

# Nerve Sprouting in Innervated Adult Skeletal Muscle Induced by Exposure to Elevated Levels of Insulin-like Growth Factors

Pico Caroni and Pedro Grandes

Brain Research Institute, University of Zurich, CH-8029 Zurich, Switzerland

**Abstract.** Partial denervation or paralysis of adult skeletal muscle is followed by nerve sprouting, probably due to release of diffusible sprout-inducing activity by inactive muscle. Insulin-like growth factors (IGF1 and IGF2) are candidates for muscle-derived sprouting activity, because (a) they induce neurite growth from peripheral neurons *in vitro*; and (b) their mRNA levels in adult skeletal muscle increase severalfold after denervation or paralysis. We sought to determine whether the presence of elevated levels of IGFs in innervated adult skeletal muscle was sufficient to produce intramuscular nerve growth.

Low concentrations of IGFs induced massive neurite growth from enriched embryonic chick motoneurons *in vitro*. Half-maximal responses required 0.2 nM IGF2 or IGF1, or 20 nM insulin. Similar hormone binding properties of motoneuron processes *in vitro* were observed.

Exposure of adult rat or mouse gluteus muscle *in vivo* to low quantities of exogenous IGF2 or IGF1 led to intramuscular nerve sprouting. Numbers of sprouts in IGF-exposed muscles were 10-fold higher than in vehicle-exposed or untreated muscles, and 12.2% of the end plates in IGF-exposed muscle (control: 2.7%) had sprouts growing from them. The nerve growth reaction was accompanied by elevated levels of intramuscular nerve-specific growth-associated protein GAP43. Additional properties of IGF-exposed muscle included modest proliferation of interstitial cells and elevated interstitial J1 immunoreactivity.

These results suggest that elevated levels of IGFs in denervated or paralyzed muscle might trigger coordinate regenerative reactions, including nerve sprouting and expression of nerve growth-supporting substrate molecules by activated interstitial cells.

**I**N the central and peripheral nervous systems of adult vertebrates, functional denervation leads to growth of processes from surrounding axons; i.e., to a sprouting reaction (Purves and Lichtman, 1985). In spite of its significance for repair processes and for our understanding of plasticity in the adult nervous system, molecular components that trigger the process have not yet been identified.

The best studied and most accessible sprouting system is the inactivated adult neuromuscular junction (Brown et al., 1981; Grinnell and Herrera, 1981; Brown, 1984; Wernig and Herrera, 1986). At the neuromuscular junction, partial denervation leads to sprouting of remaining intact intramuscular nerves. Sprouting appears to be due to lack of muscle activation, as electrical stimulation of denervated muscle suppresses a major part of the sprouting reaction, and as the latter is also observed if the muscle is paralyzed without nerve lesion.

Sprouting is a local reaction (Brown, 1984) that probably involves triggering and supporting components (Brown et al., 1980). One signal for sprouting is, in all likelihood,

Pico Caroni's present address is Friedrich Miescher Institute, Postfach 2543, CH-4002 Basel/Switzerland. Pedro Grandes' present address is Department of Neurosciences, Faculty of Medicine, Universidad del Pais Vasco, E-48940 Lejona, Vizcaya/Spain.

diffusible activities released from inactive muscle (Slack and Pockett, 1981; Pockett and Slack, 1982; Torigoe, 1985; Kuffler, 1989). Such activities have been partially purified from denervated muscle, and it has been shown that inactive muscle contains elevated amounts of sprouting activity, as measured *in vitro* on spinal cord neurons (Henderson et al., 1983). In addition to the putative diffusible sprouting activity, substrate components known to support and promote neurite growth *in vitro* are expressed in inactive muscle (Sanes and Covault, 1985). These components include the cell adhesion molecules N-CAM and tenascin (J1) (Rieger et al., 1985; Covault and Sanes, 1986; Sanes et al., 1986). One source of nerve outgrowth-promoting substrates in inactive muscle appears to be activated muscle fibroblasts (Gatchalian et al., 1989).

Recent work by Ishii and co-workers points to attractive candidates for diffusible sprouting promoting activities in inactive muscle: the insulin-like growth factors (IGFs)' IGF1 and IGF2. These growth factors have been shown to promote neurite outgrowth from sympathetic and sensory neurons *in vitro* (Recio-Pinto et al., 1986). Neurite outgrowth was inhibited. *Abbreviations used in this paper:* AChR, acetylcholine receptor; E, chick embryonic day; IGF, insulin-like growth factor; N-CAM, cell adhesion molecule; ZIO, zinc iodide solution mixed 1:4 with osmium tetroxide.

duced at very low concentrations of the somatomedins, e.g., 0.1 nM IGF2 for half-maximal response of cultured chick sensory neurons (Recio-Pinto et al., 1986), and was presumably through IGF1-type receptors on the sprouting neurons (Recio-Pinto et al., 1984; Rechler and Nissley, 1985). Most significantly, Ishii recently demonstrated that denervation or Botulinum toxin-induced paralysis of adult rat skeletal muscle led rapidly to elevated levels of IGF2 and IGF1 mRNA in the treated muscles (Ishii, 1989; Glazner, G. W., and D. N. Ishii, unpublished observations). IGF2 and IGF1 mRNA levels only declined to low control levels if reinnervation was allowed to take place. In addition, a close correlation between declining levels of IGF2 and IGF1 mRNA in muscle and the time course of synapse elimination during normal development was observed (Ishii, 1989).

The present study was undertaken to test the hypothesis that IGF2 and IGF1 might be candidates for the diffusible sprouting activity in adult inactivated muscle. First, responsiveness of motoneurons to IGFs was investigated *in vitro*. IGF2 and IGF1 induced neurite outgrowth in embryonic motoneurons *in vitro*, and the processes of the same neurons possessed somatomedin binding sites, presumably IGF1 receptors. We then determined the effect of IGFs on *in situ* muscle preparations. The presence of low amounts of added somatomedin was sufficient to induce local sprouting in adult rodent gluteus muscle. Combined with the recent findings of Ishii (Ishii, 1989; Glazner, G. W., and D. N. Ishii, unpublished observations) our findings suggest that IGF2 and IGF1 might be diffusible sprout-triggering activities in injured muscle.

## Materials and Methods

### Cell Culture

Motoneurons were prepared from embryonic day 6 (E6) chick spinal cord by the method of Dohrmann et al. (1986) with minor modifications. Briefly, E6 lumbar and brachial spinal cord was carefully dissected free of meninges and dorsal root ganglia and cells were then dissociated by trypsin treatment followed by mild trituration with a siliconized Pasteur pipette. Low density motoneurons were recovered on the top of a 23.5% Ficoll cushion in Hepes-buffered L15 medium. Cells were cultivated at densities of  $2-10 \times 10^4$  cells/ml on laminin-coated wells of four-well (Greiner GMBH, Nürtingen, FRG) dishes. The basic culture medium was enriched L15 (Mains and Patterson, 1973) with 10% horse serum. In this medium more than 95% of neurons died within 48 h unless a soluble extract of E19 chick hindlimb muscle proteins was added. The muscle extract consisted of a 100,000-g, 30-min supernatant of Polytron-homogenized muscle in L15 (10% wt/vol). The extract could be stored with minor activity losses at  $-20^\circ\text{C}$  and final protein concentrations of 20  $\mu\text{g}/\text{ml}$  were routinely used for the neurite outgrowth experiments. Under these conditions most neurons in the culture grew large multipolar cell bodies bearing long processes with characteristically thick original segments. A fraction of these neurons could consistently be retrogradely labeled when rhodamine isothiocyanate crystals were placed in the embryo hindlimb 18 h before isolation (Dohrmann et al., 1986; Thanos and Bonhoeffer, 1984). The crystals were placed in the hindlimb muscles of E 51/2 chick embryos and the lumbar spinal cord, with attached injected hindlimbs, was incubated for 18 h at  $37^\circ\text{C}$  in 1 liter of oxygenated DME (7.68 g/liter NaCl, 1.85 g/liter  $\text{NaHCO}_3$ ) supplemented with 10% horse serum, 2.5% chick embryo extract, glutamine, pyruvate, and antibiotics (Calof and Reichardt, 1984). Subsequently, motoneurons were prepared and cultivated as described above. Under these conditions,  $\sim 10\%$  of cells in the culture were intensely labeled. Rhodamine-labeled cells had characteristic large cell bodies with thick initial process segments and represented  $\sim 95\%$  of cells in our cultures. Numbers of recovered retrogradely labeled neurons were comparable to the ones described by Dohrmann et al. (1986). Nonneuronal cells were typically between 1 and 3% of cells in the motoneuron fraction. Under the culture conditions described above, these

nonneuronal cells were  $\sim 10\%$  of cells in the culture after 7 d *in vitro* (see also Fig. 1). Our culture therefore, as the one described by Dohrmann et al. (1986) from which the method was derived, seemed to consist for  $>90\%$  of muscle extract-dependent motoneurons. The correct identity of motoneurons prepared by this protocol is confirmed by the similar properties of motoneurons isolated by sorting of cells that had been retrogradely labeled *in situ* (Calof and Reichardt, 1984; O'Brien and Fischbach, 1986).

### Experiments with Insulin-like Growth Factors *In Vitro*

Highly purified human IGF2 was a kind gift of G. Haselbacher and R. Humbel (University of Zurich, Switzerland). Recombinant human IGF1 and purified  $^{125}\text{I}$ -IGF2 ( $\sim 10^8$  cpm/ $\mu\text{g}$ ) were kind gifts of J. A. Fischer (University of Zurich, Switzerland) and C. Torresani and E. Froesch (University of Zurich, Switzerland), respectively. Bovine pancreatic insulin was obtained from Sigma Chemical Co. (St. Louis, MO).

In neurite outgrowth experiments, insulin and insulin-like growth factors were diluted 20–100-fold from 0.1% BSA (Sigma Chemical Co.; RIA grade) containing stock solutions into cell-containing wells. Neurite outgrowth was evaluated from photographs of living cultures.

In binding experiments, 0.1 nM  $^{125}\text{I}$ -IGF2 (specific activity  $\sim 300$   $\mu\text{Ci}/\text{nmol}$ ) was added to 2-d-old cultures. Binding was for 1 h at  $37^\circ\text{C}$ ; cultures were then rinsed three times with medium and fixed at  $37^\circ\text{C}$  for 1 h with 3% glutaraldehyde in PBS. Dishes with fixed and labeled cells were then dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), exposed for 7 d, developed, and examined for silver grains under dark-field illumination.

### *In Vivo* Experiments

3-mo-old BALB/c mice (respectively, Lewis rats) were anesthetized with ether and injected subcutaneously with 100–200  $\mu\text{l}$  of 0.1% BSA in PBS with (IGF-exposed) or without (vehicle) 100 ng of hormone. Injections were applied directly on top of the gluteus muscle and special care was taken not to contact the underlying tissue with the injection needle. Routinely, one gluteus was exposed to hormone while the contralateral muscle was exposed to vehicle alone.

At the end of the injection protocols animals were killed with ether, and glutei were removed and immediately processed. Before removal, muscles were carefully examined for signs of lesions. The latter were observed in  $\sim 2\%$  of the animals. Such animals were not considered in the studies described below. Each experiment was routinely performed on at least three animals. Controls included noninjected contralateral glutei, as well as non-treated animals of the same age. Glutei from hormone-treated animals were macroscopically indistinguishable from untreated muscles.

Botulinum paralysis was produced by subcutaneous injection of 500 pg (mice) or 5 ng (rats) of Botulinum toxin A (Sigma Chemical Co.) on the gluteus muscle. Paralysis under these experimental conditions developed after 24–48 h and was restricted to one leg. Glutei were collected 7 d after the paralyzing injection.

### Histological Techniques

To monitor nerve sprouting, glutei were fixed and stained for 16 h at room temperature in a freshly prepared unbuffered zinc iodide solution, mixed 1:4 with osmium tetroxide (ZIO) (Akert and Sandri, 1968). Muscles were then rinsed in  $\text{H}_2\text{O}$ , fibers were carefully teased under the microscope, positioned on gelatin-coated cover slips, dehydrated, and mounted for microscopy. The fasciae was left on the muscles during ZIO staining to minimize precipitate formation on the muscle. Mounted stained muscles were systematically screened for signs of sprouting. Thin, unmyelinated, intensely stained processes of  $>5$   $\mu\text{m}$  were classified as sprouts. Sprouts emerged from either end plates (terminal sprouting), or from nerve in the vicinity of end plates (sprouts whose origin could not be traced to end plates were classified as nodal sprouts).

Cell division was compared in treated and in control mouse muscles, by injecting twice intraperitoneally 50  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (methyl  $-^3\text{H}$  thymidine, 20 Ci/mmol; New England Nuclear, Boston, MA). Injections were applied 24 and 3 h before the mice were killed. The first thymidine injection was 3 h after the last subcutaneous hormone injection. Muscles were fixed in 4% paraformaldehyde in PBS and 11  $\mu\text{m}$  cryostat sections were cut. Emulsion (NTB-2; Eastman Kodak Co.) exposure was for 14 d and developed sections were counterstained with cresyl violet. Sections were examined under bright-field illumination and labeled nuclei were counted.

For EM, animals were perfused with 1% paraformaldehyde and 2.5% glutaraldehyde in 100 mM sodium phosphate, pH 7.4. Glutei were dis-

sected, postfixed in 1% phosphate buffered (0.1 M, pH 7.4) OsO<sub>4</sub> for 2 h at room temperature, dehydrated, and embedded flat in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined on a microscope (EM 201; Philips Electronic Instruments Inc., Mahwah, NJ).

### **Detection of Muscle Components by Immunological and Ligand Binding Techniques**

GAP43 levels in rat gluteus muscle were detected on Western blots of Polytron-homogenized tissue (in 160 mM NaCl, 20 mM Hepes, 5 mM EDTA, 100 U/ml trasylol, 0.2 mM  $\beta$ -mercaptoethanol, pH 7.4). 200  $\mu$ g of muscle protein were applied per gel lane (10% SDS-PAGE) and separated proteins were transferred in the presence of 0.05% SDS as described by others (Jacobson et al., 1986). GAP43 was detected using mouse antiserum produced against gel-purified rat GAP43. The antiserum was highly specific for GAP43 on Western blots, and produced the characteristic neuron-specific staining pattern on cryostat sections and on fixed permeabilized cultured cells, as described by others (see, e.g., Benowitz and Routtenberg, 1987). 11- $\mu$ m cryostat sections of 4% paraformaldehyde-fixed tissue were incubated for 5 h at room temperature in the presence of 0.5% NP-40 and 1:100-fold dilutions of the mouse anti-rat GAP43 antiserum in 160 mM NaCl, 20 mM Hepes, 1% BSA, pH 7.4. Bound antibody was detected with FITC-labeled sheep anti-mouse (Serotec, Oxford, UK). In some experiments 1:200-fold diluted rabbit anti-rat neurofilament protein antiserum (kind gift of A. Matus, FMI, Basel, Switzerland) was added together with anti-GAP43. Bound antineurofilament antibody was detected with rhodamine isothiocyanate-labeled goat anti-rabbit (Cappel Laboratories, Malvern, PA). In control experiments each first antibody was omitted separately to verify signal specificity.

Antibodies to N-CAM and J1 (antiserum recognizing tenascin (J1) and J1-160/180 [Gatchalian et al., 1989]) were a kind gift of M. Schachner, Zürich, Switzerland. To detect N-CAM in muscle, 11- $\mu$ m cryostat sections of frozen tissue were processed as described by Sanes et al. (1986). Antibodies were diluted 1:100 in 160 mM NaCl, 20 mM Tris, 1% BSA, pH 7.4. J1 was detected similarly, but on 11- $\mu$ m cryostat sections of 4% paraformaldehyde-fixed tissue. In both cases, bound antibodies were detected with appropriate FITC-labeled second antibodies.

Acetylcholine receptor (AChR) clusters were detected in teased fiber preparations by incubation with FITC- $\alpha$ bungarotoxin (Sigma Chemical Co.; 2  $\mu$ g/ml in 160 mM NaCl, 10 mM Tris, 1% BSA, pH 7.4) as described (Rieger et al., 1985).

## **Results**

### **Insulin-like Growth Factors Induce Neurite Growth from Embryonic Motoneurons In Vitro**

E6 chick spinal cord motoneurons were cultivated on laminin-coated substratum in the presence of 20  $\mu$ g/ml of E19 chick muscle protein extract (see Materials and Methods). Under these conditions, neurons rapidly extended thick processes which tended to fasciculate. One such culture after 4 d in vitro is shown in Fig. 1 *a*. When concentrations of IGF2 as low as 0.1 nM were added to the culture medium, substantial enhancement of neurite growth, as detected by total neurite length and number of branching points per neurite, was observed (Fig. 1 *b*). Half-maximal effects were observed at  $\sim$ 0.2 nM IGF2 (Table I), whereas concentrations of IGF2 higher than 5 nM appeared to have toxic effects on the neurons. Typical responses of the neurons to increasing concentrations of IGF2 in the medium are shown in Fig. 1, *b-d*. Neurite growth effects of 0.5 nM IGF2 were quantitated in low density cultures after 1 d in vitro (Table I). Data with and without the addition of hormone were each obtained by examination of 500 neurons from three independent experiments. IGF2 could be substituted with IGF1, and both somatomedins displayed similar dose-response effects. Insulin was much less effective in producing neurite growth and half-maximal effects of insulin were observed at concentrations

of  $\sim$ 20 nM. A similar behavior has been observed for chick sensory neurons in vitro (Recio-Pinto et al., 1986), and suggests the involvement of an IGF1 receptor in mediating the neurite outgrowth response (Rechler and Nissley, 1985). Neurite growth stimulation by somatomedins was rapid, independent of culture density, and appeared to require the constant presence of hormones: obvious effects were observed after 12 h in vitro, and removal of the hormones after 1 d in vitro appeared to prevent further stimulated neurite growth (data not shown). Also, addition of hormone to a 2-d-old culture rapidly (unambiguous detection after 12 h) induced additional neurite growth.

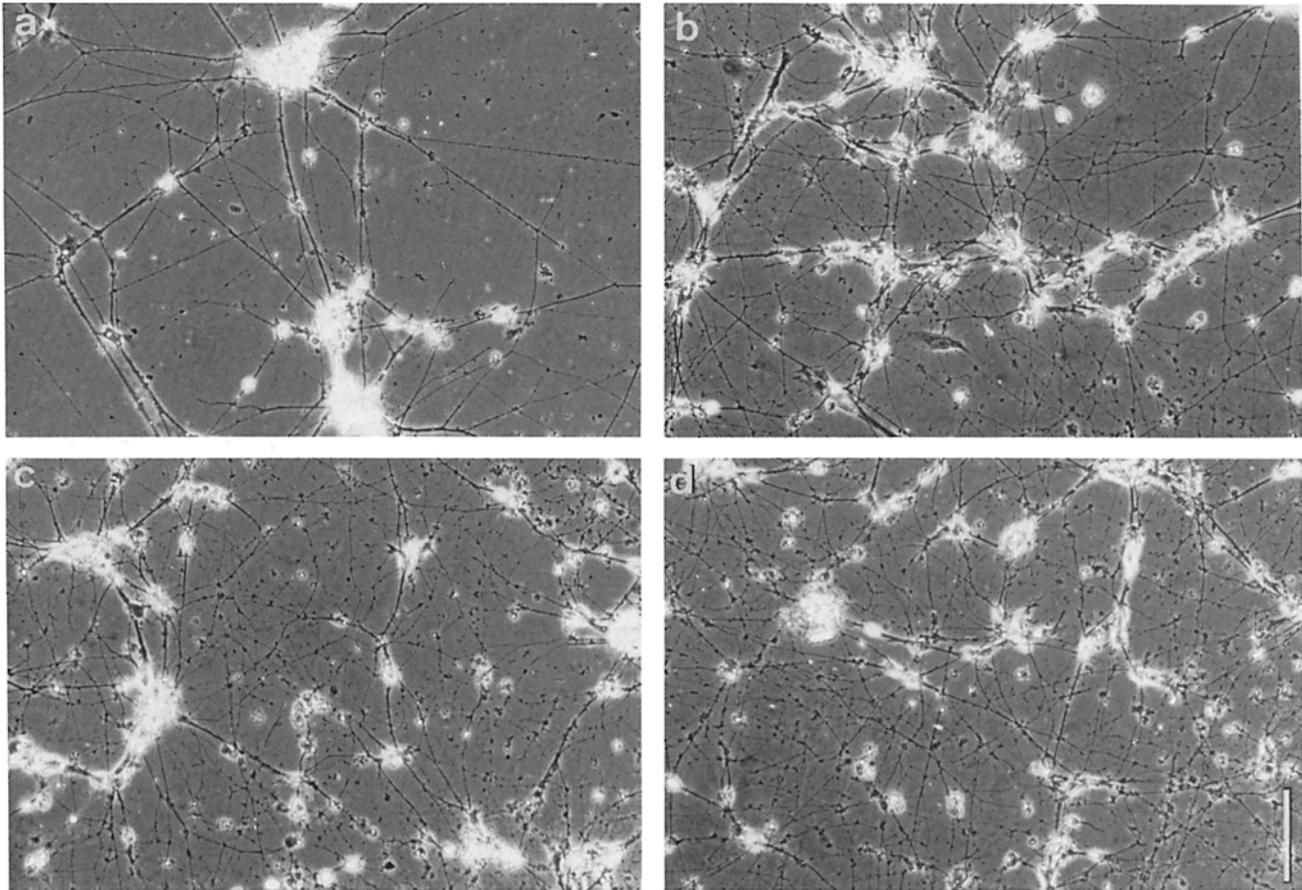
While these observations ruled out an indirect effect of the IGFs due to induction of elevated numbers of nonneuronal cells, stimulated production of neurite outgrowth-inducing factors by contaminating cells could not be excluded. We therefore determined whether embryonic motoneurons in vitro possessed high affinity binding sites for IGFs. Cultures were incubated for 1 h in the presence of 0.1 nM of <sup>125</sup>I-IGF2, washed, fixed, and treated for in situ autoradiography. As shown in Fig. 2, neurons in the culture bound IGF2. As was expected from the widespread distribution of IGF receptors (Rechler and Nissley, 1985), nonneuronal cells in the culture also bound hormone. Since essentially all neurons in the culture bound hormone, and since >90% of the neurons were motoneurons (see Materials and Methods), it is concluded that in vitro motoneurons could bind hormone. Binding was specific, as it could be displaced by higher concentrations of cold IGF2 or IGF1. 10 nM of unlabeled IGF2 or IGF1 produced a detectable reduction (by an estimated 50%) in the density of label observed on thin neurites, while insulin was without effect at this concentration (data not shown). Significantly, binding sites appeared to be present on the entire surface of the neurons, including the fine endings of neuronal processes (Fig. 2, *c* and *d*), consistent with a possible local mode of action of the IGFs on neurite growth.

From these observations we conclude that the potent and rapid induction of neurite growth from embryonic motoneurons in vitro by IGF1 and IGF2 is likely to be due to the direct activation of IGF1 receptors on the motoneurons.

### **Sprouting of Adult Intramuscular Nerves in the Presence of Insulin-like Growth Factors**

We have determined whether the addition of IGF2 or of IGF1 to otherwise untreated adult muscle is sufficient to induce intramuscular nerve sprouting. We selected the mouse (and rat) gluteus muscle as an experimental system, since it is located superficially and is only four to five muscle fibers across. This should permit nonlesioning subcutaneous application of substances under favorable diffusion conditions. The data shown in Table II demonstrate that IGF1 efficiently diffused into and through gluteus muscle under our experimental conditions. Contralateral muscle remained essentially free of added hormone. The data also show that adult mouse gluteus muscle specifically removed injected IGF1, but not reduction-inactivated hormone. Therefore, our subcutaneous injection procedure exposed gluteus muscle locally to exogenous IGFs.

Daily subcutaneous injections of 100 ng of IGF2 in 100  $\mu$ l of PBS with 0.1% BSA on the gluteus muscle of 3-mo-old mice produced marked terminal and nodal sprouting (Fig. 3,



**Figure 1.** IGF2-stimulated neurite growth from E6 chick spinal cord motoneurons in vitro. Neurons were plated on laminin-coated polyornithine in the presence of 10% horse serum and 20  $\mu\text{g}/\text{ml}$  E19 chick muscle extract. Representative sections of the cultures were photographed after 4 d in vitro. Quantitative data on IGF-induced neurite growth in vitro are presented in Table I. Additions to the culture medium were (a) no additions; (b) 0.15 nM IGF2; (c) 0.3 nM IGF2; and (d) 0.5 nM IGF2. Bar, 100  $\mu\text{m}$ .

Table III). Control contralateral muscles were injected with vehicle and were indistinguishable from muscles in untreated animals. Sprouting was detected by ZIO staining, a method particularly suited for detection of thin unmyelinated

**Table I.** IGF-stimulated Neurite Growth in Embryonic Motoneuron-enriched Cultures

Hormone added to culture medium	Neurite length* (process branching points <sup>‡</sup> ) per neuron		
	IGF1	IGF2	Insulin
nM	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$
none	42.4 (0.28)	—	—
0.1	67.6 (0.47)	63.5 (0.42)	—
0.3	118.3 (0.67)	101.4 (0.71)	40.8 (0.26)
0.5	151.2 (1.10)	135.2 (1.16)	—
2	158.8 (1.25)	151.7 (1.45)	59.2 (0.33)
10	95.3 (0.80)	102.5 (0.71)	85.1 (0.75)

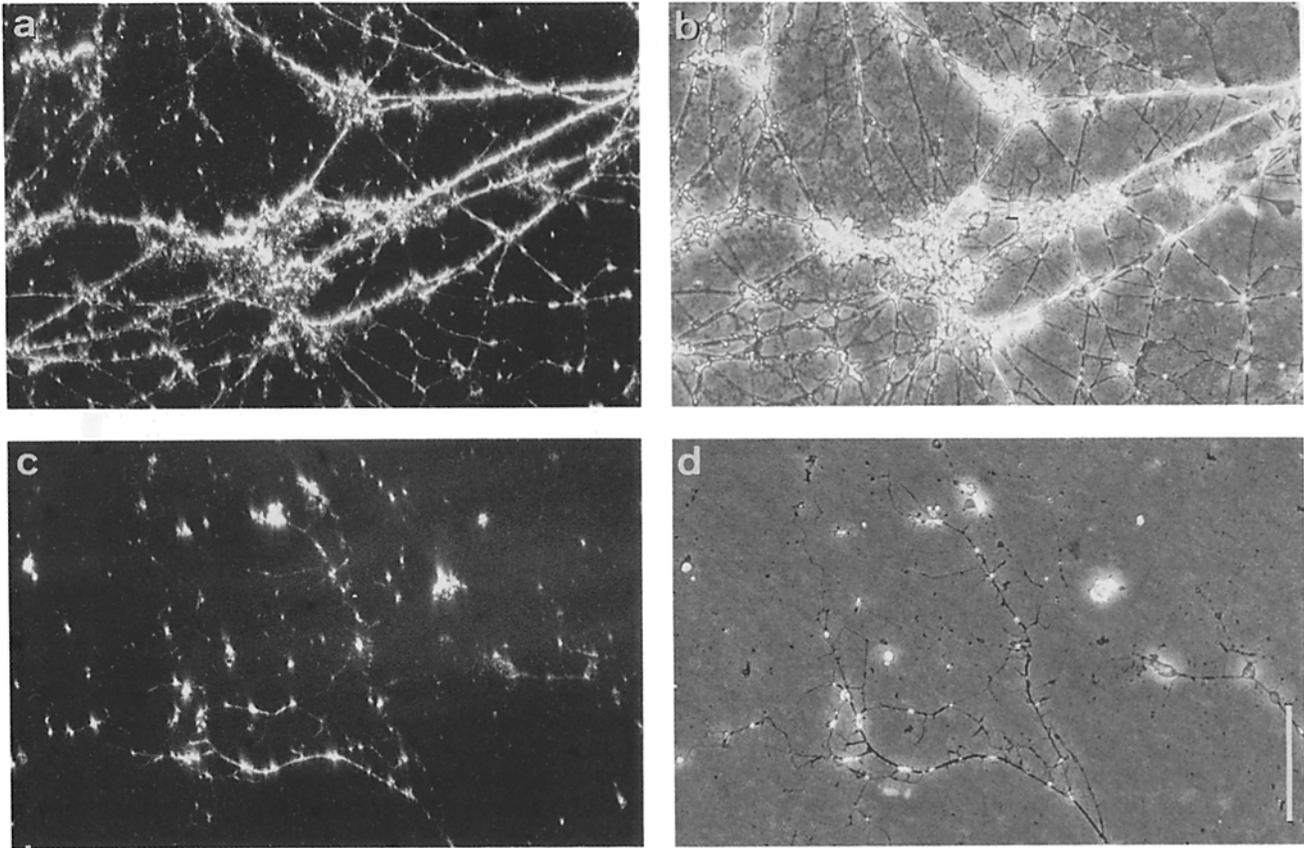
Low density cultures were photographed after 1 d in vitro. Fields of comparable cell density were selected and all neurons with their processes within the field were scored.

\* The total length of the neuritic tree was determined for each neuron and the mean value is given. A total of  $\sim 500$  neurons from three independent experiments was considered for each experimental point given in the table. Standard errors varied between 15 and 30%.

‡ The total number of process branching points per neuron was determined and a mean value is given in the table (values in parentheses). Mean values  $< 1$  were due to the absence of branching points in several neurons after 1 d in vitro. Sample size was as described above and standard errors varied between 12 and 25%.

nerve processes (see, e.g., Holland and Brown, 1981). Short sprouts could already be detected after 3 d of treatment, and were well developed after 1 wk. At this time, sprouts could be observed to originate from  $12.2 \pm 1.5\%$  of the end plates (Table III). Terminal sprouting in vehicle-injected contralateral muscle was observed in  $2.7 \pm 0.8\%$  of the end plates. This value was similar to untreated control muscles. Numerous sprouts were found to branch from nerves in the vicinity of end plates. In mouse gluteus maximus after 7 d of treatment, the total number of nodal sprouts, i.e., of thin unmyelinated processes of more than 5  $\mu\text{m}$  not emerging from end plates, were  $182 \pm 28$  for the IGF2-exposed muscle and  $15 \pm 7$  for the contralateral vehicle-injected muscle (Table III). Sprouts in IGF2-treated muscles increased in length with time. After 7 d of treatment, sprouts  $> 50 \mu\text{m}$  long were frequently ( $> 40\%$  of times) observed. Varicosities were common and the processes often followed a convoluted path. Similar results were obtained when IGF1 was applied instead of IGF2.

Intramuscular nerve growth in the presence of insulin-like growth factors was also detected indirectly by monitoring GAP43 levels in treated muscles. GAP43 is a nerve-specific, intracellular, growth-associated protein, whose levels in axons strongly correlate with axonal growth phases (for reviews see Benowitz and Routtenberg, 1987; Skene, 1989). Adult levels of GAP43 in peripheral nerves are low, and regeneration of peripheral nerve lesions has been shown to be



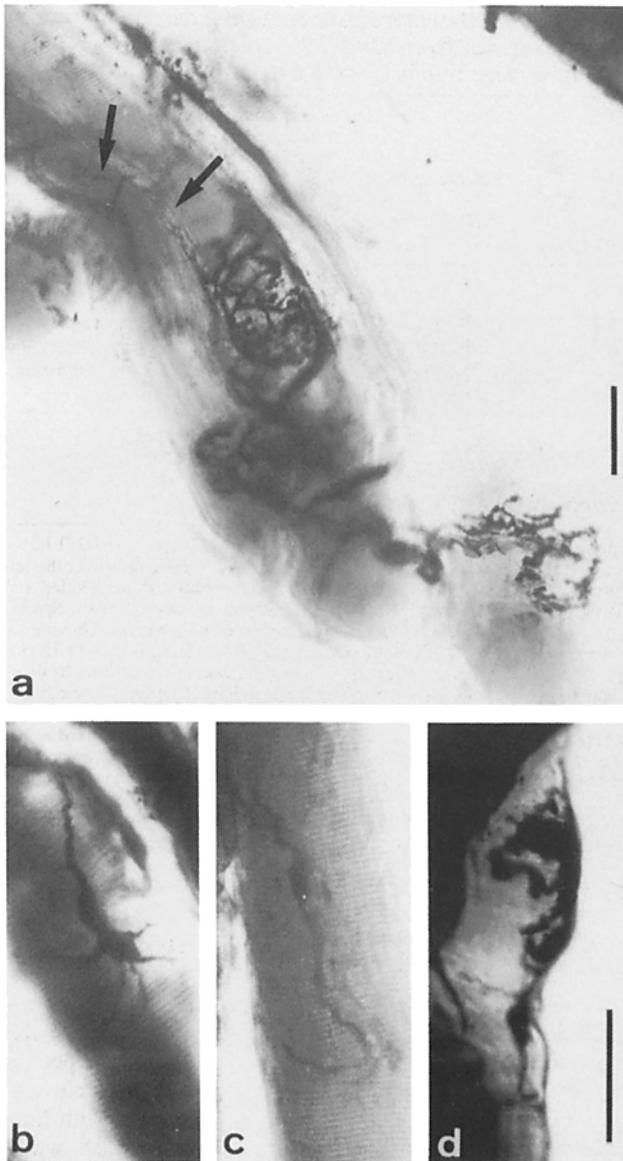
**Figure 2.** Binding of  $^{125}\text{I}$ -IGFs by the processes of embryonic chick motoneurons in vitro. E6 spinal cord motoneurons (3 d in culture) were exposed to 0.1 nM of  $^{125}\text{I}$ -IGF2 ( $\sim 10^8$  cpm/ $\mu\text{g}$  of protein) for 1 h. Cultures were exposed to emulsion for 1 wk. (a and c) Dark-field; (b and d) corresponding phase-contrast images. (a and b) Central part of the culture; and (c and d) IGF2 binding to fine processes of isolated neuron (cell body to lower right). The selected fields are representative and virtually all neurons in the culture bound  $^{125}\text{I}$ -IGF2. Bar, 100  $\mu\text{m}$ .

**Table II. Local Diffusion and Specific Removal of Subcutaneously Injected  $^{125}\text{I}$ -IGF1 in Adult Muscle**

	6 h		12 h		24 h	
	Native	Reduced	Native	Reduced	Native	Reduced
	<i>cpm</i> $\times 10^{-3}$					
<b>Ipsilateral</b>						
Gluteus maximus	350	800	85	700	80	750
Underlying hip muscle	110	600	15	550	12	700
<b>Contralateral</b>						
Gluteus maximus	<1	<1	<1	<1	<1	<1
Underlying hip muscle	<1	<1	<1	<1	<1	<1
<b>Liver</b>	650	200	105	65	55	50

Subcutaneously injected  $^{125}\text{I}$ -IGF1 efficiently diffused into and through gluteus maximus muscle. Hormone that had been inactivated by boiling under reducing conditions was not removed from muscle, suggesting that decline of native hormone levels was due to specific receptor-mediated binding.  $^{125}\text{I}$ -IGF1 (100 ng in 100  $\mu\text{l}$  of vehicle;  $\sim 5 \times 10^6$  cpm of protein-incorporated counts) was injected subcutaneously on top of the gluteus muscle of 3-mo-old mice. IGF1 was reduced by boiling iodinated protein in the presence of  $\beta$ -mercaptoethanol. Unfixed tissues were dissected and radioactivity was quantitated directly in portions of approximately equal volume. All portions from each tissue were counted to determine the tissue-associated radioactivity. Extraction experiments confirmed that >90% of counts in muscle 6 h after injection were precipitable with TCA. Data are the average of two animals per time point.

accompanied by elevated levels of this axonal protein (e.g., Verhaagen et al., 1988). After 7 d of daily subcutaneous IGF2 injections, gluteus maximus GAP43 levels were markedly elevated when compared to contralateral vehicle-injected muscle (Fig. 4). No differences were detected between contralateral vehicle-injected muscles, and muscles from untreated animals. Elevated GAP43 immunoreactivity in IGF-exposed muscles was detected in regions that specifically bound antineurofilament antibody, a finding consistent with the neuron-specific distribution of GAP43 (Fig. 4). IGF-induced elevated GAP43 immunoreactivity was much more prominent in thin nerve profiles than in large intramuscular nerves, where changes were difficult to detect. This might reflect the rapid transport of GAP43 out of cell bodies and through axons, and its retention in growing regions of nerves (Skene, 1989). In experiments aimed at producing a strong sprouting reaction in the absence of injury, Botulinum toxin was injected subcutaneously over adult rat gluteus muscle. The toxin causes paralysis and intramuscular sprouting by blocking acetylcholine release at the neuromuscular junction. Gluteus muscle GAP43 levels 7 d after Botulinum toxin treatment were elevated with respect to the ones in control untreated muscles (Fig. 4), demonstrating that this sprouting reaction is accompanied by biochemically detectable changes in intramuscular nerves. Significantly, GAP43 levels in IGF-exposed muscles were consistently higher than



**Figure 3.** Sprouting in adult mouse gluteus muscle exposed to IGF2. 100 ng of IGF2 was injected subcutaneously on top of 3-mo-old mouse gluteus muscle, each day for 7 d. Dissected gluteus maximus muscle was stained in Z10 solution and fibers were subsequently teased in small bundles. Terminal sprouting (*a*, arrow; *d*) was observed from ~10% of the end plates. Sprouts often appeared to emerge from nerves in the vicinity of end plates and might represent nodal sprouts (*b* and *c*). Corresponding quantitative data are given in Table III. Bars, 30  $\mu$ m.

in toxin-paralyzed muscle, possibly reflecting a direct inductive effect of IGFs on motoneuron GAP43.

We conclude that insulin-like growth factors can induce intramuscular sprouting in adult, otherwise intact muscles, and that the sprouting reaction is accompanied by elevated levels of intramuscular nerve-specific growth-associated protein GAP43.

#### **Additional Reactions in IGF-treated Adult Skeletal Muscle**

Insulin-like growth factors can produce mitogenic and differ-

entiation effects on essentially all intramuscular cells (Nissley and Rechler, 1984; Shimizu et al., 1986) and as a consequence, the sequence of events leading to IGF-induced intramuscular sprouting is difficult to establish. Our attempts to unambiguously demonstrate high affinity uptake and retrograde transport of  $^{125}$ I-IGFs from muscle to motoneuron cell bodies have so far failed. Therefore, while a direct mechanism of action could not be further substantiated, we have characterized the IGF-induced sprouting reaction in terms of its correlation with possible inflammatory reactions and with proliferation reactions in the muscle. We also examined treated muscles for additional characteristic reactions known to be associated with functional denervation.

It is well established that muscle lesions and intramuscular inflammatory reactions can cause nerve sprouting in adult muscle (Jones and Vrbova, 1974; Brown et al., 1978). For this reason, particular caution was exerted to avoid lesioning of gluteus muscle during subcutaneous injections. However, diluted IGF-containing solutions had to be supplemented with carrier protein to minimize adsorption losses. Invading monocytes were frequently observed on the external surface of the muscle fasciae, probably as a consequence of co-injected BSA (Table IV). These monocytes, however, did not penetrate the muscle tissue, thus confirming the nonlesioning properties of the drug application protocol. Activated macrophages have been shown to secrete a number of growth factors and it was important, therefore, to determine whether such a mechanism might explain the observed sprouting reaction. As shown in Table IV, invading mononucleated cell numbers varied among different muscles examined in a manner that was independent of the presence of IGF1. Induction of sprouting, however, was consistently and exclusively observed when insulin-like growth factors were included in the injection mixture. The mobilization of mononucleated cells is therefore not correlated with the observed sprouting reaction.

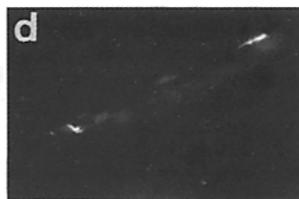
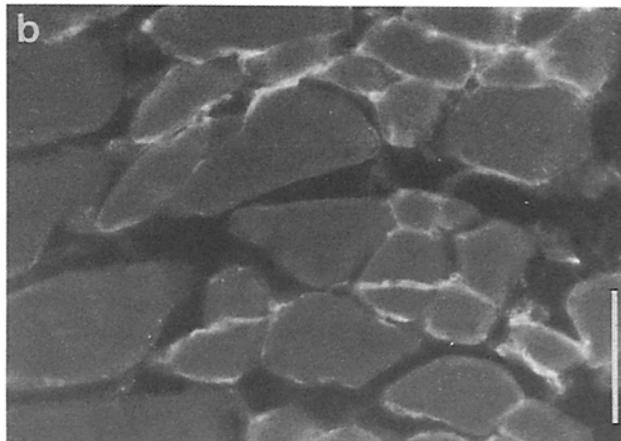
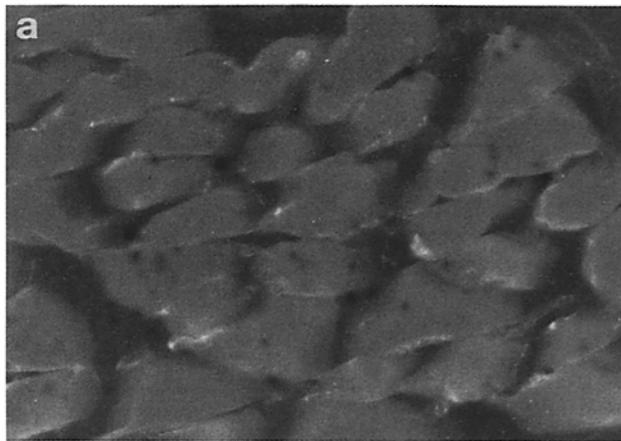
We also examined treated muscles for signs of cell division using  $^3$ H-thymidine incorporation. As shown in Table IV, induction of cell proliferation in treated muscles could be detected. The major zone of cell proliferation in treated muscles was found at the level of the mobilized superficial mononucleated cells, where cell division appeared to be independent of added IGFs. Also, the number of invading cells

**Table III. Nerve Sprouts in Adult Mouse Gluteus Muscle after Local Injections of IGF2**

	End plates with sprouts	Sprouts not traced to end plates*
	%	
IGF2	12.2 $\pm$ 1.5	182 $\pm$ 28
Vehicle	2.7 $\pm$ 0.8	15 $\pm$ 7
Untreated animals	2.4 $\pm$ 0.7	11 $\pm$ 5

3-mo-old mice were subjected to daily subcutaneous injections of IGF2 over one gluteus muscle and of vehicle over the contralateral muscle. After 7 d animals were killed and muscles were stained with Z10 as described in Materials and Methods. Teased fibers were mounted, and slides were coded and examined for thin unmyelinated processes longer than 5  $\mu$ m, which were scored as sprouts. After examination slides were decoded and average values were calculated. Sample sizes: muscles from eight treated animals and from three untreated animals were processed; number of analyzed end plates: IGF2, 458; vehicle, 357; untreated, 65.

\* Average number per muscle.



**Figure 4.** Elevated levels of neuron-specific growth-associated protein GAP43 in IGF1-exposed adult rat gluteus muscle. After 7 d of daily IGF1 (*b*) or vehicle (*a*) injections dissected muscles from the same animal were fixed and 11- $\mu$ m cryostat sections were reacted with specific antiserum to rat GAP43. While GAP43 was essentially undetectable in untreated and contralateral vehicle-injected muscle, nerve staining could be observed in IGF1-exposed muscle. The GAP43 immunoreactivity in IGF-exposed muscle (7 d) was associated with nerves; this is supported by double-staining experiments

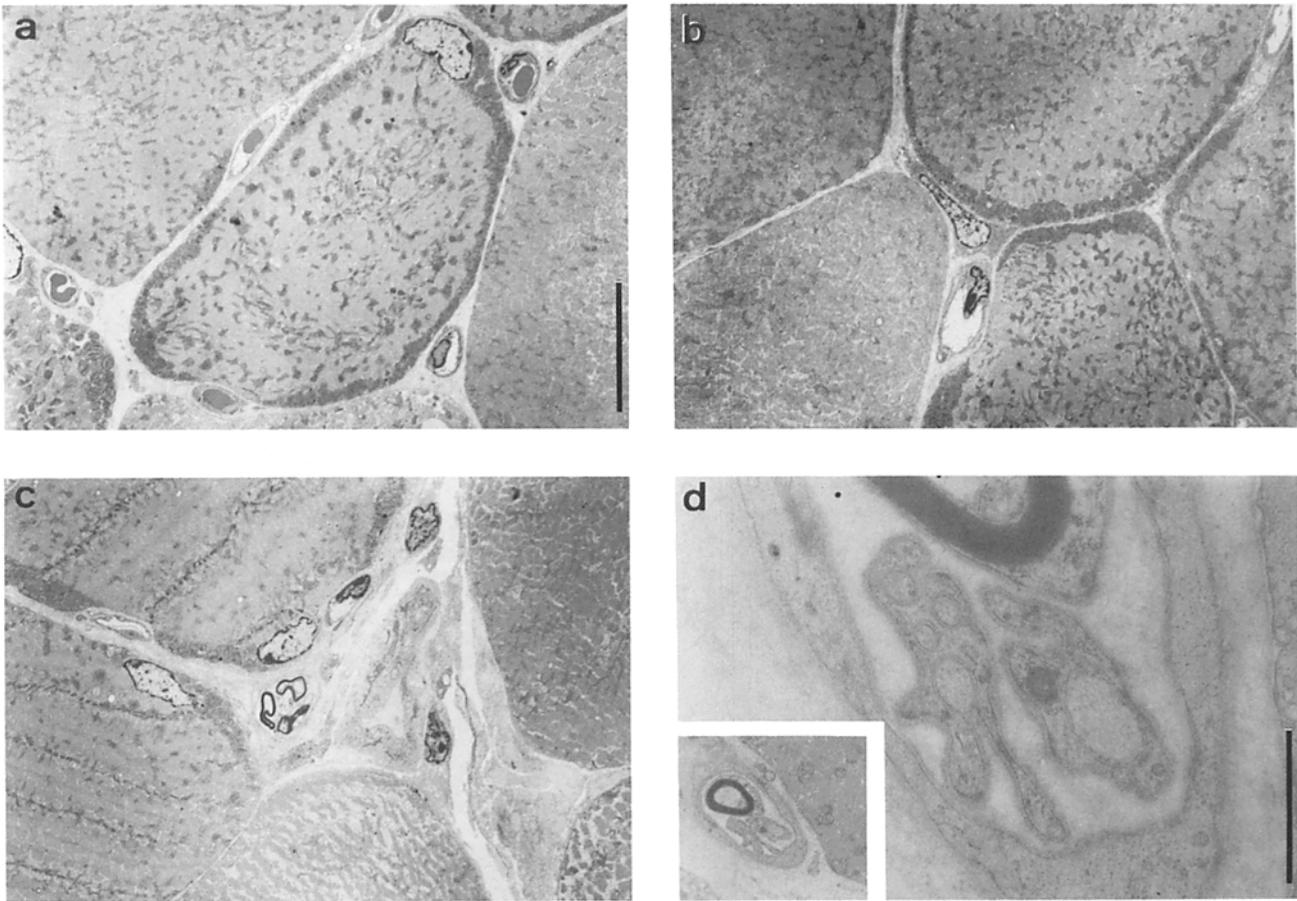
**Table IV.** Intramuscular Nerve Sprouting and Proliferation of Intramuscular Interstitial Cells Are Specifically Induced by Insulin-like Growth Factors

Duration of IGF1 treatment	Nerve sprouting		Intramuscular interstitial cells incorporating thymidine		Mononucleated cells adhering to muscle fasciae	
	IGF side	Vehicle side	IGF side	Vehicle side	IGF side	Vehicle side
			%	%		
4 d	+	-	0.8	0.6	++	++
7 d	++	-	4.5	0.6	++	+
7 d	++	-	3.8	0.6	$\pm$	+++
14 d	++	$\pm$	6.1	0.7	++	+
No injections 1	-		>0.5		-	
No injections 2	-		>0.5		-	

3-mo-old mice received daily subcutaneous injections of 100 ng of IGF1 in vehicle over the right gluteus muscle (IGF side) and of vehicle only over the left gluteus muscle (vehicle side). After indicated times animals were killed and gluteus maximus examined. Each horizontal line represents one animal. Sprouting (-) <8 thin unmyelinated processes of >5  $\mu$ m (defined in the following as sprouts) detected per 30% of gluteus muscle; ( $\pm$ ) 8-15 sprouts; (+) 15-50; and (++) >50 sprouts. Nuclei with silver grain counts higher than 20 times background were counted as  $^3$ H-thymidine incorporating cells. Muscle fiber-associated nuclei, as well as nuclei obviously belonging to nerve or blood vessels, were not counted and remaining intramuscular nuclei were defined as belonging to interstitial cells. About 500 interstitial nuclei were examined per animal. Small round, fasciae-adhering cells were intensively stained by cresyl violet and were collectively classified as mobilized mononucleated cells. About 15% of these cells incorporated thymidine, irrespective of IGF1 presence. Approximate cell numbers were (-) <10 per mm of fasciae; (+) 11-30; (++) 31-60; and (+++) 61-150.

apparently had no effect on thymidine incorporation by intramuscular cells. No attempt was made to identify intramuscular mitotic cells in the presence of IGF1, although a significant proportion of these cells appeared to have the characteristic elongated morphology of fibroblasts. Specific stimulation of cell division by IGF1 in gluteus muscle was moderate: ~5% of interstitial cells were labeled with hormone, 0.5% were positive in the absence of hormone, while up to 20% of superficial mobilized cells incorporated significant levels of  $^3$ H-thymidine (Table IV) under our label-

with specific antineurofilament antibody. (*c* and *d*) IGF exposed muscle; (*e* and *f*) corresponding vehicle-exposed muscle; (*c* and *e*) antineurofilament; and (*d* and *f*) anti-GAP43. These findings were confirmed on Western blots of gluteus muscle protein. As a positive control, Botulinum toxin paralyzed muscle (*1*), i.e., muscle in which a substantial sprouting reaction is known to occur, contained elevated levels of GAP43, when compared to control, vehicle-injected muscle (*3*). IGF1-exposed muscle (*2*) reproducibly contained markedly elevated GAP43 levels (about sixfold higher, by comparison of different exposure times), thus confirming that elevated levels of intramuscular IGFs lead to nerve growth-associated reactions in otherwise intact adult skeletal muscle. Equal amounts of muscle protein were applied to each gel lane, whereas 200 times less newborn rat brain homogenate protein was applied to the control lane (*4*). Note that GAP43 contents in IGF-treated muscle were more than a thousand times lower than in newborn rat brain, consistent with the neuron-specific localization of GAP43. Bars, 50  $\mu$ m. Bar in *b* applies to *a* and *b*; bar in *f* applies to *c-f*.



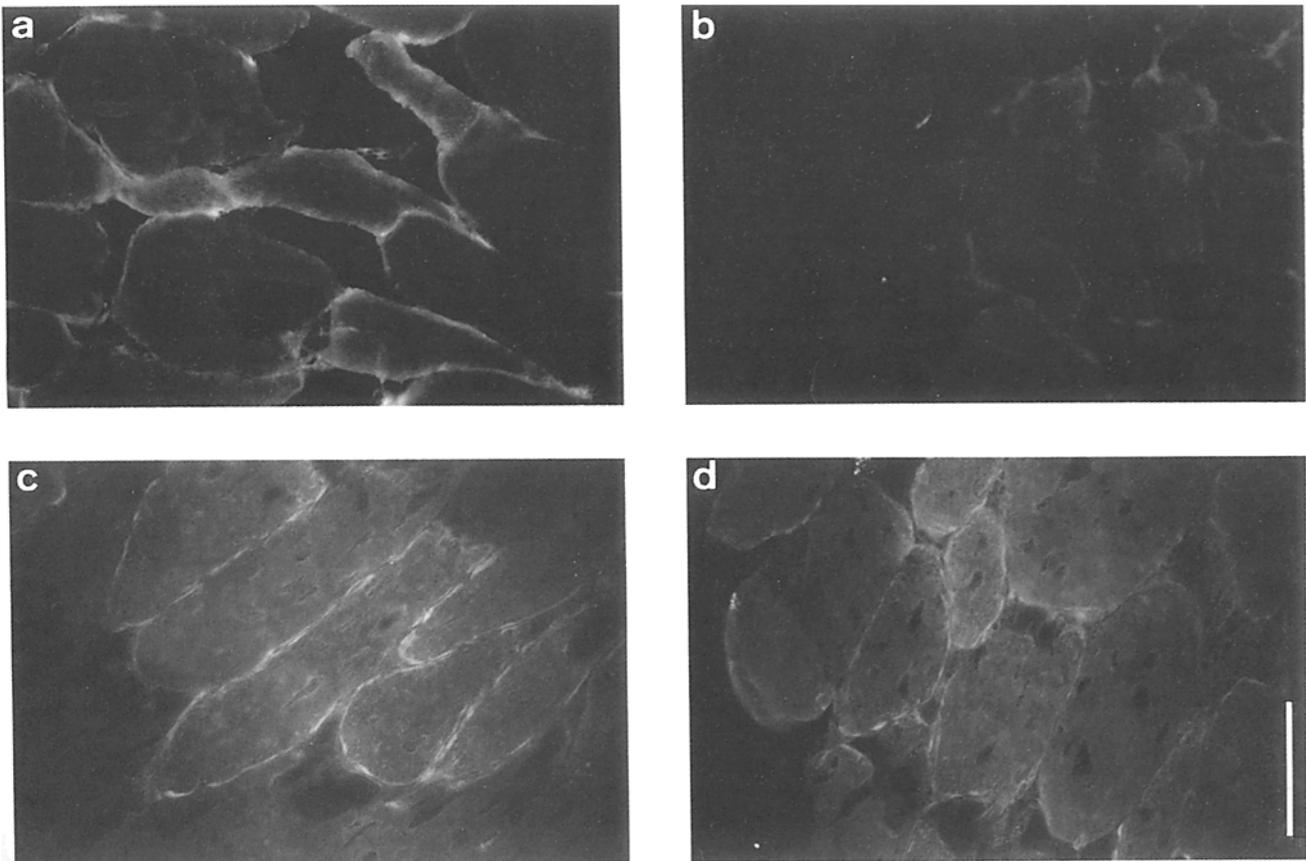
**Figure 5.** Interstitial spaces in IGF-exposed gluteus muscle. Width of interstitial spaces in mouse gluteus muscle exposed to daily injections of IGF1 for 14 d (*a*, *b*, and *d*), was not significantly different from vehicle-injected control muscle (*c*). At higher magnification, IGF-exposed muscle displayed about twice as many fibroblasts, with apparently more extended processes. In addition, unmyelinated nerve profiles of more than 0.4  $\mu\text{m}$  of diameter were seen more frequently in IGF-exposed muscle, in particular within perineurium sheets (*d*). The latter nerve profiles might represent nodal sprouts (see also Hopkins and Slack, 1981). (*a*–*c*) Same magnification; bars: (*a*) 5  $\mu\text{m}$ ; (*d*) 2  $\mu\text{m}$ .

ing conditions. The latter incorporation levels were hormone independent. In keeping with the observed low levels of stimulated mitosis, electron microscopic examination of gluteus muscle sections did not reveal major hormone-induced histological changes (Fig. 5): width of extracellular spaces in hormone-injected muscles did not differ significantly from control and no elevated mast cell, or macrophage levels could be detected. Four sections of IGF-exposed and vehicle-exposed muscle for each of six experimental animals were systematically examined. Half of the animals for EM were treated for 7 d, the other half for 14 d. Observed changes in IGF-injected muscles were a reproducible increase in unmyelinated, Schwann cell-associated axonal profiles (e.g., Fig. 5 *d*), and an approximately twofold increase in fibroblast numbers. In addition, a distinct increase in the mean diameter (minor axis of ellipsoid) of unmyelinated axonal profiles in intramuscular nerves was observed in gluteus muscles exposed to IGF1 for 14 d. Mean diameters were  $0.32 \pm 0.05$  (45 axons, 3 animals) in control muscle and  $0.68 \pm 0.11$  (68 axons, 3 animals) in contralateral IGF-exposed muscles. Similar changes have been observed in partially denervated mouse gluteus maximus muscle (Hopkins and Slack, 1981; Hopkins and Brown, 1982), where the appearance of

large unmyelinated axonal profiles was attributed to the presence of sprouts.

In the final part of this study we examined the distribution of three muscle components that have been shown to undergo profound changes after denervation or muscle paralysis. These components are N-CAMs, tenascin, and AChR. Levels of the cell adhesion molecule N-CAM are dramatically elevated upon denervation of adult muscle and changes in N-CAM correlate with the susceptibility of muscle to innervation (Sanes et al., 1986). Elevated levels of N-CAM are found on denervated myofibers, and are expressed on the surface of regenerating nerves (Rieger et al., 1985; Covault and Sanes, 1985). This expression creates favorable conditions for the growth of neurites on muscle fibers (Bixby et al., 1987). In addition, dividing fibroblasts contribute modestly to the elevated interstitial N-CAM in denervated muscle (Gatchalian et al., 1989). We failed to observe substantial elevation of N-CAM in IGF-treated gluteus muscle (Fig. 6), and only interstitial N-CAM, if at all, was elevated.

Levels of tenascin (J1) (Chiquet and Fambrough, 1984; Chiquet-Ehrismann et al., 1986) are markedly elevated in interstitial spaces in the vicinity of denervated end plates (Sanes et al., 1986), due to local stimulation of fibroblast prolifera-



**Figure 6.** Elevated levels of J1 immunoreactivity in IGF-treated adult muscle. The gluteus muscles of a rat were exposed to IGF1 (*a* and *c*) on one side and to vehicle (*b* and *d*) on the contralateral side for a total of 7 d. J1 immunoreactivity was essentially undetectable in vehicle-injected contralateral muscle (*b*), while interstitial immunoreactivity, apparently associated with connective tissue could be detected in IGF1-exposed muscle (*a*). N-CAM immunoreactivity in IGF1-exposed muscle (*c*) was only marginally elevated with respect to vehicle-exposed muscle (*d*). Bar, 50  $\mu\text{m}$ .

tion (Gatchalian et al., 1989). We also found significant elevation of J1 immunoreactivity in IGF-treated muscle (Fig. 6). This finding correlates qualitatively and quantitatively with the extent of interstitial cell proliferation we observed in IGF-treated muscle, particularly when compared to the corresponding reactions in denervated muscle (Murray and Robbins, 1982; Connor and McMahan, 1987; Gatchalian et al., 1989). It seems plausible to conclude, therefore, that exposure to exogenous IGFs resulted in moderate stimulation of muscle interstitial cells, including fibroblasts. This response was also accompanied by moderately elevated levels of J1 immunoreactivity.

We examined lastly the appearance and distribution of AChR clusters in IGF-treated muscle. Light microscopic appearance of  $\alpha$ -bungarotoxin binding sites on teased fiber preparations of gluteus muscle exposed to daily injections of IGFs for periods of up to 15 d did not differ significantly from control, and no signs of extrajunctional clusters could be detected (data not shown).

### Discussion

We have shown that IGF2 promotes neurite growth from embryonic motoneurons in vitro. The mechanism is probably by direct binding of the hormone to IGF1 receptors on neu-

ronal processes. We further showed that addition of insulin-like growth factors to otherwise untreated adult skeletal muscle was sufficient to induce a substantial nerve sprouting reaction in the muscle. The sprouting reaction was not correlated with an inflammatory response. On the other hand, IGFs treatment also led to proliferation of muscle interstitial cells, including fibroblasts, and to elevated levels of J1 immunoreactivity. These latter reactions might contribute to sprouting by providing favorable substrate conditions.

IGF-mediated induction of neurite growth from embryonic spinal cord motoneurons in vitro displayed a similar dose response and relative hormone profile as previously described for sympathetic and sensory neurons (Recio-Pinto et al., 1986). The simplest explanation for the IGF1, IGF2, and insulin responses (in the subnanomolar range for the IGF and 20-nM range for insulin) is the activation of IGF1 receptors by these hormones (see, e.g., Rechler and Nissley, 1985). The IGF1 receptor-activated neurite outgrowth response appears to be a frequent feature of embryonic neurons in vitro and it will be important to determine whether sprouting in adult neurons might also be induced by insulin-like growth factors.

Motoneurons in vitro specifically bound hormone to their processes, with relative efficiencies characteristic of binding to the IGF1 receptor (Rechler and Nissley, 1985). In addi-

tion, the sprouting reaction was independent of culture density, required the constant presence of hormone (data not shown), and nonneuronal cells in the culture were not frequent (Fig. 1). It seems plausible to assume that neurite outgrowth was, therefore, due to insulin-like growth factor binding to the processes of motoneurons. Such a mode of action would be of significance, as it would be compatible with the anticipated local mode of action of the naturally occurring sprout inducer in inactivated muscle.

IGFs did not support motoneuron survival *in vitro* (data not shown), and the addition of proteins from a muscle extract was necessary to perform the sprouting experiments. Extracts were made from E19 chick hindlimb muscle. In the animals from which these tissues were taken, IGFs should have reached low adult levels, assuming that, as has been found in the rat (Ishii, 1989), IGF1 and IGF2 levels in chick muscle would decrease in parallel with the elimination of polyinnervation. Serum was included in the culture medium to provide the cells with optimal culture conditions to minimize possible nutritive effects of the tested hormones. Although serum does contain significant levels of insulin-like growth factors, the latter are found in an essentially inactive form, being associated with high affinity binding proteins (Nissley and Rechler, 1984). As a consequence, effects of low amounts of added IGFs could be detected, as has also been found by others (see, e.g., Recio-Pinto et al., 1986), irrespective of the presence of serum in the culture medium.

The demonstration of IGF-stimulated neurite growth from embryonic motoneurons *in vitro* adds plausibility to the hypothesis of an IGF-induced sprouting reaction in functionally denervated adult skeletal muscle. It is, however, important to point out that embryonic motoneurons *in vivo*, but especially adult motoneurons, might not respond or might respond differently to the hormones. Adult spinal cord motoneurons, on the other hand, are difficult to isolate and therefore no attempts were made to perform similar experiments with those neurons.

The presence of exogenous IGFs in skeletal muscle was sufficient to observe nerve sprouting *in vivo*. The first signs of intramuscular sprouting could be detected as early as 3 d after beginning treatment, and long twisting unmyelinated processes with frequent varicosities were observed after 1 wk of treatment. While sprouts emanating from endplates were frequently observed, a larger number of long processes seemed to originate from intramuscular nerves and thus possibly corresponded to nodal sprouts. The relatively low frequency of terminal sprouting observed appears to be consistent with results from Brown (1984). These authors have proposed that active muscle fibers might discourage sprouting from their end plates by failing to provide favorable substrate conditions on their surface (Brown, 1984). Some growth of terminal sprouts on the surface of innervated muscle fibers has however been documented unambiguously (Törigoe, 1988). It is possible that activation of intramuscular cells by IGFs might lead to improved substrate conditions in interstitial spaces, and possibly also in intramuscular nerves. Significant improvement of substrate conditions on muscle fibers in the vicinity of end plates might require functional denervation.

Exposure to IGFs induced a mitotic response, especially in interstitial cells. Stimulated cell division was less than has been described after partial denervation, but features of the

proliferation reactions elicited by the two different protocols were similar (Murray and Robbins, 1982; Connor and McMahan, 1987; Gatchalian et al., 1989). Proliferation after partial denervation is particularly prominent in the vicinity of inactive end plates and predominantly involves muscle fibroblasts. These activated fibroblasts are the main source of interstitial N-CAM and J1 in denervated muscles (Gatchalian et al., 1989). In IGF-exposed muscles we observed proliferation of interstitial cells (Table IV) and slightly elevated levels of muscle fibroblasts. We also observed significantly elevated levels of J1 immunoreactivity in hormone-treated muscles. N-CAM expression in activated fibroblasts has been shown to be relatively low (Gatchalian et al., 1989), which may explain why we did not detect changes in N-CAM immunoreactivity in IGF-exposed muscles. It is conceivable that the elevated levels of intramuscular IGFs that follow partial denervation or Botulinum toxin-induced paralysis (Ishii, 1989; Glazner, G. W., and D. N. Ishii, unpublished observations), might induce local proliferation of interstitial cells. These cells could then facilitate reinnervation by providing favorable substrate conditions. Massive proliferation upon partial denervation might be caused by additional mitogenic signals due to the presence of degenerating nerve terminals.

It is attractive to speculate that muscle-released IGFs might directly induce both intramuscular sprouting and interstitial cell activation, leading to a conducive milieu for nerve growth through the production of adhesive molecules. The possibility exists, however, that the observed sprouting reaction was entirely due to indirect effects of the IGFs. *In vitro* experiments have established that IGFs can produce neurite growth by a direct mechanism (Recio-Pinto et al., 1984). On the other hand, direct stimulation of nerve growth *in situ* by IGFs requires the presence of IGF receptors on responding neurons. While the latter has not yet been conclusively demonstrated, the existence of a neuronal variant of the IGF1 receptor has been reported (Burgess et al., 1987). The latter study also provided some evidence for the presence of IGF1 receptors on adult neurons *in situ*. Our attempts to demonstrate high affinity uptake and retrograde transport of <sup>125</sup>I-IGF2 by motoneuron processes *in situ* have so far failed. While, on theoretical grounds, there is no need to postulate retrograde transport of IGFs to explain the induction of a local sprouting reaction, the accumulation of retrogradely transported IGF1 at the site of a sciatic nerve ligature was recently reported (Hansson et al., 1987). The issue of whether IGFs directly stimulate intramuscular nerve growth by binding to IGF1 receptors on motoneuron processes cannot be decided by the experiments presented in this study. Our data, however, clearly demonstrate that the presence of low amounts of IGF2 (or of IGF1) in adult skeletal muscle is sufficient to induce a series of reactions, including intramuscular sprouting, that are observed upon partial denervation or muscle paralysis. Taken together with Ishii's data on muscle IGF mRNA levels (Ishii, 1989), our data suggest that IGFs might play a role in triggering regenerative reactions in denervated and in paralyzed muscle. These reactions include nerve growth and the production of a supportive environment for nerve growth by activated interstitial cells.

Partial denervation or paralysis, in addition to triggering intramuscular nerve growth also leads to inactive muscle fibers becoming receptive to innervation. Although molecular components leading to muscle susceptibility to innerva-

tion have not yet been identified, extrajunctional AChR and N-CAM correlate with the receptive state of muscle. Expression of the latter components in nonjunctional regions of muscle fibers is repressed by muscle activity (Fontaine and Changeux, 1989; Covault and Sanes, 1985; Rieger et al., 1985). We have examined the distribution of N-CAM and the appearance of AChR clusters in IGF-treated muscles. Receptor clusters appeared to be unchanged in shape and size after up to 15 d of daily hormone injections. In addition, no evidence for extrajunctional AChR clusters could be obtained (data not shown). Similarly, IGF-exposed muscle failed to display the dramatically elevated myofiber N-CAM levels that have been detected after denervation. Therefore, IGFs did not bring about inactivation-associated changes in muscle fibers. These findings are consistent with the limited degree of terminal sprouting from active end plates in partially denervated muscle reported by Brown (1984). Sprouting factors may induce cellular events leading to improved substrate conditions in interstitial and perineurial spaces, but not on the surface of innervated muscle fibers, with the result that sprout growth from end plate regions is restricted. In paralyzed muscle, on the other hand, the presence of both sprouting factor(s) and modified substrate conditions on muscle fibers would lead to massive terminal sprouting.

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