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Differential adaptations of insulin-like growth factor-I, basic fibroblast growth factor, and leukemia inhibitory factor in the plantaris muscle of rats by mechanical overloading: an immunohistochemical study

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Abstract We investigated changes in several growth factors in the rat plantaris muscle produced by mechanical overloading by ablation of synergists using immunohistochemistry. At 1 and 3 days post surgery, the insulin-like growth factor-I (IGF-I) level was slightly increased in the cytosol and markedly increased in the invading cells of the extracellular space. Thereafter, the IGF-I immunoreactivity evoked by overloading rapidly decreased to the normal level. The level of leukemia inhibitory factor (LIF), which was not shown to change at 1 day post surgery, was increased in the cytosol at 3, 5, 7 and 10 days and at 2 weeks. Basic fibroblast growth factor (bFGF) immunoreactivity did not change during the entire period of overloading (1 day–3 weeks post surgery). These results indicate that the elevations of the levels of IGF-I and LIF show differential time course in the plantaris muscle subjected to functional overload. Furthermore, bFGF appears not to be related to the compensatory hypertrophy produced by overloading.

Key words Mechanical overloading · Insulin-like growth factor · Fibroblast growth factor · Leukemia inhibitory factor · Muscle hypertrophy

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Introduction

When a skeletal muscle acting together with other muscles is deprived of these cooperative synergists, the muscle markedly increases in weight [3]. This increase, which is referred to as compensatory hypertrophy, can be easily produced in animals. Such an animal model has been utilized in many studies on the regulation of muscle metabolism and growth. This response includes quantitative and qualitative remodeling of muscle proteins, leading to increases in mass and cross-sectional area with corresponding changes in mechanical properties [27, 28, 30]. However, the mechanism that links the mechanical stimulus resulting from increased loading to the hypertrophy response has not been clearly elucidated. Although it is well known that growth hormone, testosterone and insulin are essential for the normal postnatal growth of skeletal muscle, recently attention has been focused on the roles of local growth factors such as fibroblast growth factor (FGF), insulin-like growth factor-I (IGF-I) and transforming growth factor-β in muscle growth regulation [10, 11]. The overloaded muscle probably undergoes compensatory hypertrophy in hypophysectomized (endocrine-deficient) rats in a manner similar to that in intact animals [1, 9].

Leukemia inhibitory factor (LIF), which is related to ciliary neurotrophic factor (CNTF) and interleukin-6 (IL-6) owing to their predicted structural similarities, has recently been implicated in both neural and myogenic regulation. LIF, which is suggested to play a major role in the developing and regenerating neuromuscular system [8, 33], is up-regulated in muscle damaged by nerve transection [21] and crush injury [22]. Additionally, subcutaneous LIF injection increases the rate of regeneration of crush-traumatized muscle [5]. All of these results indicate that LIF seems to play a crucial role in muscle regeneration.

Several recent studies [6, 25] imply that a break in the integrity of the plasma membrane would be an essential element for muscle hypertrophy, because chemical mediators (growth factors) of remodeling stored in the cell cytosol (e.g. FGF) are released from this location through

plasma membrane disruption. If this hypothesis is true, the muscle hypertrophy elicited by mechanical stress would include a damage-regeneration (remodeling) phase, in which LIF would be up-regulated and would play an important role in muscle regeneration. However, no study has been conducted to investigate whether functional overload changes LIF levels.

IGF-I peptide and mRNA are widely expressed in regenerating muscle [18], and increase of their levels has been shown in muscle tissue subjected to mechanical overloading [1, 9]. Additionally, a study by Yamada et al. [32] using Western blot analysis has revealed that the level of acidic and basic FGF (bFGF) is also promoted by functional overload. However, less information is available as to the site(s) of the elevations of IGF-I and FGF in the overloaded muscle. It is possible that the functional overload increases the levels of these growth factors in the cytosol, in the extracellular space, or in both regions.

This prompted us to design an experiment to investigate whether functional overload increases the level of LIF. In addition, we were interested in examining in detail by immunohistochemical methods the changes in IGF-I and bFGF likely to be evoked by overloading.

Materials and methods

Experimental animals

Twenty-one male Wistar rats (4 weeks of age) with body weights of 90–110 g were used in the experiments. The rats were housed in a temperature (22 ± 2°C)- and humidity (60 ± 5%)-controlled room regulated to provide alternating 12-h periods of light and darkness. They were allowed to feed (commercial rat chow) and drink ad libitum.

Surgical procedures

Compensatory enlargement of the plantaris muscle was induced in one leg of each rat by surgical removal of the ipsilateral soleus and gastrocnemius muscles. While the rat was under pentobarbital anesthesia (50 mg/kg intraperitoneally), an incision was made through the skin and fascia on the caudal aspect of the leg over the calf musculature. The tendons of the soleus muscle and the lateral and medial heads of the gastrocnemius muscle were exposed and severed, and the muscles were carefully peeled up and away from the intact plantaris muscle. Care was taken to avoid rupturing vessels and nerves, and bleeding in the surgical field was minimal. The soleus muscle and the two heads of the gastrocnemius muscle were then clamped off with a hemostat near their origins and ablated. The wound was cleansed, and the fascia and skin were then sutured with surgical silk. A sham operation was performed on the contralateral leg after the rat was anesthetized by intraperitoneal injection of sodium pentobarbital. All rats awoke from anesthesia within 2 h, and were observed to use both hindlimbs immediately, and all rats survived for the duration of the experiment. These procedures were carried out according to the method of Armstrong et al. [3].

Tissue removal and preparation

Using excess pentobarbital administration, rats were killed in groups of three at 1, 3, 5, 7 and 10 days and 2 and 3 weeks post surgery. The plantaris muscles of both legs were rapidly dissected and freed of fat and connective tissue, and the wet mass was determined. Each tissue sample, after being immediately frozen in isopentane cooled to –150°C, was stored at –80°C until preparation for immunohistochemistry.

Primary antibodies

The antibodies employed in the present study were as follows: monoclonal anti-human IGF-I antibody (BM23, Cymbus Bioscience); affinity-purified goat polyclonal antibody to bFGF (FGF2, C18, Santa Cruz Biotechnology); and affinity-purified goat polyclonal antibody to LIF (N18, Santa Cruz Biotechnology).

The IGF-I antibody detects mouse, rat and human IGF-I and the cross-reactivities with IGF-II, multiplication stimulating activity and porcine insulin at 1%, 0.05% and 0.0001%, respectively. The LIF antibody is raised against a peptide corresponding to residues 23–40 mapped at the amino terminus of human LIF precursor (identical to corresponding mouse and rat sequences). Goat anti-bFGF is raised against a peptide corresponding to residues 123–141 mapped at the carboxyl terminus of the human FGF-2 precursor (identical to corresponding mouse and rat sequences). This antibody detects mouse, rat and human bFGF and does not cross-react with other members of the FGF family.

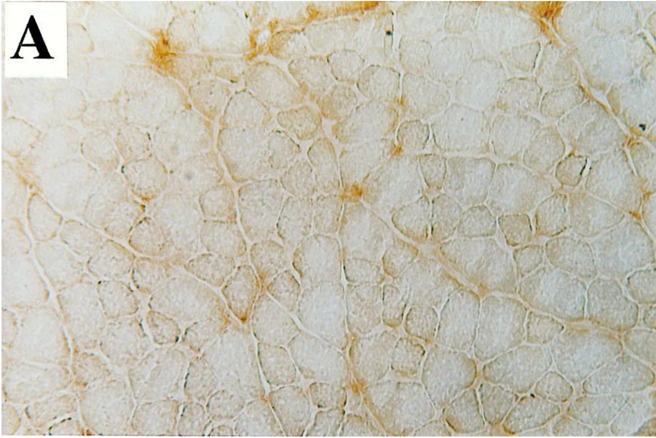
Immunohistochemistry

Serial 8-µm transverse cryostat (Bright: OT-FAS) sections were mounted on silanized slides (Dako Japan), fixed by cold acetone (4 min), rinsed cold (–20°C) phosphate-buffered saline (PBS) for 5 min. To inhibit endogenous peroxidase, these slides were preincubated with 0.3% hydrogen peroxide in absolute methanol for 20 min at room temperature (RT). Sections were blocked with 130 µl of 1.5% horse serum in PBS at RT (30 min). The primary antibodies to the IGF-I, bFGF and LIF were applied to the sections (1:50 dilution in PBS) for 60 min at RT. The sections were washed in PBS for 10 min, after which 130 µl of a 1:500 dilution (PBS) of biotinylated anti-mouse (for IGF-I) or anti-goat (for bFGF and LIF) IgG (Vector Laboratories) was applied for 30 min at RT. The sections were then rinsed in PBS (15 min), and incubated for 30 min with peroxidase-streptavidin conjugate (Vector Laboratories). The binding of primary antibodies was visualized with diaminobenzidine (0.1 mg/ml; Aldrich, Milwaukee, USA) and 0.02% hydrogen peroxide. Negative control slides with omission of the primary antibodies were included in the immunostaining procedures in each instance.

Results

Overloading induced marked increases in the mass of the plantaris muscles during the entire post-surgical period (1 day–3 weeks). The mass at 1 day showed a 41.1% increase over the control value. The percent hypertrophy at 3, 5, 7 and 10 days was 56.2%, 40.9%, 60.7%, and 57.7%, respectively, and that at 2 and 3 weeks was 47.5% and 23.1%, respectively.

Fig. 1 Immunohistochemical staining of IGF-I in the sections ▶ from the control (**A**, **C**, **E**, **G**) and mechanical overloaded (**B**, **D**, **F**, **H**) plantaris muscles of rats. Light photomicrographs showing cryosections stained immunohistochemically for IGF-I at 1 (**A**, **B**), 3 (**C**, **D**), 5 (**E**, **F**) and 10 (**G**, **H**) days post surgery. In the overloaded muscle, IGF-I immunoreactivity was slightly increased in the cytosol and markedly increased in the invading cells of the extracellular space at 1 (**B**) and 3 (**D**) days post surgery. Primary antibodies were visualized by a standard avidin-biotin peroxidase method (*IGF-I* insulin-like growth factor-I). *Bar* = 100 µm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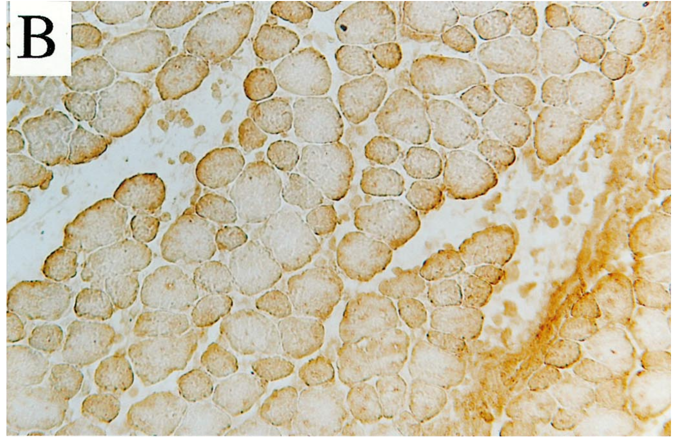
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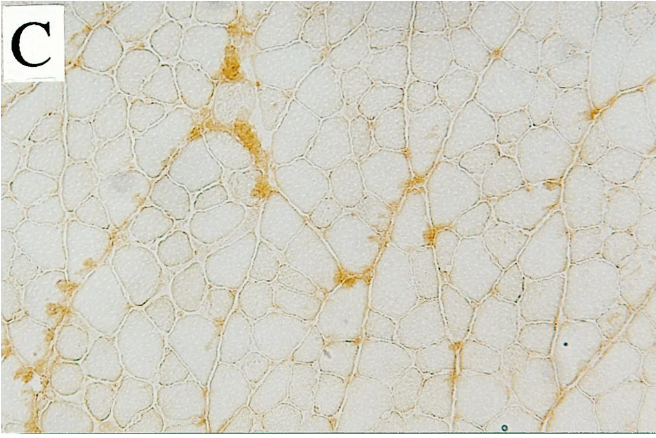
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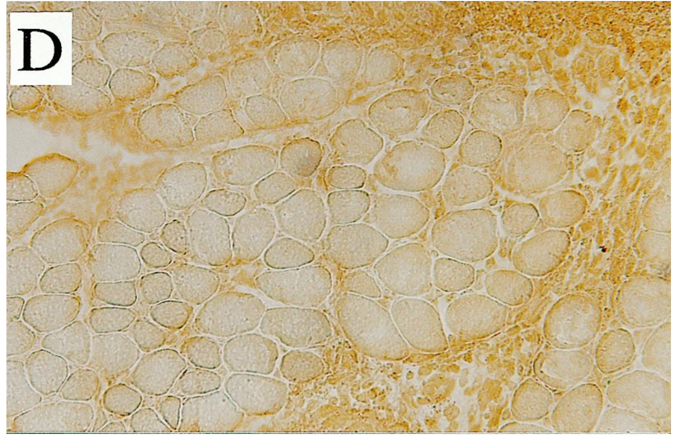
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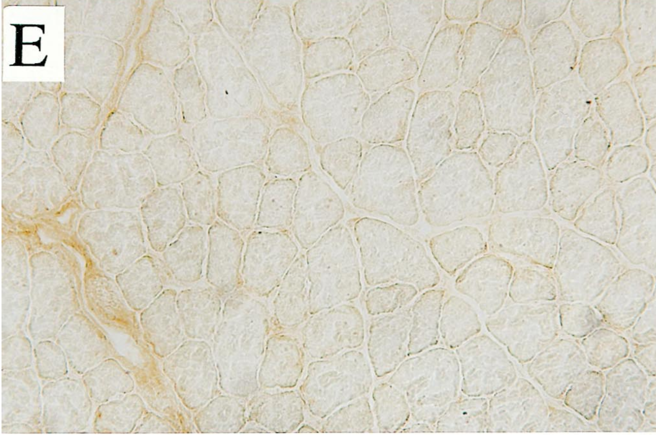
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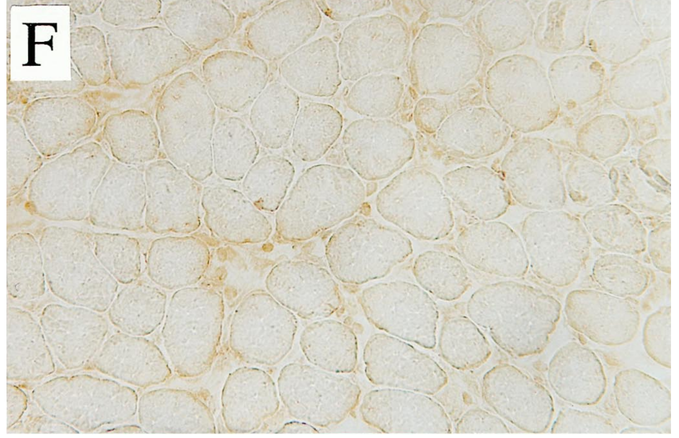
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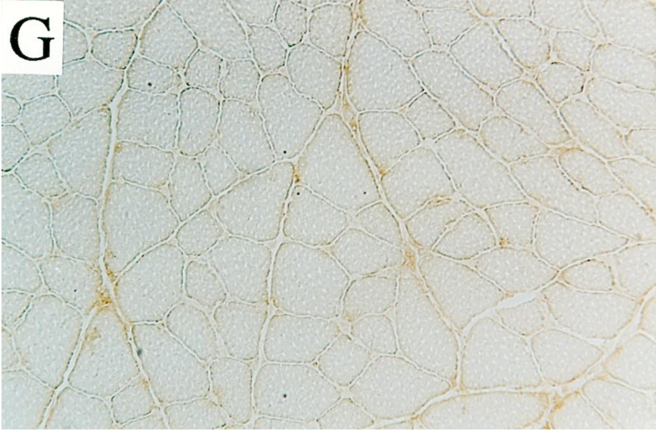
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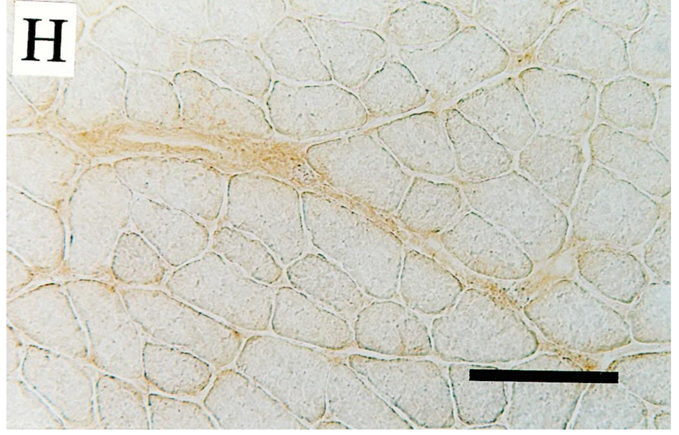
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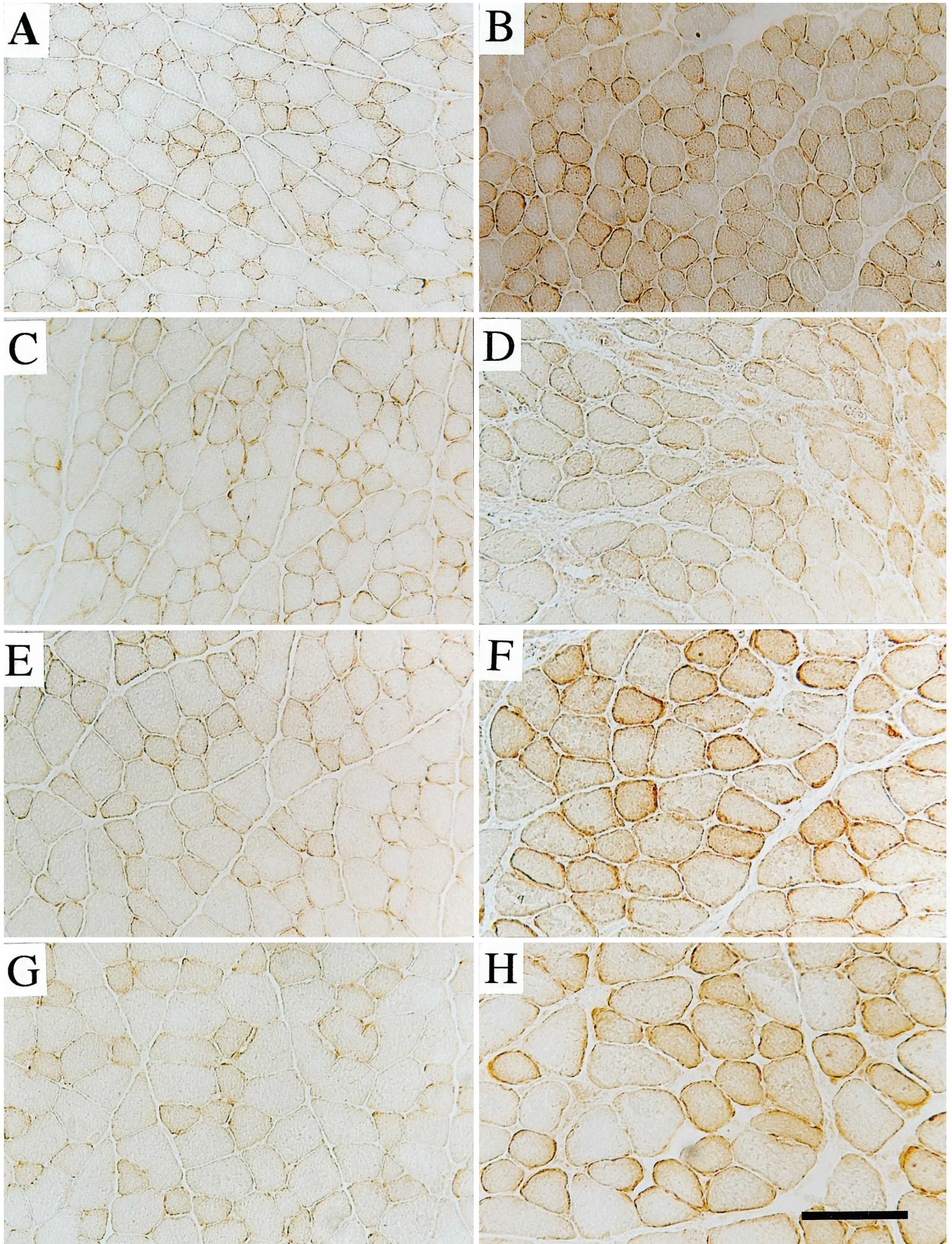
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◀ **Fig. 2** Sections from the plantaris muscles prepared for immunohistochemistry using LIF antibody in the control (**A**, **C**, **E**, **G**) and overloaded (**B**, **D**, **F**, **H**) rats. Light microphotographs showing cryosections at 3 (**A**, **B**), 7 (**C**, **D**), 10 (**E**, **F**) days and 2 weeks (**G**, **H**) post surgery. In the overloaded muscle, LIF immunoreactivity was markedly increased in the cytosol at 3 (**B**), 7 (**D**), 10 (**F**) days and 2 weeks (**H**) post surgery. Primary antibodies were visualized by a standard avidin-biotin peroxidase method (*LIF* leukemia inhibitory factor). *Bar* = 100 μm

The results for the pattern of IGF-I expression in the controls and the mechanically overloaded plantaris muscles are shown in Fig. 1. In the contralateral plantaris muscle, no specific IGF-I immunoreactivity was demonstrated on the plasma membrane or in the cytosol at 1 (Fig. 1A), 3 (Fig. 1C), 5 (Fig. 1E), 7 and 10 days (Fig. 1G) and at 2 and 3 weeks (data not shown) after the surgical procedure. The neurites in intramuscular nerves and muscle spindles were lightly stained, as were scattered Schwann cells. On the other hand, at 1 (Fig. 1B) and 3 days (Fig. 1D) post surgery, the IGF-I immunoreactivity was slightly increased in the cytosol and markedly increased in the infiltrating cells of the extracellular space compared to that of the contralateral plantaris muscle. The enhanced IGF-I levels in the overloaded muscle thereafter subsided, resulting in staining pattern similar to that of the control plantaris muscle at 10 days post surgery (Fig. 1H). These results are consistent with the findings obtained by Adams and Haddad [1] and Devol et al. [9] using Northern blot and immunoassay analyses.

As illustrated in Fig. 2, in the cytosol as well as on the sarcolemma, slight LIF immunoreactivity was observed in the slow-twitch oxidative and fast-twitch oxidative fibers in the control plantaris during the entire post-surgical period (Fig. 2A, C, E, G). We found pronounced LIF immunoreactivity in the cytosol of the overloaded plantaris muscle at 3 (Fig. 2B), 5 (data not shown) and 7 (Fig. 2D) days, although the expression was not different from the control value at 1 day (data not shown). That at 10 days (Fig. 2F) and 2 weeks (Fig. 2H) was increased compared with the immunoreactivity in the contralateral muscle.

Fig. 3 Immunohistochemical analysis of bFGF localization in control (**A**, **C**) and hypertrophied (**B**, **D**) plantaris muscles. The sections were stained with anti-bFGF at 3 (**A**, **B**) and 10 (**C**, **D**) days post surgery. Compared to the control muscle (**A**, **C**), in the surgically treated muscle the pattern of bFGF immunoreactivity did not change at 3 (**B**) and 10 (**D**) days post surgery. Primary antibodies were visualized by a standard avidin-biotin peroxidase method (*bFGF* basic fibroblast growth factor). *Bar* = 100 μm

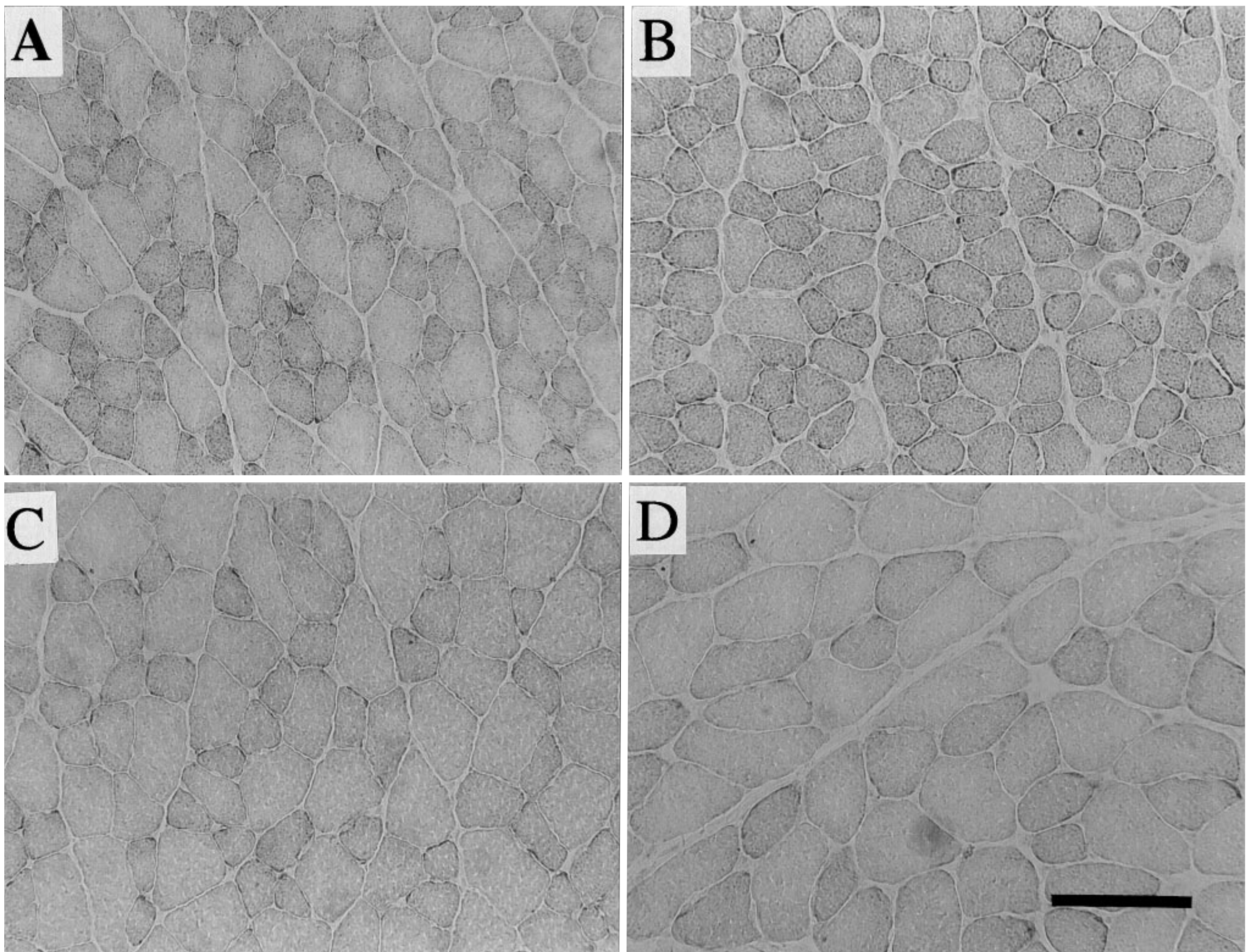

Figure 3 shows transverse sections of the control and mechanically overloaded muscles prepared for bFGF antibody staining. In the control plantaris muscle, we observed faint positive staining in the cytosol and marked immunoreactivity on the sarcolemma (Fig. 3A, C). No bFGF immunostaining was recognized in the extracellular space, e.g., on neurites in intramuscular nerves or muscle spindles. These results accord with those reported by Yamada et al. [32], who found bFGF immunoreactivity to be distributed continuously along the surface of muscle fibers. Compared to the control plantaris muscle, in the surgically treated plantaris muscle the pattern of immunostaining for bFGF antibody did not change during the period examined (Fig. 3B, D).

The cytosol, sarcolemma and the extracellular space in the negative control slides (with omission of primary antibodies) showed no immunoreactivity (data not shown).

Discussion

The major finding of the present study was that functional overload of the plantaris muscle gives rise to the elevation of LIF expression, but elicits no change of bFGF immunoreactivity. Furthermore, in the overloaded plantaris, the level of IGF-I expression increased not only in the cytosol but also in the extracellular space, although this phenomenon was not observed for LIF expression.

Leukemia inhibitory factor in hypertrophied muscle

To our knowledge, this investigation is the first immunohistochemical study that indicates that LIF is expressed in the overloaded plantaris muscle. Functional overload elevated the expression of LIF peptide at 3 and 10 days and 2 weeks post surgery. One possible interpretation of the enhancement is related to remodeling of muscle tissues damaged by mechanical stress, since surgical removal of synergistic muscles appears to bring about wide-spread inflammation and leukocyte invasion [3]. The large number of effects described for LIF in the nervous system and in muscle suggest that LIF plays a major role in the developing and regenerating neuromuscular system [8, 21, 33]. LIF, like IGF-I [18], bFGF [13] and hepatocyte growth factor [19], is up-regulated in the muscle following nerve transection [21] and crush injury [22]. Additionally, LIF, when applied exogenously, is found to enhance the rate of regeneration of crush-traumatized muscle [5].

Another possibility is that LIF is directly associated with muscle hypertrophy following mechanical overload. CNTF, which is related to LIF and IL-6 given their predicted structural similarities, is suggested to possess a direct trophic action on skeletal muscle, independent of innervation [15]. Helgren et al. [15] have demonstrated that subcutaneously injected CNTF attenuates the muscle fiber atrophy following denervation. Since LIF and CNTF, sharing the gp130 signal-transducing subunit as a common receptor component [16], have been shown to act on cells via the same signaling pathway [20], one may assume that LIF also has a direct trophic action on muscle fibers undergoing compensatory overload. This assumption might be supported by the observation that LIF stimulates the proliferation of human and murine myoblasts in vitro [4].

Changes in insulin-like growth factor following functional overload

IGF-I is suggested to increase myonuclei and myofiber size in tissue culture [31]. In the transgenic mouse, Coleman et al. [7] found that overexpression (47-fold increase) of IGF-I in muscle leads to hypertrophy of muscles but not of other tissues. On the other hand, newborn mice homozygous for a targeted disruption of IGF-I exhibit growth deficiency and marked hypoplasia [23]. Therefore, IGF-I seems to play a crucial role in the formation and hypertrophy of muscle fibers. The present result that the IGF-I peptide levels were increased at 1 and 3 days of overloading and thereafter subsided is consistent with that reported by Adams and Haddad [1]. Using radioimmunoassay and Northern blot analyses, they found that, in the plantaris muscle of rats, IGF-I peptide and mRNA were markedly increased during the early phase (3 and 7 days) and decreased rapidly from 1 to 2 weeks after functional overloading. Taken together, these results suggest that IGF-I would be involved in the hypertrophic adaptation of muscle in response to increased mechanical loading.

Satellite cells are a population of muscle precursor cells located outside the plasmalemma but beneath the basal lamina [24]. Satellite cells seem to have an important role not only in muscle fiber regeneration [29], but in fiber hypertrophy, because the prevention of satellite cell division by gamma irradiation restricts fiber hypertrophy following functional overload [26]. IGF-I, which is expressed in the satellite cells of regenerating muscle [17], has been shown to stimulate satellite cell proliferation and fusion in primary rat satellite cell culture [2]. These results imply that IGF-I is concerned with hypertrophic adaptation of muscle via activation of satellite cells.

In the overloaded plantaris muscle, slightly increased immunoreactivity of IGF-I in the cytosol may be due to presence of edema. Since edema might contain IGF-I and since the plasma membranes would be wounded in the mechanically overloaded muscle, sarcoplasmic positivity might be the consequence of non-specific uptake of edematous fluid present in the endomysium.

In the extracellular space as well as in the cytosol, we found enhanced immunoreactivity of IGF-I in the overloaded plantaris. We are unsure how to interpret this result. However, it was demonstrated that IGF-I is expressed by the Schwann cells of intramuscular nerves early in the post-ischemic phase as part of promotion of muscle regeneration [14]. This may provide us with a clue to understanding the overloading-induced IGF-I elevation in the extracellular space because of the occurrence of

similar regenerative adaptations after marked inflammation and damage produced by functional overload [3].

Basic fibroblast growth factor
in mechanically overloaded plantaris

bFGF is a potent endothelial, fibroblast, and muscle (smooth, skeletal, and cardiac) growth- and/or multiplication-inducing factor [12]. Thus, its local release at sites of mechanical stress may stimulate several essential activities of tissue remodeling/reinforcement: fibroblast hyperplasia and consequent rebuilding of the extracellular matrix; and increase in muscle cell size and/or numbers. However, as demonstrated in this study, the intensity of bFGF immunostaining remained unaltered during the entire post-surgical period from 1 day to 3 weeks. This result is discordant with the findings of Yamada et al. [32], whose investigation with Western blot analysis demonstrated that mechanical overloading of the plantaris muscle elevates the bFGF levels. This discrepancy may be due to the differences in surgical procedures. In our study, compensatory hypertrophy in the plantaris muscle was produced by complete ablation of the gastrocnemius and soleus muscles. In contrast, this was carried out with transection at the tendons of the gastrocnemius by Yamada et al. [32].

In conclusion, mechanical overloading-induced muscle hypertrophy results in increased expression of IGF-I and LIF, but not of bFGF. The function of LIF in the overloaded plantaris, however, remains to be elucidated in further studies.

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