereas this did not occur with an implanted scaffold. Conclusions: A fibrotic discontinuity, such as an implanted collagen scaffold, delays muscle regeneration in skeletal muscle. This model is suitable to study skeletal muscle regeneration in the presence of a fibrotic lesion and to evaluate new treatment modalities for muscle strain injuries. Key Words: MUSCLE STRAIN INJURIES, FIBROSIS, MUSCLE HEALING, SATELLITE CELLS

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uscle strain injuries occur regularly in professional athletes as well as in the general population (14). The hamstring is the most common muscle group affected and is characterized by a recurrence rate of 30% within the first year after injury. This indicates that full recovery of a hamstring strain injury is often not obtained (7,34,35). Magnetic resonance imaging analysis shows that during the healing of a hamstring injury, fibrotic tissue is formed, preventing full recovery (10,38).

In muscle strain injuries, the muscle is sheared, which results in a total rupture of the myofibers and their plasma membrane (21,24). At this site, necrosis of the myofibers begins but is restricted to the injury site by contraction bands inside the myofibers (19). After injury, satellite cells, which are located between the sarcolemma and the basal lamina of the muscle fibers (29,32), are released, activated, and migrate to the site of injury. There they proliferate, differentiate, and fuse to each other or to damaged myofibers to regenerate the skeletal muscle (8,37). However, blood vessels are also torn, and a hematoma is formed, filling the gap between the damaged muscle ends. This forms a primary matrix not only for inflammatory cells but also for fibroblasts, which synthesize extracellular matrix components (21,24). These fibroblasts firstly produce fibronectin, followed by collagen type III, and finally collagen type I (22). This might lead to a fibrotic tissue that inhibits growth of muscle fibers and thus impairs regeneration and muscle function (15,18,24,25). It has been shown that recurrent muscle strains occur in proximity of this fibrotic discontinuity, probably because of its different stiffness and contractility properties (34,38). Furthermore, recurrent injuries are also more severe and take a longer time to heal than primary strain injuries (7,27). It is therefore important to prevent or to minimize the formation of such a fibrotic discontinuity to reduce the risk of recurrence.

To reduce fibrosis and to optimize muscle regeneration, several strategies have been evaluated. The injection of growth factors such as insulin-like growth factor, fibroblast growth factor 2, nerve growth factor, and granulocyte...
colony-stimulating factor improves muscle regeneration (25,36,39). More importantly, the administration of decorin, an inhibitor of transforming growth factor β, reduces fibrosis (12,36). The direct delivery of isolated muscle cells is another approach (9,16,31). Although the latter yields promising results, a major problem is the poor cell survival and limited migration of the injected cells (3,11). Alternatively, several different scaffold materials have been used for improving muscle regeneration but with varying results (5,17,26,41). However, a model to study impaired healing in the presence of a fibrotic lesion is not yet available. Therefore, the aim of this study is to establish an in vivo model for a fibrotic discontinuity in healing skeletal muscle by implanting a collagen scaffold.

MATERIALS AND METHODS

Animals. All animal experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre in accordance to the Dutch laws and regulations on animal experiments, which conform to the American College of Sports Medicine animal care standards. Twenty-four 5-wk-old male Sprague–Dawley rats (Janvier, Le Genest, France) were used for the experiments. The rats were housed under normal laboratory conditions, but in the first week after the experimental procedure, they were housed individually. All the rats were fed normal rat chow and water ad libitum. Before the start of the experiments, the rats had been acclimatized to the animal facility for 1 wk.

Preparation of the collagen scaffolds. The collagen scaffolds were prepared and chemically cross-linked as previously described (6). Briefly, a 1% (w/v) homogenized collagen suspension was prepared using insoluble type I collagen from bovine Achilles tendon (Sigma Chemical Co., St. Louis, MO). The collagen suspension was degassed to remove air bubbles, frozen overnight at −80°C, and lyophilized. The dried collagen scaffolds were sterilized by immersion in 70% ethanol for 1 h and then washed three times with sterile phosphate-buffered saline (PBS). The animals were divided into four groups of six rats according to the suturing method and the presence of a collagen scaffold: A) knot suturing without collagen scaffold, B) knot suturing with collagen scaffold, C) continuous suturing without collagen scaffold, and D) continuous suturing with collagen scaffold. The easiest method for suturing is with one continuous suture around the muscle. However, if this one suture breaks, the wound opens and the scaffold might be lost. To be sure, we also used a method with multiple sutures. However, none of the sutures had broken, and there was no different response between the two suturing methods. We therefore decided to group the animals together (A + C and B + D). The fascia and the skin were closed with 5-0 Polysorb and 5-0 Vicryl sutures (Johnson-Johnson, Langhome, PA), respectively. To minimize muscle tension, the paw was splinted with an aluminum strip at an angle approximately 45° with respect to the tibia for 1 wk. In group B, the paws were swollen and reddish when the aluminum strips were removed. These rats therefore received 1 mg·kg⁻¹ enrofloxacin two times a day (Bayer Healthcare, Brussels, Belgium) for 7 d. After 14 d, the rats were sacrificed according to the standard CO₂/O₂ protocol.

Histology and immunohistochemistry. After sacrifice, the left (wound) and the right (intact control) musculus soleus of three rats of each group were fixed in freshly prepared 4% paraformaldehyde in PBS for 4–6 h and processed for paraffin embedding. The left and the right musculus soleus of the other three rats of each group were immediately frozen in optimal cutting temperature compound (OCT) embedding compound (CellPath, Newton, UK) using isopentane precooled in liquid nitrogen. The muscles were cut longitudinally, and 5-μm sections were collected on superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). For general morphometry, paraffin sections were stained with hematoxylin and eosin (H&E).

Paraffin sections were also stained with the following antibodies: mouse anti-alpha-smooth muscle actin (α-SMA; Sigma), rabbit anti-Ki67 (Research Diagnostics Inc., Flanders, NJ), mouse anti-ED1 (CD68, Serotec; DPC, Breda, The Netherlands), and mouse anti-MyoD (DAKO; Dakopatts, Glostrup, Denmark). Briefly, the sections were deparaffinated, rehydrated, treated with 3% H₂O₂ for 20 min to inactivate endogenous peroxidase, and postfixed with 4% formaldehyde in PBS. For α-SMA and ED1 staining, the sections were heated in citrate buffer (pH 6.0) for 10 min at 70°C. For Ki67 and MyoD staining, the sections were heated to 100°C for 10 and 40 min, respectively. After rinsing with 0.075% glycine in PBS, the sections were preincubated with 10% normal donkey serum (NDS; Chemicon, Temecula, CA) followed by the antibodies against α-SMA (1:1600), ED1 (1:100), Ki67 (1:50), or MyoD (1:25) for 60 min. Subsequently, the biotinylated secondary antibodies goat-anti-mouse immunoglobulin G (IgG; H + L) (1:500; Jackson Labs, West Grove, PA) for α-SMA, ED1, and MyoD and goat-anti-rabbit IgG (H + L) (1:500; Jackson Labs) for Ki67 were added. The bound antibodies were visualized using a...
preformed biotinylated horse radish peroxidase and avidin complex (Vector Laboratories, Burlingame, CA).

The frozen sections were double stained with the antibodies rabbit anti-collagen IV (Euro-Diagnostica BV, Arnhem, The Netherlands) and mouse anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa City, CA). Briefly, the sections were dried in air overnight and postfixed with 1% paraformaldehyde in PBS for 10 min. After rinsing with 0.05% Triton X-100 in PBS, the sections were preincubated with 10% NDS followed by rabbit anti-collagen IV (1:100) for 60 min. Collagen IV was then detected using the biotinylated donkey anti-rabbit IgG (H + L) (1:500; Jackson Labs) for 60 min and an AlexaFluor-488-labeled avidin (1:500; Molecular Probes, Eugene, OR) for 60 min. Thereafter, the sections were again preincubated with 10% NDS and then incubated with mouse anti-Pax7 (1:100) overnight at 4°C. Pax7 was detected using an AlexaFluor-594-labeled goat-anti-mouse IgG (H + L) (1:200; Molecular Probes). All sections were photographed with the Zeiss Imager.Z1 together with the AxioCam MRc5 camera using the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Jena, Germany).

**Statistical analysis.** The numbers of Pax7- and MyoD-positive cells were counted in 1) the control muscle (C), 2) the cutting zone without the collagen scaffold (W), 3) the cutting zone with the collagen scaffold (W + S), 4) inside the collagen scaffold (S), and 5) in the noninjured muscle tissue of the wounded musculus soleus (NI). To count the Pax7-positive cells, the images were divided into 50 squares. In five random squares, the total number of Pax7-positive cells and DAPI-stained nuclei was counted. The total number of MyoD-positive cells and nuclei of every group was determined in three different fields of an overview image. The numbers of Pax7- and MyoD-positive cells were expressed as a percentage ± SD of the total number of cells. The differences in the percentages of Pax7- and MyoD-positive cells were tested for significance using a Kruskal–Wallis one-way ANOVA on Ranks followed by Dunn’s method. A value of $P < 0.05$ was considered to be significant.

**RESULTS**

Of the 24 rats, 1 rat in group A without a collagen scaffold did not survive the surgery. After an initial growth arrest, all rats in every group had gained about 25% body weight at the 10th day. The groups were not significantly different. The immobilization of the left hind leg did not affect the growth of the rats. Macroscopically, the wounded muscle adhered partly to the surrounding tissues. Furthermore, the collagen scaffolds were not visible anymore and appeared to be integrated into the muscle tissue. The sutures did not break, and the different suturing methods had no effect on muscle morphology and gave the same results regarding muscle regeneration. Therefore, the animals of groups A and C and groups B and D were grouped together.

**General histology.** H&E staining (Fig. 1A) revealed properly arranged longitudinal myofibers in the controls (C) but not in the wounded muscles. Within the wounds, regenerating myofibers were present, indicated by centrally located nuclei (Fig. 1A, magnification). Some myofibers in the cutting zone had fused properly in the group without the collagen scaffold. On the contrary, the implantation of a collagen scaffold prevented fusion of the myofibers (W + S). The collagen scaffolds were surrounded by giant cells and a cell layer (an interphase).

**Immunostainings.** Paraffin sections were stained with antibodies against α-SMA, ED1 (CD68), Ki67, and MyoD to identify blood vessels and myofibroblasts, inflammatory cells, proliferating cells, and activated satellite cells, respectively (Fig. 1B). In the controls (C), ED1-positive inflammatory cells and Ki67-positive proliferating cells were present. However, the controls hardly contained any MyoD-positive nuclei (indicated by arrows). As expected, α-SMA–positive cells were not present in the muscle tissue of the controls but only in blood vessels. Without a collagen scaffold (W), there was an increase in the number of ED1- and Ki67-positive cells of which the majority surrounded the sutures. More importantly, many MyoD-positive nuclei were present (a few are indicated by arrows). The number of α-SMA–positive blood vessels was also increased, and there were also α-SMA–positive cells in the muscle tissue. The implantation of a collagen scaffold (W + S) caused an infiltration of ED1-positive giant cells and other inflammatory cells, which surrounded the scaffold. Even inside the scaffolds, inflammatory cells were present. Proliferating Ki67-positive cells were present in the muscle tissue, in the interphase, and also inside the collagen. Again, the muscle tissue around the scaffold (W + S) contained many MyoD-positive nuclei (a few are indicated by arrows), but all the cells inside the scaffolds were negative for MyoD. The expression pattern of α-SMA was similar to the wounds without the scaffold (W). Inside the scaffolds, α-SMA–positive blood vessels were also found.

To identify the resident satellite cells, cryosections were stained with the Pax7 antibody (Fig. 2). In the controls (C), only a few satellite cells were present, but around the cutting zone in the wounded muscle tissue with (W + S) or without (W) a collagen scaffold, the number of satellite cells was increased. However, no satellite cells were present within the collagen scaffolds.

**Quantifications.** The percentage of MyoD- and Pax7-positive cells were determined on the paraffin (Fig. 1B) and cryosections (Fig. 2), respectively (Fig. 3). The controls contained only a low number of Pax7-positive satellite cells (2.7% ± 0.4%), which significantly ($P < 0.05$) increased to 7.2% ± 0.6% and 6.2% ± 0.6% in the wounded tissue without (W) or with (W + S) the collagen scaffold, respectively. The number of MyoD-positive cells also significantly ($P < 0.05$) increased from 6.2% ± 1.1% in the controls to 16% ± 4.3% and 15.9% ± 4.9% in the wounds without (W) or with (W + S) the collagen scaffold,
FIGURE 1—Histology of the musculus soleus at 14 d after surgery. A. H&E staining of the control (C), wound without the collagen scaffold (W; group C), and wound with the collagen scaffold (W + S; group D) revealed the disruption of the aligned myofibers at the cutting zone after laceration. The implanted collagen scaffold is surrounded by an interphase and prevented myofiber fusion. B. Immunohistochemistry of the control (C), wound without the collagen scaffold (W; group C), and wound with the collagen scaffold (W + S; group D) with antibodies directed against ED1, Ki67, MyoD, and α-SMA. Only a few ED1-, Ki67-, and MyoD-positive cells (indicated by arrows) and α-SMA–positive blood vessels are present in the control (C). In the wound (W), the number of these cells is higher and α-SMA–positive cells are present. The collagen scaffold (W + S) is surrounded by an ED1-positive interphase. In the wounded muscles and around the scaffold, many Ki67-positive, MyoD-positive (a few are indicated by arrows), and α-SMA–positive cells are present. The scaffold also contains ED1-, Ki67, and α-SMA–positive cells and blood vessels but no MyoD-positive cells.
Discussion

The successful treatment of muscle strains in sports medicine is still a problem. Fibrotic lesions are often formed during muscle regeneration, causing incomplete functional recovery. More importantly, recurrent muscle injuries may occur near this fibrotic tissue (10,34,38). Because fibrotic tissue consists mainly of collagen type I (20,23), we developed an in vivo model for a fibrotic discontinuity by implanting a type I collagen scaffold between the lacerated muscle ends. Using this method, it is possible to standardize the wounds with a collagen scaffold, but it is important to be aware that this is an extreme version of a muscle strain. In this model, we evaluated muscle regeneration after a 2-wk healing period. The numbers of Pax7- and MyoD-positive (activated) satellite cells or myoblasts were increased about twofold in the wounded muscle tissue and around the collagen scaffolds compared with the control muscle. This indicates that the muscle fibers were regenerating and that the scaffold did not inhibit the activation of satellite cells in the adjacent muscle tissue. However, inside the collagen scaffold, these cells were absent. Thus, in the presence of a fibrotic discontinuity, the skeletal muscle cannot regenerate properly because activated satellite cells do not migrate into the fibrotic tissue. Similar to our results, others have also shown that after a strain injury, inflammation occurs, followed by the production of fibrous tissue, which could eventually develop into a fibrotic lesion (14,33). Another study on rectus femoris strain in humans showed a chronic inflammation and a mixture of regenerating muscle fibers and fibrotic tissue in the wound (40). Although muscle regeneration was only evaluated after 2 wk in this initial study, collagen scaffolds can persist in the muscle tissue for up to 50 d (28). Therefore, our model can be used to evaluate treatment strategies for recurrent muscle strains.

Optimal treatment should diminish or prevent the formation of fibrotic tissue and reduce the risk of recurrence. We and others (1,30) observed that suturing the lacerated muscle ends directly together allows full regeneration of the muscle. Currently, the treatment principle of muscle strains consists of rest, ice, compression, and elevation (22). With specific compression, which could serve as a splint, it may also be possible to bring the muscle ends to each other and diminish the onset of fibrosis. Surgical treatment to suture the muscle ends together is only indicated in cases with extensive injury to the muscle (22). If a fibrotic tissue...
from a previous injury is already present, additional treatment with matrix metalloproteinase 1 (MMP-1) might offer a solution. Previous research has shown that treatment with injection of MMP-1 improves muscle regeneration and that a fibrotic lesion can be partially resolved (4, 23). Thus, combining the injection of MMP-1 with specific compression therapy might diminish a preexisting fibrotic discontinuity or minimize the risk of a secondary fibrosis.

In this study, the musculus soleus in rats is used as a wound model because all the myofibers run parallel. However, the musculus soleus consists mainly of type I (slow) fibers (42), whereas the hamstring, which is the most common muscle group affected in muscle strains, consists of type II (fast) fibers (13). It has been shown that type II muscles regenerate better than type I muscles, which more often develop fibrotic lesions (2). This indicates that the results obtained in this study may differ from a hamstring injury, in which the regeneration process could be more efficient. However, it also demonstrates that the musculus soleus is a good model to study the effects of the presence of a fibrotic discontinuity on muscle regeneration.

In this study, we only analyzed 14 d after surgery because satellite cell activation is a relatively early event in muscle healing (8, 37). In future studies, analysis at later time points is necessary to exclude the possibility that implantation of a collagen scaffold only delays muscle regeneration. In addition, it is important that functional studies are performed to further evaluate this model.

In conclusion, we generated a model for the regeneration of skeletal muscle in the presence of a fibrotic discontinuity. This model can be used to evaluate new treatment strategies for recurrent muscle strains.

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REFERENCES