Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle

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SUMMARY

Using muscle as an in vivo model system, we have tested the hypothesis that basic fibroblast growth factor is released from a cytoplasmic storage site into the extracellular environment via diffusion through survivable, mechanically-induced plasma membrane disruptions. Normal and dystrophic (mdx) mouse muscle were studied. Strong immunostaining for bFGF was detected in the cytoplasm of myofibers of uninjured muscle fixed in situ by perfusion. By contrast, myofibers did not stain cytoplasmically for bFGF after suffering lethal disruptions of their plasma membranes caused by freezing and thawing followed by sectioning. Sub-lethal, transient disruptions of myofiber plasma membranes - termed plasma membrane ‘wounds’ - were shown to be induced by needle puncture or exercise of muscle. Quantitative image analysis revealed that these wounded fibers contained significantly reduced levels of bFGF. Dystrophic exercised and unexercised muscle was found to possess an ~6-fold higher proportion of wounded myofibers than does normal muscle under equivalent conditions. Release of bFGF at a rate that is a direct function of the frequency of myofiber wounding may explain in part how a muscle adjusts its growth to meet changing mechanical demand as well as the pathological hypertrophy characteristic of certain stages of muscular dystrophy.

Key words: bFGF, myofibers, wounded myofibers, muscle, dystrophic muscle

INTRODUCTION

An important function of many tissues in the mammalian body is to produce and/or withstand mechanical force. When mechanical stress is imposed on various tissues in vivo, transient, survivable disruptions are created in the plasma membranes of constituent cells. We have termed this form of cell injury ‘cell wounding’ to distinguish it from lethal injuries such as are caused by irreversible disruption of the plasma membrane. We have shown that cells of gut (McNeil and Ito, 1989), skin (McNeil and Ito, 1990), aortic endothelium (Yu and McNeil, 1992) and skeletal muscle (McNeil and Khakee, 1992) frequently suffer survivable plasma membrane wounds in vivo under physiological and pathological conditions of mechanical stress. Another frequent consequence of the imposition of mechanical stress on a tissue is the activation of remodeling or repair processes. Muscle, for example, hypertrophies under certain regimens of exercise and is capable of repairing extensive pathological (i.e. cut or crush) injuries. The mitotic, locomotory and other cellular responses that accomplish stress-induced remodeling and repair are believed to be activated by polypeptide growth factors.

One such molecule, basic fibroblast growth factor (bFGF), is a potent mitogenic, chemoattractant and angiogenic polypeptide now believed to be involved in regulating muscle growth. Although bFGF is presumed to mediate these cellular responses by binding to cell surface receptors, it lacks the classical signal peptide sequence believed to be necessary for protein secretion via the exocytotic pathway (Abraham et al., 1986). Thus the mechanism by which bFGF is released into the extracellular environment by producing cells has been unclear.

One solution to this puzzle is that bFGF is exported by a novel ‘secretory’ mechanism yet to be discovered (Muesch et al., 1990). Encouragement for this possibility comes from recent studies of the muscle lectin, L-14. This 14.5 kDa polypeptide, which lacks a signal peptide sequence, is first concentrated in discrete patches on the cytoplasmic aspect of the plasma membrane and is shed when this segment of membrane becomes separated by blebbing from the parent plasma membrane (Cooper and Barondes, 1990). Furthermore, because cell death is demonstrably not necessary for bFGF export (Mignatti et al., 1991, 1992), it has been suggested that there must exist a cell-injury independent mechanism of bFGF secretion. To date, however, no evidence such as that available for L-14, has been forthcoming that would elucidate the mechanistic nature of such a bFGF ‘secretory’ mechanism.

A second possibility is that bFGF behaves as a ‘wound
hormone’ that escapes from the cytoplasm of producing cells when they suffer plasma membrane disruptions - 'wounds' - induced by mechanical stress and other injurious agents. In support of this, our work, discussed above, documents the occurrence of cell wounding in vivo and therefore establishes the widespread existence of this route in vivo, both under physiological and pathological conditions of mechanical stress. Moreover, our studies of cultured cells demonstrate that bFGF is efficiently released from its cytosolic storage site through mechanically-induced plasma membrane disruptions, including membrane disruptions survived by cells (McNeil et al., 1989; Muthukrishnan et al., 1991). Chemical injuries too cause bFGF release from endothelial cells (Gajdusek and Carbon, 1989; Brooks et al., 1991). Before this ‘wound hormone’ mechanism of bFGF release can be accepted, however, it must be demonstrated in vivo that physiologically and pathologically relevant plasma membrane wounding events lead to loss of bFGF by wounded cells.

For both technical and biological reasons, muscle is an attractive tissue in which to attempt such a demonstration. First, cell wounding and bFGF immunostaining are readily quantified by image analysis of the large and orderly cellular (myofiber) profiles characteristic of cross-sectioned muscle (McNeil and Khakee, 1992). Therefore, we could quantitatively compare the bFGF content of wounded and undisturbed cells. Second, cell wounding and bFGF have recently both been implicated in muscle growth and disease. For example, the physiological levels of mechanical force generated by exercise wound the myofibers of normal muscle (McNeil and Khakee, 1992) and such exercise promotes muscle growth (hypertrophy). Myofiber necrosis, hypertrophy and, ultimately, muscle wasting characterize the Duchenne muscular dystrophy disease state (Emery, 1988), and these pathologies may be initiated by the mechanical fragility of the dystrophic myofiber plasma membrane. bFGF is present at elevated levels in both dystrophic muscle (McNeil and Khakee, 1992) and such exercise promotes muscle growth (hypertrophy). Myofiber necrosis, hypertrophy and, ultimately, muscle wasting characterize the Duchenne muscular dystrophy disease state (Emery, 1988), and these pathologies may be initiated by the mechanical fragility of the dystrophic myofiber plasma membrane. bFGF is present at elevated levels in both dystrophic muscle (McNeil and Khakee, 1992) and such exercise promotes muscle growth (hypertrophy). Myofiber necrosis, hypertrophy and, ultimately, muscle wasting characterize the Duchenne muscular dystrophy disease state (Emery, 1988), and these pathologies may be initiated by the mechanical fragility of the dystrophic myofiber plasma membrane. bFGF is present at elevated levels in both dystrophic muscle (McNeil and Khakee, 1992) and such exercise promotes muscle growth (hypertrophy). Myofiber necrosis, hypertrophy and, ultimately, muscle wasting characterize the Duchenne muscular dystrophy disease state (Emery, 1988), and these pathologies may be initiated by the mechanical fragility of the dystrophic myofiber plasma membrane.

We here provide quantitative data derived from image analysis of immunostained muscle that wounded myofibers contain less cytoplasmic bFGF than do those that have not suffered recent survivable plasma membrane disruptions. We found this to be the case regardless of whether the mechanical force causing myofiber wounding was generated by contraction of the muscle itself or was imposed upon the muscle by a frank injury. Moreover, we show that myofiber wounding is elevated in dystrophic muscle and similarly results in diminished bFGF staining of myofiber cytoplasm.

MATERIALS AND METHODS

Animals
Mice, mdx (Bulfield et al., 1984) and B10 co-isogenics, were the kind gift of Dr L. Kunkel, Harvard Medical School, Boston, MA. Male Sprague Dawley rats (250 g) were obtained from Charles River Laboratories, Wilmington, MA. All animal care and experimentation was carried out in accordance with Harvard Medical School and Medical College of Georgia guidelines.

Antibodies and reagents
A rabbit polyclonal antibody directed against bFGF was the kind gift of Dr D. Gospodarowicz, University of California, San Francisco. The mouse monoclonal antibody (MAB-78) directed against a partial amino-acid sequence of recombinant human bFGF and the synthetic peptide fragment of human bFGF, PepI (Seno et al., 1989), were kindly donated by the Takeda Chemical Industries Ltd., Osaka, Japan. Fixable fluorescein dextran (FDxLys) (M, ~10,000), a highly branched carbohydrate to which lysine and fluorescein residues are covalently linked, was made as described by Gilmich and Braun (1985).

Preparation of bFGF from mouse muscle
Fresh B10 triceps muscle (0.1 g wet weight) was ground under liquid nitrogen and homogenized (Brinkman homogenizer for 20 seconds at full speed) in 1 ml of 100 mM Tris-HCl (pH 7.0), containing 5 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotonin and 100 µg/ml L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (protease inhibitors were purchased from the Boehringer Mannheim Corp., Indianapolis, IN). The homogenate was centrifuged at 8,000 g for 15 min and the supernatant was incubated for 4 hours at 4°C with constant agitation with 50 µl of pre-equilibrated heparin-Sepharose slurry (Pharmacia-LKB, Uppsala, Sweden). The heparin-Sepharose was collected by centrifugation and the supernatant was frozen away for later use as a bFGF-depleted muscle extract for antibody pre-absorption. The heparin-Sepharose pellet was washed twice with 1 ml of 100 mM Tris-HCl (pH 7.0) and twice with 1 ml of 1 M NaCl and then boiled for 4 minutes in 50 ml of 0.5 M Tris-HCl (pH 6.8) containing 2% SDS, 20% β-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue.

SDS-PAGE
Proteins were separated on polyacrylamide gels (12%) as described by Laemmli (1970). Non-heparin-binding protein was loaded at 50 µg per gel lane, heparin-binding proteins at 15 µg per lane. Total protein per sample was determined using a commercial protein assay kit (Bio-Rad Laboratories, Richmond, CA). Recombinant human bFGF (R&D Systems, Inc., Minneapolis, MN) (10 µg per lane) and aFGF (the kind gift of Dr Pat D’Amore, Childrens Hospital, Boston, MA.) (50 µg per lane) were also run on the same gel along with prestained molecular weight markers (low molecular weight range; Bio-Rad Laboratories).

Immunoblotting
Proteins were transferred to a PVDF membrane (Bio-Rad Laboratories) using a semi-dry transfer cell (Bio-Rad Trans-Blot SD) and a Trit/c-amino-n-caprylic acid transfer buffer (pH 10.5). The PVDF membrane was incubated in 100 mM Tris-buffered saline, pH 7.2 (TBS) containing 10% BSA for 16 hours at room temperature and then for one hour (also at room temperature) with rabbit anti-bFGF polyclonal antibody prepared in a blocking buffer containing 4% BSA, 0.05% Tween 20 and 10 µg/ml of bFGF-depleted muscle extract made up in TBS (pH 7.2). Primary antibody binding was visualized using a peroxidase-conjugated anti-rabbit biotinylated antibody kit (Vector Laboratories, Burlingame, CA.).

Running experiments
Age matched (12-week-old), mdx and B10 mice were injected intraperitoneally (i.p.) with 0.25 ml of PBS containing 400 mg/ml FDxLys. One hour later the animals were placed on a treadmill...
and the triceps muscle was punctured 8 times along its length with a 22 gauge hypodermic needle. One hour later, the animals, while still under anesthesia, were fixed by perfusion. Muscle puncture experiments

B10 mice (12 weeks old) were injected i.p. with 0.25 ml of PBS containing 400 mg/ml of FDxLys. Four hours later, the B10 mice were deeply anesthetized (i.p. injection, 200 mg/kg pentobarbital) and the triceps muscle was punctured 8 times along its length with a 22 gauge hypodermic needle. One hour later, the animals, while still under anesthesia, were fixed by perfusion.

In situ fixation by perfusion

Animals were deeply anesthetized (i.p. injection, 200 mg/kg pentobarbital) and perfused with warm PBS (37°C) containing 0.1% procaine via a cannula inserted into the left ventricle of the heart. This was followed by perfusion with a fixative solution of freshly generated 8% (w/v) paraformaldehyde in PBS. Mice were perfused using 50 ml volumes of each solution, rats with 250 ml volumes. One hour after completion of perfusion, the triceps muscles were excised from the forelimbs of the animal and immersed in fresh fixative for a further 16 hours. In order to obtain both fixed and unfixed muscle from the same animal, the right front limb was ligated in some experiments at the shoulder joint using suture thread and the triceps muscle of this limb was quickly dissected out. The remainder of the animal was perfused as described above, while the unfixed triceps was immersed in Tissue Tek OCT embedding compound (Miles Scientific, Naperville, IL) and immediately snap-frozen in liquid nitrogen-cooled isopentane.

Frozen sectioning

Muscle tissue that had been fixed in situ by perfusion was gradually infiltrated with sucrose (15% w/v, 30% w/v and 60% w/v made up in PBS) over a period of 48 hours. The belly of the muscle was dissected out and placed in Tissue Tek OCT for a further 3 hours and then mounted on a stub and snap-frozen in liquid nitrogen-cooled isopentane. Frozen sections (10 µm) were cut on a Microm HM 500 OM microtome cryostat (Carl Zeiss Inc., Thornwood, NY) and were picked up onto Superfrost Plus glass slides (Erle Scientific, Portsmouth, NH). The sections were immediately placed in fixative solution (4% w/v paraformaldehyde and 0.075% w/v glutaraldehyde in PBS) for 10 minutes and then washed using three changes of PBS over 20 minutes. Unfixed tissue was sectioned in a similar manner and fixed after sectioning as above or placed directly into PBS. Triceps muscles from mdx and B10 control animals destined for comparative image analysis were mounted on the same stub and sectioned simultaneously to ensure that immunostaining conditions were identical. Those sections that did not require immunostaining were immediately mounted in an aqueous mounting media (Johnson and Arujo, 1981) containing the anti-bleaching agent, p-phenylenediamine, in preparation for fluorescence microscopy and image analysis.

Immunostaining

Tissue sections were incubated in PBS containing 10 mM ammonium chloride for 10 minutes, washed in three changes of PBS over 10 minutes and permeabilized in 0.1% Triton X-100 in PBS for 5 minutes. The sections were then placed for 30 minutes in 2 changes of a blocking buffer containing 4% heat-inactivated goat serum and 0.05% Triton X-100 in PBS (for anti-serum albumin staining, 4% heat-inactivated sheep serum was substituted for goat serum). Sections were incubated with appropriate primary antibody for 1 hour: rabbit anti-bFGF polyclonal antibody was used at a concentration of 70 µg of total protein/ml of blocking buffer containing 10 µg/ml bFGF-depleted muscle extract; MAb 78 was used at a concentration of 5 µg of total protein/ml of blocking buffer; horseradish peroxidase-conjugated sheep anti-serum albumin antibody (Cappel Research Products, Durham, NC.) was used at a concentration of 20 µg total protein/ml of blocking buffer. Sections were then washed using three changes of PBS over a period of 20 minutes and incubated with appropriate secondary antibodies: rabbit polyclonal antibody binding was detected using a rhodamine-conjugated goat anti-rabbit IgG antibody (Fisher Scientific, Pittsburgh, PA.); mAb 78 binding was detected using a peroxidase-conjugated rabbit anti-mouse IgG antibody (Cappel Research Products, Durham, NC.). Peroxidase-conjugated antibody binding was visualized using the horseradish peroxidase-diaminobenzidine (DAB) reaction as described by Adams (1977) with the exception that cobalt chloride enhancement was omitted. Specificity of the rabbit anti-bFGF polyclonal antibody for bFGF in muscle tissue was demonstrated by preincubation of the primary antibody with a 20-fold molar excess of recombinant bFGF at 37°C for 2 hours, which resulted in abolition of staining. Specificity of mAb-78 for bFGF in muscle tissue was demonstrated by pre-incubation of the primary antibody with a 40-fold molar excess of the synthetic polypeptide, Pep-1, for a period of 2 hours at 37°C, which resulted in abolition of staining. Pep-1 has previously been shown to specifically inhibit the binding of MAB-78 to bFGF (Seno et al., 1989).

Image analysis

Quantification of myofiber immunofluorescence staining intensity was carried out using an Image 1 (Universal Imaging Corporation, West Chester, PA) or Tracor Northern 8500 (Tracor Inc., Middleton, WI) image analysis system. The methods for random acquisition of digitized transmitted light and fluorescence images of muscle sections, to delimit for analysis the cytoplasmic portion of the myofiber and for quantitatively analyzing the data thus generated, were as previously described by McNeil and Khakee (1992) with the following modifications.

For quantification of myofiber wounding using the fluorescent probe, FDxLys, sections were viewed under fluorescent illumination using a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, Germany) and fluorescent images were acquired through a Hamamatsu camera (Hamamatsu Systems Inc., Walton, MA) into a Tracor Northern image analysis system. After subtracting background fluorescence from each digitized fluorescent image, the FDxLys intensity (expressed as a gray level value) in the cytoplasm of ~22,000 individual myofibers from six eccentrically exercised mdx and normal B10 co-isogenic control triceps muscles were measured and expressed on a linear gray scale. An average gray level value was calculated from a sample of 200 myofibers which, by microscopic examination, were judged not to be labeled with cytoplasmic FDxLys. Myofibers that had a gray level value above this ‘threshold’ value were deemed as having been wounded at their plasma membrane.

For the quantification of bFGF staining (rhodamine) and FDxLys (fluorescein) labeling of individual myofibers in co-stained muscle sections, digitized images of identical regions were acquired into the Image 1 analysis system by selection of the appropriate filter set. After background subtraction the images were displayed side by side so that paired densitometric measurements of average rhodamine and fluorescein fluorescence intensities could be acquired from each myofiber. These values...
were plotted as x,y co-ordinates of a scatter plot and then subjected to regression analysis.

The frequency of serum albumin-positive myofibers in the triceps muscles of sedentary mdx and B10 mice was determined by image analysis exactly as described elsewhere (McNeil and Khakee, 1992).

RESULTS

Immunolocalization of bFGF in muscle

The polyclonal anti-bFGF used in this study recognized human recombinant bFGF but not aFGF in western blots. The antibody recognized a heparin-binding protein in muscle extract with a $M_r$ of ~2 kDa less than that of human recombinant bFGF, and this protein was not detected in the non-heparin-binding fraction of muscle extract (Fig. 1). In addition, two other antibodies to bFGF (a mouse monoclonal, DE6; and another rabbit polyclonal, CR2) similarly recognized an identical protein in mouse muscle extract (data not shown).

Previous immunostaining studies suggested that bFGF is located predominantly in the extracellular matrix of adult muscle (Anderson et al., 1991; DiMario et al., 1989). We first re-examined the question of where bFGF is located in adult skeletal muscle. Normal mouse (B10) triceps muscles were either excised and snap-frozen without fixation, or the contralateral muscle was fixed in situ by perfusion, before sectioning and immunofluorescence staining for bFGF. In perfusion-fixed muscle, staining for bFGF was present in the cytoplasm of the myofibers, and no discernible extracellular staining was observed (Fig. 2A). In snap-frozen muscle not fixed in situ but perfused but fixed immediately after frozen sectioning, cytoplasmic bFGF staining was uneven in nature and reduced in intensity (Fig. 2B). Finally, in unfixed, snap-frozen muscle, cytoplasmic bFGF staining was virtually abolished and the bFGF staining that remained was limited primarily to the fiber periphery and/or extracellular matrix (Fig. 2C), consistent with previous studies that used unfixed muscle (Anderson et al., 1991; DiMario et al., 1989). Specificity of the primary antibody for bFGF in this tissue was demonstrated by pre-incubation of the primary antibody with a 20-fold molar excess of recombinant bFGF, which resulted in complete abolition of staining. Substitution of the primary antibody with non-immune serum resulted in no significant staining of the tissue.

We observed the same pattern of bFGF staining in mdx muscle as in B10 muscle: predominantly cytoplasmic in the myofibers of muscle fixed in situ by perfusion (Fig. 2D) and diminished staining in unfixed muscle (Fig. 2E and F). However, staining of the extracellular matrix was observed in heavily fibrotic areas of dystrophic muscle (not shown). Moreover, the intensity of bFGF staining in the cytoplasm of mdx myofibers appeared to be greater than in B10 myofibers. Using a mouse monoclonal antibody, MAB 78, to stain rat muscle fixed in situ, we also observed cytoplasmic staining for bFGF (Fig. 3).

These results suggest that bFGF is a cytoplasmic molecule in both the normal and dystrophic myofiber and that a gross injury to the myofiber brings about loss of cytoplasmic bFGF.

Trauma-induced myofiber wounding

Although the above results are consistent with the wound hormone hypothesis for bFGF release, the gross (i.e. unfailingly and uniformly lethal) nature of the muscle injury caused by frozen sectioning is unlikely to have relevance in vivo for either the normal or pathological responses of muscle to mechanical stress. As a more direct test therefore of a muscle’s response to mechanical stress in vivo, we pathologically stressed (i.e. injured) muscle mechanically by puncturing it with a syringe needle. Myofibers suffering survivable plasma membrane wounds during the needle puncture were identified by the entry into their cytoplasm of FDxLys, which was administered to the animal by i.p. injection 4 hours prior to the injury. FDxLys has previously been used in this manner as a marker of survivable myofiber wounding in vivo (McNeil and Khakee, 1992), as well as survivable plasma membrane wounding in vitro (Clarke and McNeil, 1992). In cross-sections of B10 triceps muscle, the track of such a puncture wound is lined by large numbers of myofibers heavily labeled in their cytoplasm with FDxLys (Fig. 4A). These FDxLys-positive myofibers possessed only weak, or no cytoplasmic bFGF staining (Fig. 4B). Quantitative image analysis (Fig. 4C) revealed that there was a significant negative correlation ($P < 0.01$) between the intensity of myofiber FDxLys and anti-bFGF staining.

Exercise-induced myofiber wounding

We have previously demonstrated that ~4% of the myofibers in the triceps of the sedentary, unexercised rat are labeled with markers for survivable sarcolemma wounds and that the frequency of such myofiber wounding is greatly (~7-fold) increased after eccentric exercise of this muscle (McNeil and Khakee, 1992). In order to determine whether this physiologically-induced membrane wounding occurs in mice and whether it could be responsible for release of
bFGF from myofibers, we here have similarly identified wounded myofibers in the eccentrically exercised mouse triceps and asked whether their staining for bFGF is reduced relative to non-wounded myofibers. The triceps of animals were eccentrically exercised by downhill running on a treadmill, and FDxLys, as above, was used as a marker of ‘acute’ myofiber wounding. The mdx animals collapsed after no more than 5 min of this exercise, at which time the B10 controls were also removed from the treadmill. Eccentrically exercised muscle of both normal (Fig. 5A) and dystrophic (Fig. 5C) animals possessed FDxLys-labeled myofibers. Quantitative image analysis (Fig. 6) revealed that the number of wounded myofibers in eccentrically exercised mdx triceps muscle was 6.5-fold greater ($P < 0.005$) than that detected in eccentrically exercised normal muscle. Wounded myofibers in both normal and dystrophic muscle (Fig. 5A and C) appeared to stain less strongly for bFGF than did non-wounded, neighboring myofibers (Fig. 5B and D). This qualitative impression that FDxLys labeling intensity was inversely correlated to bFGF staining

**Fig. 2.** Immunofluorescence staining of bFGF in the triceps muscle of sedentary normal (left column) and dystrophic (right column) mice. (A and D) Cross-sections of muscle fixed in situ by perfusion. Anti-bFGF stains the cytoplasm of the myofibers, but not the extracellular matrix. (B and E) Cross-sections of snap-frozen muscle fixed immediately after sectioning. Cytoplasmic staining for bFGF is reduced compared to perfusion fixed muscle from the same animal. (C and F) Cross-sections of unfixed, snap-frozen muscle. Anti-bFGF staining of the myofiber cytoplasm is further reduced, and staining of the extracellular matrix and some nuclei is now apparent. (A-F) were photographed under identical conditions. Bar, 10 µm.
intensity was confirmed as statistically significant ($P < 0.01$) using quantitative image analysis (Fig. 7A and B). Thus, exercise-induced myofiber wounding, as well as that induced by needle puncture, results in loss of myofiber bFGF, and this loss is greater for a given level of exercise in the $mdx$ than in the normal animal.

**Myofiber wounding in sedentary dystrophic muscle**

Finally, we asked whether the muscle hypertrophy characteristic of the sedentary $mdx$ mouse could be linked to myofiber wounding. If so, one would predict a greater incidence of wounded myofibers in sedentary $mdx$ than in normal B-10 mouse muscle. As muscle hypertrophy is a chronic condition in the $mdx$ mouse, we chose a chronic label for cell wounding, the animal’s own serum albumin. Wounded myofibers were present in muscle from both sedentary $mdx$ and B10 animals (Fig. 8A and B). Quantitative image analysis (Fig. 9) showed, however, that the number of such wounded myofibers was 6.25-fold greater ($P < 0.025$) in sedentary dystrophic muscle than in sedentary, normal muscle. The great majority of these wounded myofibers displayed none of the characteristics of necrotic fibers, which are also, though less frequently, present in 12-week-old $mdx$ muscle. Indeed, frankly necrotic (dead) fibers are not stained with anti-serum albumin, presumably because their ‘cytoplasmic’ serum albumin is lost during the saline perfusion that precedes fixation.

**DISCUSSION**

Our data strongly suggest that bFGF is located within the cytoplasm of the myofiber, and that it is released from this location when the sarcolemma is disrupted by the imposition of mechanical force. We show that such release may occur both after the pathological injury caused by needle puncture and after the physiological injury caused by muscle exercise. According to this ‘wound hormone’ model, bFGF is a key signal molecule for transforming the mechanical stimulus of muscle exercise or injury into a chemical one that can initiate cell growth or repair. Striated and cardiac muscle, as well as skin, all are known to utilize a signal transduction system of this kind, since all these tissues respond to mechanical stress with hypertrophy or hyperplasia.

**Localization of bFGF in muscle**

A cytoplasmic localization for bFGF in skeletal muscle is consistent with previous studies of cultured cells. Cell fractionation and immunostaining both localized bFGF to the cytoplasm and the nucleus of fibroblasts (Renko et al., 1990), and to the cytoplasm of endothelial cells (Muthukrishnan et al., 1991). bFGF immunostaining of the extracellular matrix was absent in both of these studies. Moreover, bFGF has previously been localized, in line with the results of this study, to the cytoplasm of myotubes in the developing chicken embryo and myofibers of human muscle (Joseph-Silverstein et al., 1989; Cordon-Cardo et al., 1990), as well as to the cytoplasm of numerous other cells in vivo (see, for example, Adamis et al., 1991;
Loss of bFGF from wounded myofibers by Schulze-Osthoff et al., 1990; Sunderkotter et al., 1990; Spirito et al., 1991; Tateishi et al., 1990; Zagzag et al., 1990).

By contrast, in numerous other studies bFGF has been immuno-localized to the extracellular matrices of various tissues (see, for example, Gonzalez et al., 1990; Folkman et al., 1988) including muscle (Anderson et al., 1991; DiMario et al., 1989). Moreover, bFGF has been identified biochemically in the extracellular matrix left behind by cultured cells removed from their dishes by treatment with lytic concentrations of detergent or lethal doses of urea (Vlodavsky et al., 1987; Baird and Ling, 1987).

bFGF has, as is well known, a strong affinity for heparan sulfate (Klagsbrun, 1990), a common constituent of the extracellular matrix. It seems likely that a failure to adequately fix bFGF within a cell can result in the diffusion of this small (~18 kDa), soluble, cytoplasmic protein out of cell cytoplasm during tissue sectioning/processing for immunostaining. This formerly cytoplasmic bFGF would

Fig. 4. The relationship between FDxLys labeling and bFGF staining of myofibers in needle punctured mouse triceps. Photographs of the digitized, video images of a cross-section through the site of a needle puncture wound of the triceps showing the same field of view of (A) FDxLys labeled myofibers (fluorescein filter) and (B) bFGF immunostaining (rhodamine filter). Note the binary template overlay and corresponding average fluorescence intensity values for (A) FDxLys (fluorescein) and (B) bFGF immunostaining (rhodamine) from identical areas of the same myofiber. These and additional values from other regions were then plotted as the (x,y) co-ordinates of a scatter plot (C). Regression analysis revealed a significant negative correlation between the two experimental parameters (y = 33.5 – 0.67x ; R = −0.57, n = 215; P < 0.01) indicating that as the degree of membrane wounding increased, myofiber bFGF content decreased. Bar, 10 µm.
Fig. 5. The relationship between FDxLys labeling and bFGF staining of myofibers in the eccentrically exercised normal (A and B) and dystrophic (C and D) triceps. (A and C) Fluorescein fluorescence micrographs of cross-sections of eccentrically exercised B10 and mdx triceps, showing cytoplasmic labeling with FDxLys of wounded myofibers. (B and D) Rhodamine fluorescence micrographs of the same field of view showing staining for bFGF. Note that those myofibers in mdx muscle labeled with FDxLys (C) generally possessed reduced staining for bFGF (D). This relationship also appeared to be true in normal muscle (A and B) but was not as qualitatively obvious as in dystrophic muscle. Bar, 10 μm.
Loss of bFGF from wounded myofibers
then either be lost in the staining buffers or bind to the nearest extracellular matrix. In support of this, Adamis et al. (1991) show that bFGF is located within the cytoplasm of the epithelial cells of the uninjured cornea, but that upon chemical or mechanical damage to the corneal epithelium, bFGF becomes associated with the extracellular matrix of Bowman’s layer.

The importance of proper fixation in identifying the true cellular distribution of a soluble protein molecule is not, of course, a new concept. For example, the cellular site of calmodulin, as detected by immunolocalization, varies depending on whether the permeabilization step is carried out before or after fixation (Nielsen et al., 1987). Lactate dehydrogenase and serum albumin introduced into, and demonstrably present primarily within the cytoplasm of living cells, are both localized instead in the nucleus after fixation (Melan and Sluder, 1991). The choice of fixative, too, can determine the outcome of the localization (Melan and Sluder, 1991). Differential extraction/preferential retention of the soluble protein depending on the fixation/permeabilization procedure used most likely explain such artifacts. The often contradictory reports of the site(s) of bFGF localization within a given tissue may be explained in part by such fixation/extraction artifacts. Certain tissues may be particularly vulnerable in this regard. Muscle is one such tissue because it can be fixed thoroughly and rapidly only by perfusion, and because sectioning unavoidably permeabilizes (by transection) its fibers.

Myofiber wounding and dystrophic muscle
A mechanically induced plasma membrane disruption may or may not constitute a lethal (or ‘lytic’) injury to a cell. Microinjection, scrape-loading and bead-loading are, for example, three techniques for loading macromolecules into living cells that rely on the cell’s ability to reseal and thus survive a mechanically induced plasma membrane disruption (for review see McNeil, 1988). The two soluble, freely diffusible molecules we have used here for labeling wounded cells - serum albumin and FDxLys - act as vital dyes in the reverse sense of trypan blue: only living cells that have successfully resealed a membrane disruption trap and retain them in their cytoplasm. Indeed, in a previous study of eccentric exercise-induced injury of the triceps, we found no evidence that myofibers identified by rat serum albumin labeling as wounded 30 minutes after exercise subsequently (24-48 hours) became necrotic (McNeil and Khakee, 1992). Similarly we have found that those myofibers labeled with FDxLys persist for up to 72 hours after a needle puncture, and hence have retained membrane integrity for this interval (unpublished observations).

Fig. 6. Image analysis comparison of the frequency of myofiber wounding in the eccentrically exercised triceps muscles of the mdx and B10 control mice as detected by the presence of cytoplasmic FDxLys. Image analysis of ~22,000 myofibers from 6 mdx and 6 B10 control triceps revealed that the percentage of wounded myofibers detected in eccentrically exercised dystrophic muscle was 6.5-fold greater (P < 0.005, Student’s t-test, error bar = 1 s.d.) than detected in eccentrically exercised normal muscle.

Myofiber wounding and dystrophic muscle
A mechanically induced plasma membrane disruption may or may not constitute a lethal (or ‘lytic’) injury to a cell. Microinjection, scrape-loading and bead-loading are, for example, three techniques for loading macromolecules into living cells that rely on the cell’s ability to reseal and thus survive a mechanically induced plasma membrane disruption (for review see McNeil, 1988). The two soluble, freely diffusible molecules we have used here for labeling wounded cells - serum albumin and FDxLys - act as vital dyes in the reverse sense of trypan blue: only living cells that have successfully resealed a membrane disruption trap and retain them in their cytoplasm. Indeed, in a previous study of eccentric exercise-induced injury of the triceps, we found no evidence that myofibers identified by rat serum albumin labeling as wounded 30 minutes after exercise subsequently (24-48 hours) became necrotic (McNeil and Khakee, 1992). Similarly we have found that those myofibers labeled with FDxLys persist for up to 72 hours after a needle puncture, and hence have retained membrane integrity for this interval (unpublished observations).

Finally, it has been possible to directly demonstrate that endothelial cells of the aorta must survive membrane disruptions in order to stain in their cytoplasm with an anti-serum albumin antibody (Yu and McNeil, 1992). All available data would therefore indicate that the myofibers labeled in this study with FDxLys or anti-serum albumin had suffered survivable plasma membrane disruptions, as contrasted with the lethal membrane disruptions suffered by the myofibers of unfixed muscle that was excised, frozen, transsected and then thawed.

The muscles of humans suffering from Duchenne mus-
cular dystrophy, and of mdx mice, lack the protein dystrophin (Hoffman and Kunkel, 1989). The consequences for dystrophic muscle are well known (Emery, 1988; Hoffman and Gorospe, 1991). It contains an abnormally high proportion of frankly necrotic and hypertrophic fibers, as well as large inter-fiber zones of fibrotic connective tissue.

Two functional roles have been ascribed to dystrophin. The first is as a key structural element of the membrane cytoskeleton that acts to mechanically stabilize the myofiber plasma membrane. Dystrophin exhibits significant homologies with the red blood cell (RBC) protein spectrin (Hoffman et al., 1987), known to be essential for the maintenance of normal RBC shape and mechanical properties (reviewed by Branton et al., 1981). Like spectrin, dystrophin is found on the cytoplasmic surface of the plasma membrane, and recent evidence suggests that dystrophin associates with a glycoprotein complex that spans the plasma membrane and, hence, may be involved in force transduction across it (Ibraghimov-Beskrovnaya et al., 1992). Finally, it has been shown that mdx muscle fibers are more susceptible to rupture when stressed mechanically than are normal muscle fibers (Weller et al., 1990; Menke and Jockusch, 1991; Petrof et al., 1993). In intact muscle, this increased susceptibility is dependent on the force exerted on the muscle by contraction and not on the number of contractions (Weller et al., 1990; Petrof et al., 1993).

A second proposed role for dystrophin is in regulating intracellular Ca$^{2+}$ homeostasis. In support of this role, abnormal Ca$^{2+}$ entry into cultured mdx muscle fibers has been measured (Franco and Lansman, 1990). In addition, these authors showed that normal and mdx myofibers are equally prone to rupture in response to applied suction pressure than are normal myofibers (Franco and Lansman, 1990).

Our data is most consistent with the membrane stabilizing role for dystrophin. This is because metabolically- and/or calcium influx-initiated injuries sufficient to compromise membrane integrity are lethal to cells including muscle cells (Jones et al., 1984; Weinberg et al., 1991; Lemasters et al., 1987). As elaborated above, mechanically induced membrane disruptions are, by contrast, demonstrably survivable by cells. We have documented that sublethal disruptions of membrane integrity occur more frequently in mdx than in normal muscle. Given its otherwise seemingly normal locomotory behavior and exercise tolerance at low levels of activity (Hudecki and Pollina, 1990; Weller et al., 1991), one surprising observation made here was the remarkable sensitivity of the mdx mouse to eccentric exercise: it collapsed after no more than five minutes of downhill running. Eccentric exercise, in which the muscle contracts while lengthening, exerts higher levels of mechanical force on a muscle than does concentric exercise (Armstrong et al., 1983). Our observation is in line with subsequent studies by Hudecki et al. (1993) who found mdx mice to be significantly weaker than normal mice at higher levels of activity than originally studied, and is further evidence consistent with mechanical force being the injurious agent that dystrophic muscle is most vulnerable to.
Wound-induced loss of bFGF

We have shown that bFGF is lost from myofibers wounded by muscle needle puncture, and that it is similarly lost from myofibers wounded by eccentric exercise. bFGF thus released from myofibers would be expected to bind to surrounding extracellular matrix. As mentioned above, this appeared to be the case for un-fixed, frozen-sectioned muscle. We did not however observe any bFGF labeling of extracellular matrix in exercised or needle-injured muscle. There may be several reasons for this: (1) the proportion of myofibers injured and the average extent of the individual myofiber injury induced were far less than that induced by frozen sectioning; (2) the antibody we used may not recognize with sufficiently high affinity bFGF bound to heparan sulfate; and/or (3) released bFGF may have been carried away from the site of release in the blood or in the extensive pre-fixation saline perfusion.

A considerable body of recent evidence suggests a role for bFGF in regulating muscle growth. bFGF promotes satellite cell proliferation (DiMario and Strohman, 1988) and delays myocyte fusion (Gospodarowicz et al., 1976; Rapraeger et al., 1991) in vitro. Mechanical loading of muscle tissue initiates the satellite cell proliferation (Schultz, 1989) believed to be involved in muscle hypertrophy (Yamada et al., 1989). Exercise-conditioning increases the levels of bFGF (Morrow et al., 1990) and bFGF mRNA (Guthridge et al., 1992) in muscle tissue. While its role in muscle repair in vivo remains unproven, bFGF is directly implicated in the repair of injuries to other tissues (Broadley et al., 1989). We suggest that the adjustment of a muscle’s growth state to changing mechanical demand is achieved in part by loss of bFGF from myofiber cytoplasm in direct proportion to the frequency of myofiber wounding, followed by bFGF stimulation of satellite cell proliferation. We have shown here and elsewhere (McNeil and Kharee, 1992) that eccentric exercise increases myofiber wounding. Hence this mechanism for modulating bFGF release can explain the muscle hypertrophy that follows such exercise. Conversely, unused normal muscle may atrophy due to a reduction in myofiber wounding below a basal level, characteristic of the sedentary animal.

We show here that the proportion of wounded myofibers is elevated in dystrophic relative to normal muscle under comparable exercise conditions, and that as a consequence more bFGF is lost from the mdx myofiber for a given level of exercise. Others have shown that bFGF is elevated in dystrophic muscle (Anderson et al., 1990; DiMario et al., 1989). We suggest therefore that bFGF release in dystrophic muscle is abnormally elevated both due to this tissue’s increased susceptibility to myofiber wounding and to its higher endogenous levels of bFGF. This could explain why dystrophic muscle hypertrophies at levels of mechanical demand far below those required for inducing hypertrophy of normal muscle.

In summary, the work described here provides the first in vivo evidence that bFGF is lost from the cytoplasm through plasma membrane disruptions caused by physiologically generated mechanical force. Release of bFGF through plasma membrane disruptions can, as we have previously stated (McNeil et al., 1989; Muthukrishnan et al., 1991), explain how bFGF is accurately targeted both in time and in space to sites of mechanical injury. Local binding, to extracellular matrix (Folkman and Klagsbrun, 1987), of bFGF released in this manner could further enhance a localized action of this growth factor in promoting cellular repair responses such as locomotion, chemotaxis and mitosis (for review see Rifkin and Moscatelli, 1989). The ‘wound hormone’ hypothesis for explaining bFGF export is, however, possibly less attractive in other biological contexts. bFGF is, for example, believed to be involved in regulating early development (Amaya et al., 1991; Kimmelman et al., 1988; Slack et al., 1987) but there is no evidence that cell wounding occurs in the early embryo. Moreover, recent evidence (Mignatti et al., 1991, 1992) shows that a ‘secretory’ mechanism independent of cell death must exist for bFGF. We propose that survivable plasma membrane wounding induced by mechanical stress may provide such a mechanism in muscle. Clearly, further work will be required for determining what exactly are the additional routes of bFGF release in vivo, and whether the wound hormone mechanism of release of bFGF operates in other tissues as well as muscle.

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