ORIGINAL ARTICLE

Angiogenic effect of intramuscular administration of basic fibroblast growth factor in atrophied muscles: an experimental study in the rat

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Background: Although angiogenic therapy using recombinant growth factors holds much hope for the treatment of ischaemic diseases, there are still many unanswered questions, including its effectiveness on atrophic muscles.

Objective: To evaluate the angiogenic effects of intramuscularly administered basic fibroblast growth factor (b-FGF) on normal gastrocnemius muscles of rats and atrophic gastrocnemius muscles after tenotomy.

Methods: Forty rats were divided into groups as follows: group A, controls; group B, injected with 1 µg b-FGF; group C, tenotomy performed on the right gastrocnemius muscle; group D, tenotomy and 1 µg b-FGF. Mouse anti-rat CD31 antibody was used to evaluate the number of blood vessels present in histological preparations.

Results: There was a significant ($p < 0.01$) decrease in the number of blood vessels compared with the controls in the atrophic muscles of group C. This was similar to the decrease in muscle weight in this group. However, there was a significant ($p < 0.01$) increase in the number of blood vessels compared with the controls in groups B and D. Similarly, there was a significant ($p < 0.01$) increase in the number of blood vessels in group D compared with the atrophic muscles in group C.

Conclusion: Intramuscular administration of b-FGF increases angiogenesis in both normal and atrophic rat gastrocnemius muscles at the injection area.

Skeletal muscle atrophy occurs in a variety of situations, but it is most commonly associated with lack of mechanical load on the musculoskeletal system. It typically occurs in the elderly and patients under bed rest immobilisation after traumatic injury or surgery. It is also known that tenotomy causes atrophy of gastrocnemius muscle.¹ In all these human and animal systems, removal of mechanical load from skeletal muscles has been directly implicated in initiation of the muscle atrophic response.²

Angiogenesis is the generation of endothelial cells from pre-existing microvessels and their subsequent growth into new vessels. It normally occurs principally during embryonic growth, but it can also take place under certain conditions during adult life. Formation of new blood vessels in the endometrium of reproductive women during their monthly menstrual cycle is such an example.³ The mechanisms underlying the capillary growth process in skeletal muscle are not known, but reduced oxygen tension and the related metabolic consequences have been suggested as possible stimuli.⁴ Over the last few years, growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factor have been proposed to be important in angiogenic processes.^{5–8}

The use of recombinant growth factors such as FGFs for treatment of various clinical conditions represents a promising strategy for restoration of the angiogenic process.^{9,10} Garcia-Martinez *et al*¹¹ injected a recombinant adenovirus containing basic FGF (b-FGF) and achieved expression of functional isoforms of b-FGF in the hindlimb muscles of mdx mice. Immunohistological analysis showed an increased number of large calibre blood vessels in the treated muscles compared with control muscles. Their results show that adenovirus mediated transfer of the human b-FGF gene can induce angiogenesis in muscle. Similarly, others have

investigated the effects of acidic FGF and b-FGF on muscle microcirculation using isolated arterioles and intact cremaster muscles of the rat and suggested that FGFs modulate blood flow in the skeletal muscle by acting on the endothelium of arterioles. The signalling mechanism of FGF induced vasodilation involves the synthesis of nitric oxide by arteriolar endothelium.¹² Although angiogenic therapy holds much hope for the treatment of various diseases, there are still many unanswered questions, including the method of administration, the appropriate dose of these factors, and the effectiveness on muscle atrophy.

b-FGF is a 154 amino acid polypeptide that is expressed in brain, pituitary, myocardium, kidney, and liver as well as in macrophages and endothelial and muscle cells. This study aimed to evaluate its angiogenic effects in gastrocnemius muscle of rats atrophied by tenotomy, producing a disuse model which simulated bed rest during injury.

MATERIALS AND METHODS

Design

Forty Wistar male rats weighing 280–300 g were used for this study. During the experimental period, the animals lived under stable conditions of temperature and a reverse light cycle programme. They were allowed to eat *ad libitum*. The animals were divided into four groups of equal numbers as follows (table 1). Group A comprised controls which, under ether anaesthesia, were injected intramuscularly with 0.1 ml saline into the right gastrocnemius muscle every three days for a total period of 15 days. Group B rats were injected intramuscularly with 1 µg of the angiogenic factor b-FGF into the right gastrocnemius muscle every three days for a total period of 15 days. Tenotomy was performed on the right gastrocnemius muscle of the animals in groups C and D. The level of atrophy had been evaluated by measuring the weight

Table 1 Number of blood vessels per optical field in the right gastrocnemius muscle of the groups of rats studied

Group	Treatment	Number of blood vessels
A	Control	17.39 (3.16)
B	b-FGF	20.92 (0.55)*
C	Tenotomy	13.36 (0.72)*
D	Tenotomy + b-FGF	24.43 (0.56)*†

Values are mean (SD).

* $p < 0.01$ compared with controls.

† $p < 0.01$ compared with group C.

b-FGF, Basic fibroblast growth factor.

loss of the muscle in several pilot experiments (animals killed after five, 10, and 15 days). Maximum weight loss was observed after 10 days. The animals in group C received 0.1 ml saline as described for group A, and those in group D received 1 μ g b-FGF, as described for group B, after 10 days (maximum atrophy).

At the appropriate time, rats were killed and the right gastrocnemius muscle removed. The weight of the muscle was determined as a criterion of atrophy after tenotomy. The effect of b-FGF was assessed by evaluating blood vessel density in the removed muscle tissues through histological examination.

Histological examination

The gastrocnemius muscle samples from the four animal groups were cut into 3 mm thick slices, fixed in 10% neutral buffered formaldehyde at 4°C for 24 hours, and processed for routine paraffin embedding. The tissue sections were routinely stained with haematoxylin & eosin stain and then immunohistochemistry was performed to evaluate the density of the blood vessels.¹³ The blood vessels were detected by using a monoclonal mouse anti-rat antibody (CD31 (platelet endothelial cell adhesion molecule-1); Dako, Glostrup, Denmark), which reacts with surface antigen CD31 present on mouse endothelial cells. The immunoperoxidase method was used for the detection of the antigens. Sections were deparaffinised in 70% alcohol, and endogenous peroxidase was blocked with 3% methanol solution of H₂O₂. Sections were then preincubated in 20% serum from the species from which the secondary antibody was raised, and the primary antibody was applied at a dilution of 1:40. After 45 minutes incubation at room temperature, the secondary biotinylated antibody was applied for 30 minutes. Control slides were incubated for the same period with normal mouse serum. After several 10 minute washes in phosphate buffered saline, samples were elaborated with a peroxidase LSAB kit (labelled streptavidin-biotin method; Dako), which detects the primary antibody. The slides were briefly counterstained with Mayer's haematoxylin, mounted, and examined with an Olympus BX40 microscope. Tissue sections (5 μ m) were deparaffinised, rehydrated, and treated with 0.3% H₂O₂ for five minutes to quench endogenous peroxidase activity. Non-specific binding was blocked by applying serum for 10 minutes. Slides were then incubated for 30 minutes with the monoclonal antibodies (1:40 dilution) mouse anti-(human HLA-DR α chain) (Dako) and CD4 (Dako). Quantification of angiogenesis was based on measurement of the new blood vessels in muscle sections under $\times 40$ and $\times 100$ magnification. High vascularisation fields (hot spots) were selected and measurement of blood vessels was performed under $\times 200$ magnification in the three selected fields of highest vessel density. The mean number of blood vessels in these three high vascularisation fields was considered to be the final microvessel density.

Figure 1 Gastrocnemius muscle from control rats (group A). Blood vessels detected using a monoclonal mouse anti-rat antibody (CD31, clone: INN-PECAM).

Figure 2 Gastrocnemius muscle from rats injected with basic fibroblast growth factor (group B). Immunohistochemical staining using monoclonal antibody CD31 showing an increased number of blood

Figure 3 Atrophied gastrocnemius muscle after tenotomy from rats in group C. Immunohistochemical staining using monoclonal antibody CD31 showing a decreased number of blood vessels compared with controls.

Figure 4 Gastrocnemius muscle from rats injected with basic fibroblast growth factor after tenotomy (group D). Immunohistochemical staining using monoclonal antibody CD31 showing increased number of blood vessels compared with controls and group C.

What is already known on this topic

- It is known that receptors for fibroblast growth factors (FGFs) exist in skeletal muscle, and studies have shown that the use of recombinant FGFs represents a promising strategy for the restoration of angiogenesis in various conditions
- It is also known that tenotomy causes atrophy of the gastrocnemius muscle

What this study adds

- This study confirms that intramuscular administration of b-FGF increases blood vessel density in normal skeletal muscle
- Intramuscular administration of b-FGF also increases angiogenesis in muscle atrophied as the result of tenotomy

Statistical analysis

The data were analysed using the statistical package STATISTICA 4.5 for Windows (StatSoft Inc, Tulsa, Oklahoma, USA). All continuous variables are expressed as mean (SD). Because the distribution of these variables did not appear to be normal, a Shapiro Wilk test for normality was performed, and data were analysed using the Mann-Whitney U test and Kruskal-Wallis one way analysis of variance to assess differences in a continuous variable between two or more groups of rats respectively. Post hoc analyses were performed using the Mann-Whitney U test with Bonferroni adjustment. All tests were two tailed, and statistical significance was accepted at $p < 0.05$.

RESULTS

Angiogenic effects of b-FGF on muscles

The mean (SD) weight of the right gastrocnemius muscles in group A (controls) was 2.13 (0.09) g, whereas the mean weight of the same muscles in group C, after tenotomy, was 1.78 (0.09) g. This decrease was significant ($p < 0.01$), indicating atrophy of the muscles in the tenotomised animals.

As shown in table 1, there was a significant ($p < 0.01$) decrease in blood vessel density compared with the controls (fig 1) in the atrophied muscles of group C (fig 3), similar to the decrease in muscle weight in this group. However, there was a significant ($p < 0.01$) increase in blood vessel density compared with controls in groups B (treatment with b-FGF; fig 2) and D (treatment with b-FGF and tenotomy; fig 4). Similarly, a significant ($p < 0.01$) increase in blood vessel density was present in group D compared with group C.

From these results it is clear that the intramuscular administration of b-FGF increases angiogenesis both in normal rat gastrocnemius muscles and after atrophy caused by tenotomy.

DISCUSSION

A large number of various cytokines have been recognised to cause neovascularisation, but FGFs and vascular endothelial growth factor have produced the best results in experimental models^{14–15} and clinical trials.¹⁶ However, there are still many unanswered questions, one of them being about the neovascularisation effect in situations of muscle atrophy.

We have recently reported that both exercise and intramuscular administration of vascular endothelial growth factor increased angiogenesis in rat heart, even though only exercise alone increased angiogenesis significantly. The combined protocol (administration of both growth factor and exercise) led to an increase in angiogenesis in cardiac muscles.¹⁷

In the present investigation we aimed to study the angiogenic effect of b-FGF in normal and tenotomy induced atrophic muscles. We show that the intramuscular injection of b-FGF produces a significant increase in angiogenesis in normal gastrocnemius rat muscles in the area of the injection, confirming previous studies.

It was already known that the capillary density of muscular tissue is greatly decreased following the disuse produced by tenotomy or immobilisation.¹⁸ In this study, we also show a significant decrease in the number of blood vessels in atrophied muscles after tenotomy.

Our study shows that the intramuscular administration of b-FGF increases angiogenesis significantly in atrophied gastrocnemius rat muscles. Other authors have investigated the effect of FGF on atrophic muscles. Walter *et al*¹⁹ tried to determine whether FGF-1 implanted with viable nerve into atrophied muscle would stimulate formation of functional, acetylcholine-producing motor end plates. They found that FGF-1 with fibrin adhesive carrier could facilitate the reinnervation of atrophied muscle by enhancing the formation or revitalisation of motor end plates. A model of atrophic rat submandibular gland has also been used to examine the ability of b-FGF to accelerate tissue repair.²⁰ The results showed increased immunoreactivity in the damaged gland, which may be due to the difference in the response to b-FGF between damaged and normal glands. Motoyoshi *et al*²¹ evaluated the effects of b-FGF on the recovery of vocal fold movement and the attenuation of laryngeal muscle atrophy after transection of recurrent laryngeal nerve. They reported that b-FGF facilitated regeneration of the transected laryngeal nerve and attenuation of intrinsic laryngeal muscle atrophy, thereby restoring laryngeal function. In a clinical trial, Clarke *et al*²² used a terrestrial model of space flight to investigate the amount of myofibre wounding and FGF release occurring after muscle unloading. Their results suggested that mechanically induced FGF release mediated by myofibre wounding may play an important role in the aetiology of unloading induced skeletal muscle atrophy.

Our findings conclusively show that tenotomy induced atrophy causes a significant decrease in blood vessel number. Furthermore, the intramuscular administration of b-FGF increases angiogenesis in both normal and atrophic gastrocnemius rat muscles at the injection area.

In view of the high prevalence of human muscular dysfunction in various atrophic situations after bed rest during injury, the increased angiogenesis induced by b-FGF may help the rehabilitation of atrophied skeletal muscle.

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COMMENTARY

In this study the authors show that tenotomy induced atrophy of the gastrocnemius muscle in rats causes a significant decrease in blood vessel number. Furthermore, they conclude that the intramuscular administration of b-FGF increases angiogenesis in both normal and atrophic (after tenotomy) gastrocnemius muscles in rat at the injection area. Human muscular dysfunction in various atrophic situations such as bed rest because of injury is very common, leading to disability. The increased angiogenesis induced by b-FGF may help the rehabilitation of atrophic skeletal muscle, a fact that could be significant from the clinical point of view, as the number of muscle injuries is increasing through modern sports activity and road traffic accidents.

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