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# Synthesis and secretion of a high molecular weight form of nerve growth factor by skeletal muscle cells in culture

(conditioned culture medium/myoblasts/myotubes)

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ABSTRACT **Rat skeletal muscle cells and a cloned myogenic cell line synthesize and secrete in culture a molecule that is immunologically and biologically indistinguishable from the active form of nerve growth factor (NGF) from mouse submandibular gland. This protein can be detected in medium conditioned by muscle cells both before and after fusion and in the soluble fraction of muscle cell homogenates. Chromatographic data also reveal that the molecular properties of muscle cell NGF differ from those of the growth factor purified from mouse submandibular glands. Muscle cell NGF has a molecular weight between 140,000 and 160,000, whereas purified mouse gland NGF has a molecular weight of 26,000. The biologic function of muscle cell NGF is not known, although it could be that it plays some role relating to the association of nerves and muscle *in vivo*.**

Recent studies from this laboratory have shown that a variety of transformed and untransformed cells synthesize and secrete nerve growth factor (NGF) *in vitro* (1–3). Moreover, this property is not limited to established cell lines, since primary cultures of both chick (4) and human (5) fibroblasts have been shown to secrete NGF in culture. The present study was initiated to determine whether a primary cell of nonfibroblast origin also produces NGF. Skeletal muscle cells were selected for this purpose since they offer several experimental advantages. First, large numbers of cells can be collected and cultured in relatively pure form, with only minor contamination from cells of nonmuscle origin. Second, muscle cells can be examined in culture as they differentiate along the pathway from unfused myoblasts to fused myotubes.

The information presented below reveals that primary rat skeletal muscle cells as well as a cloned line of muscle cells, before and after fusion, synthesize and secrete in culture a molecule that we have been unable to distinguish immunologically from mouse submandibular gland NGF. Medium conditioned by these cells is also highly active in stimulating neurite outgrowth from chick sensory ganglia in culture. Furthermore, chromatographic studies reveal that the NGF secreted by muscle cells is a stable high molecular weight factor that differs physiochemically from biologically active forms of the NGF that have been isolated and purified from mouse submandibular glands. A preliminary report of some of this work has been presented (6).

## METHODS AND MATERIALS

**Reagents.** Eagle's minimal essential medium with Earle's balanced salts, Waymouth's medium, Earle's spinner culture salts, heat-inactivated fetal calf serum, and horse serum were obtained from Microbiological Associates; tissue culture flasks, from Falcon Plastics; blue dextran 2000 and Sephadex G-200 and G-75, from Pharmacia; D-arabinofuranosylcytosine (Ara C) and cycloheximide, from Calbiochem; bovine serum albumin (3× crystallized), trypsin, and horse heart ferricytochrome *c*, from Sigma; $^3H_2O$ and $Na^{125}I$ from New England Nuclear; and human IgG from Pentex. Rat tail collagen was prepared by the procedures of Ehrmann and Gey (7). Calf skin collagen without preservative was purchased from Worthington. NGF was isolated and purified from male mouse submandibular glands by modifications (1) of the method of Bocchini and Angeletti (8). All preparations were electrophoretically homogeneous as previously described (1).

Antisera to NGF were prepared in rabbits (1). IgG was purified by diluting serum with an equal volume of 0.15 M NaCl in 10 mM sodium phosphate, pH 7.4. This solution was brought to 50% saturation with ammonium sulfate, and the precipitate was redissolved in and dialyzed against 10 mM sodium phosphate/15 mM NaCl at pH 7.5. The solution was applied to a column of DE-52 (Whatman) in the same solvent and the unadsorbed fraction was collected and stored frozen. Prepared by these methods, anti-NGF IgG (14.5 μg/ml) totally inhibited the biological response exhibited by 10 ng of mouse NGF per ml; the same concentration of nonimmune IgG had no inhibitory effect.

**NGF Assays.** Procedures for radioimmunoassay of NGF as well as the methods of preparation of $^{125}I$-labeled NGF have been published (2). The biological activity of NGF-containing solutions was examined with cultures of 9-day chick embryo dorsal root ganglia that were grown on collagen-coated surfaces (2).

**Muscle Cell Cultures.** Cultures of fused and unfused rat skeletal muscle were prepared by modifications of the method of Yaffe (9). Unfused cell cultures were established by dispersing thigh muscles from newborn rats in a solution of 0.25% trypsin in Earle's spinner culture salts for 30 min at 37°. The cell suspension was plated in uncoated tissue culture dishes for 15 min to remove fibroblasts (10). The remaining suspension, containing predominantly myoblasts, was cultured on a surface of calf skin collagen in 150-mm plastic dishes ($2 \times 10^6$ cells in 20 ml) in a mixture of minimal essential medium and Waymouth's medium (3:1) supplemented with 10% horse serum and 1% chick embryo extract. Cells were grown for a 24-hr period

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Abbreviations: NGF, nerve growth factor; Ara C, D-arabinofuranosylcytosine.

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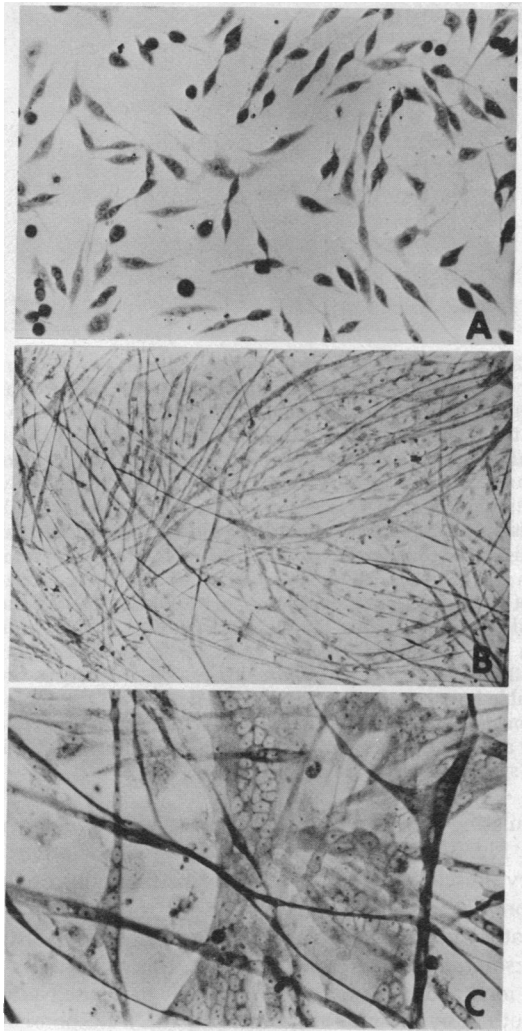


FIG. 1. Primary muscle cell cultures grown as described in the *text* and treated with Giemsa stain. (*A*) Unfused myoblasts at the beginning of the incubation period in serum-free medium. (×150.) (*B* and *C*) Low and high power views of multinucleated myotubes after 48 hr of incubation in serum-free medium. (*B*, ×96; *C* ×150.)

in a humidified atmosphere containing 5% $CO_2$. Cultures of fused cells were prepared by identical techniques through the first 24 hr after the initial plating. Following this period, the feeding solution was replaced with medium containing 20% fetal calf serum and 4% embryo extract, a solution that stimulates cell proliferation (9). After 24 hr, the medium was replaced with the initial feeding solution and cell fusion began 15 hr later, or about 60 hr after the original plating. Unfused, dividing cells were removed from the cultures by 48-hr treatment with 5 $\mu$g of Ara C per ml; the cultures were then incubated in fresh medium not containing the nucleoside for an additional 24–48 hr.

To prepare cells for assay, we washed each culture thoroughly three times with 50 ml of minimal essential medium without serum. Cultures were then incubated in fresh serum-free medium for 2–4 days, after which the medium was removed, centrifuged at 5000 × *g* at 4°, and frozen. Cells were scraped from the plates and collected by centrifugation (1500 × *g*). For NGF analyses, medium was thawed, dialyzed exhaustively against 10 mM ammonium acetate, and lyophilized. The dry residue was redissolved in and dialyzed against minimal essential medium (for biological assay) or against 0.1 M potassium phosphate, pH 7.0 (for radioimmunoassay). Muscle cells were disrupted in ground-glass homogenizers in 0.1 M potassium phosphate, pH 7.0. The homogenate was centrifuged at 1500 × *g*, and the supernatant solution was dialyzed against 0.1 M potassium phosphate for analysis by radioimmunoassay.

$L_8$ cells comprise a line of rat myoblasts originally isolated by Yaffe (11). Cells were routinely maintained in minimal essential medium supplemented with 10% horse serum and were passed twice weekly with 0.05% trypsin in spinner salt solution. For the experiments described below, it was necessary to modify these cells so that they could be maintained in the presence of low serum concentrations or in serum-free media. This was accomplished by growing cells in minimal essential medium plus 2% horse serum. Serial cloning techniques were used; the clones that produced the highest degree of fusion were isolated and cultured separately on uncoated plastic dishes. These cells were found to survive well, and their fusion characteristics were similar to the original $L_8$ line except that they formed multinucleated syncytia at much lower cell densities. Media and cells were prepared for NGF analysis by the procedures described above for primary cultures.

**Morphological Analyses of Muscle Culture.** At the beginning and again at the end of the incubation period in serum-free medium, single representative cultures were rinsed with Eagle's salt solution and fixed with methanol. After Giemsa staining, the cultures were observed by phase microscopy. The number of nuclei in several fields was counted and the total cell number per plate estimated. Fusion in each culture was quantitated by counting the number of nuclei incorporated into myotubes.

**Gel Filtration Studies.** The chromatographic behavior of NGF from muscle cell-conditioned medium was analyzed with columns of Sephadex G-200. Samples were dialyzed against and the columns equilibrated with 0.1 M potassium phosphate, pH 7.0, with or without 1 mg of bovine serum albumin per ml. Fractions were collected in weighed test tubes and elution volumes were determined by measuring the tube weight plus fraction. Void ($V_0$) and internal ($V_i$) column volumes were measured with blue dextran 2000 and $^3H_2O$, respectively. Concentrations of NGF were determined by radioimmunoassay.

## RESULTS

Fig. 1 shows cultures of primary rat muscle cells grown under the experimental conditions described above. In unfused cultures, most cells were bipolar with large central nuclei. In fused cultures, multinucleated myotubes predominated, but some polygonally shaped unfused cells were also present.

Since primary noncloned cultures of muscle cells contain a small percentage of fibroblasts and since fibroblasts are known to secrete NGF (4), it was necessary to rule out the possibility that NGF detected in muscle-conditioned medium was contributed by fibroblasts rather than muscle cells. Consequently, cultures of fused myotubes were treated with Ara C for 48 hr. This reagent prevents DNA synthesis, is lethal to dividing cells (12), and has been used to remove single cells from fused preparations of chick skeletal muscle (13, 14). In these experiments Ara C effectively reduced the number of single cells in fused cultures, although a small number of unfused cells remained.

The appearance of $L_8$ cells was similar to that of primary cultures except that the cells appeared to fuse into multinucleated syncytia rather than well-defined myotubes. The degree of fusion could be regulated and was directly proportional to

Table 1. NGF secreted by primary rat muscle cultures

| Exp. | % fusion* Day 1 | % fusion* Day 3 | NGF produced,† ng/nucleus |
|---|---|---|---|
| 1 | 0 | 20 | $3.8 \times 10^{-7}$ |
| 2 | 2 | 35 | $1.0 \times 10^{-7}$ |
| 3 | 70 | 80 | $0.8 \times 10^{-7}$ |
| 4 | 98 | 98 | $3.0 \times 10^{-7}$ |

Serum-free media conditioned by cultures of primary rat thigh muscle were collected, concentrated, dialyzed against 0.1 M potassium phosphate, pH 7.0, and examined by radioimmunoassay using NGF from mouse submandibular gland as a standard. Exps. 1 and 2 used unfused cell cultures, while Exps. 3 and 4 used fused preparations that had been treated with Ara C. Between 2 and $7 \times 10^7$ cells were used for each experiment shown.

* Percent fusion, measured at the beginning and end of the experiment, reflects the number of nuclei in the culture present in myotubes.

† NGF production is expressed as a function of the number of nuclei present at the end of the experimental incubation period.

the length of time the cells were grown in serum-containing medium. Cultures of $L_8$ cells were not treated with Ara C since the line was originally cloned from myoblasts and is not contaminated with fibroblasts.

Tables 1 and 2 present results of radioimmunoassays of concentrated serum-free medium conditioned by cultures of primary rat muscle and $L_8$ cells, respectively. The data reveal that an immunoreactive substance (based upon NGF from mouse submandibular gland as standard) is secreted both by fused and unfused cultures of muscle cells. Primary muscle cells produced approximately the same amount of NGF as $L_8$ cells, and no consistent differences were detected in the amount of NGF produced by fused and unfused cells in either culture system.

To eliminate the possibility that the immunoreactive material being measured arose from residual serum or embryo extract, we determined the total amount of NGF (intracellular plus extracellular) present in the cultures at the beginning and end of the experimental incubation period. Fig. 2 reveals that in serum-free medium the amount of NGF present (intracellular plus extracellular) in both primary and $L_8$ muscle cell cultures increases over time. Cycloheximide (5 $\mu$g/ml) completely blocks this effect (tested in cultures of $L_8$ cells). Taken together, these results demonstrate that the immunoreactive factor is being synthesized by muscle cells during their incubation in culture.

Medium conditioned by muscle cells is also biologically active

Table 2. NGF secreted by $L_8$ cell cultures

| Exp. | % fusion Day 1 | % fusion Day 3 | NGF produced,* ng/nucleus |
|---|---|---|---|
| 1 | 0 | 10 | $3.4 \times 10^{-7}$ |
| 2 | 20 | 50 | $3.0 \times 10^{-7}$ |
| 3 | 65 | 65 | $7.6 \times 10^{-7}$ |
| 4 | 60 | 85 | $4.6 \times 10^{-7}$ |

Media conditioned by $L_8$ cells were collected and prepared for NGF radioimmunoassay according to the procedures listed in the legend to Table 1. Primarily unfused cell cultures (Exps. 1 and 2) were grown in medium with serum for 1 day prior to the experimental incubation. Primarily fused cultures were prepared (Exps. 3 and 4) by allowing the cells to grow in serum-containing medium for 2–4 days. At the beginning of the test period, cultures in both groups were washed and incubated in serum-free medium. Between 1 and $2 \times 10^7$ cells were used in each experiment shown.

* NGF production is expressed as a function of the number of nuclei present at the end of the experiment.

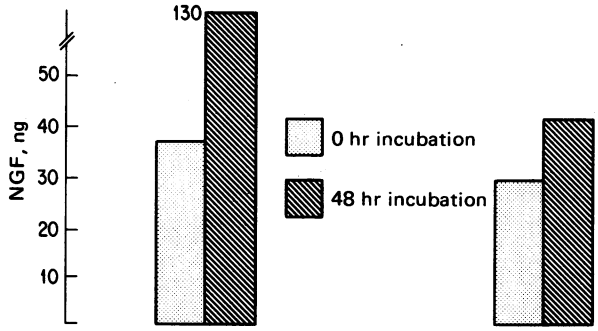


FIG. 2. Net NGF synthesis by muscle cell cultures. Cultures of primary (*Left*) and $L_8$ (*Right*) rat muscle cells were prepared as described in the *text*. At the beginning of the experiment, all dishes were washed four times in serum-free medium. The cultures in each experiment were then separated into two groups containing identical cell populations. The medium from one group was immediately collected, dialyzed against 10 mM ammonium acetate, and lyophilized. The cells were scraped from the culture dishes, centrifuged, and frozen. Cells in the other group were incubated for 48 hr in serum-free medium. For analysis, cell pellets were thawed and homogenized in 1.0 ml of 0.1 M potassium phosphate, pH 7.0. Insoluble residue was removed by centrifugation (15 min at $2000 \times g$). The supernatant fraction and the media collected from each group were dialyzed against 0.1 M potassium phosphate, pH 7.0. NGF concentrations in the soluble homogenate fraction (intracellular NGF) and in conditioned media (extracellular NGF) were determined by radioimmunoassay and these values were combined to give the total NGF in the culture system at zero time and after 48 hr.

in stimulating neurite outgrowth in the sensory ganglion assay system. Fig. 3*B* shows that ganglia treated with solutions from primary muscle cell cultures produce neurite outgrowth that is not present in ganglia treated with control media (Fig. 3*A*) and that is indistinguishable from that elicited by NGF from mouse submandibular gland. Further, this response is completely inhibited by monospecific antibody to mouse NGF (Fig. 3*D*), but not by normal rabbit IgG (Fig. 3*C*). Responses comparable to those shown in Fig. 3 were observed when ganglia were treated with media from $L_8$ cell cultures.

Recent studies in this laboratory have shown that the NGF secreted by mouse L cells in culture (1) differs physiochemically from the biologically active NGF purified from mouse submandibular glands (15, 16). For example, biologically active mouse gland NGF (molecular weight 26,000) is composed of two identical noncovalently joined polypeptide chains, each with a molecular weight of 13,259 (17). Moreover, this NGF dimer is in rapid reversible equilibrium with its constituent monomers, and biological activity in the sensory ganglion system is mediated by the monomeric form (15). In contrast, L cell NGF is a highly stable protein complex with molecular weight close to 160,000 (16). In light of these findings, we have estimated the molecular size of muscle cell NGF at concentrations that are biologically active. Fig. 4 presents the Sephadex G-200 gel filtration profiles of both intracellular and secreted muscle cell NGF and the Sephadex G-75 profile of purified mouse gland NGF. The elution volume of muscle NGF (Fig. 4 *middle* and *bottom*) is considerably less than that of mouse gland NGF, which emerges from the column at a position consistent with its monomeric mass of 13,000 g/mole (Fig. 4 *top*) (15). To estimate the masses of the intra- and extracellular muscle NGF, the chromatographs depicted in Fig. 4 were calibrated with cytochrome *c*, IgG, and bovine serum albumin. The weight average partition coefficients ($\sigma_w$) for all proteins were then computed from the relation

$$\sigma_w = (V_e - V_0)/V_t \qquad [1]$$

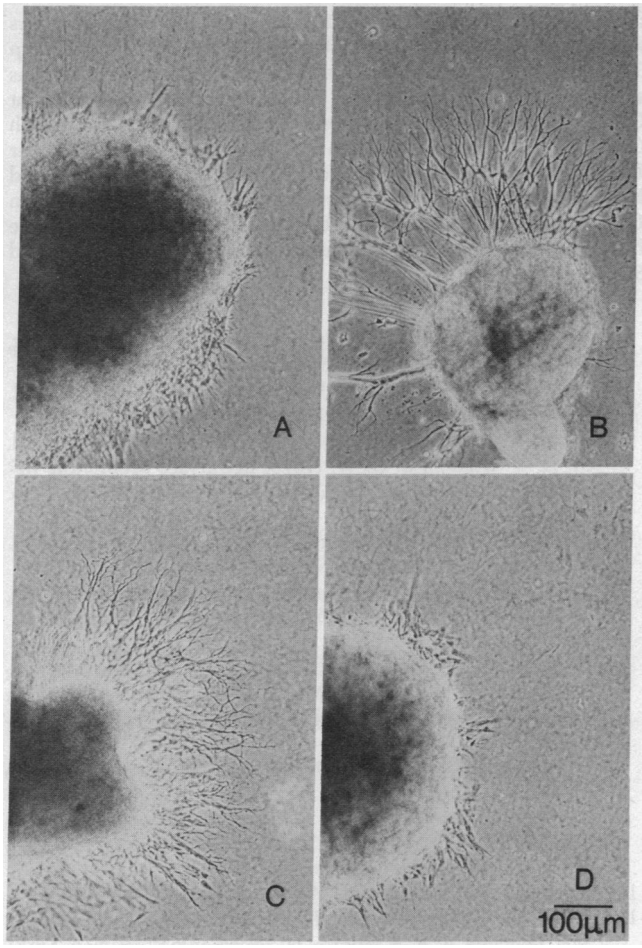


FIG. 3. Effects of muscle-conditioned medium on embryonic chick dorsal root ganglia grown *in vitro*. All ganglia were incubated in minimal essential medium containing 10% fetal calf serum. Concentrated conditioned medium (containing 10 ng of NGF/ml) was dialyzed against minimal essential medium. The figure shows representative portions of ganglia treated with: (*A*) control medium; (*B*) medium conditioned by primary rat myotubes; (*C*) medium conditioned by primary cultures of unfused myoblasts containing 14.5 μg of purified normal rabbit IgG per ml; and (*D*) the same preparation of conditioned medium used in *C*, supplemented with 14.5 μg of rabbit anti-NGF IgG per ml. All samples were incubated for 18 hr at 37° on cover slips coated with rat tail collagen. (Phase contrast photomicrographs; ×79.)

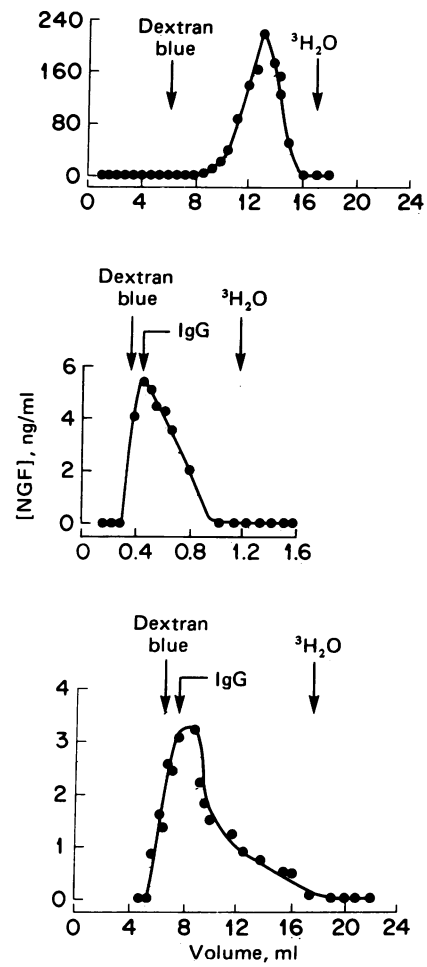


FIG. 4. Sephadex elution profiles of purified NGF from mouse submandibular gland and from muscle. (*Top*) G-175 column (1 × 23 cm) equilibrated with 0.1 M potassium phosphate, pH 7.0, containing 1 mg of bovine serum albumin per ml at 4°; 100 μl of mouse gland NGF (9.8 μg/ml) was applied. Fractions of 300 μl were collected. (*Middle*) G-200 column (0.5 × 6.5 cm); solvent: 0.1 M potassium phosphate, pH 7.0, at 25°; 100 μl of concentrated primary muscle culture medium was applied to the column and 30-μl fractions were collected. (*Bottom*) G-200 column (1 × 23 cm) equilibrated with 0.1 M potassium phosphate, pH 7.0, containing 1 mg of bovine serum albumin per ml at 4°. Fractions of 300 μl were collected. Intracellular primary muscle NGF was prepared as described in the legend to Fig. 2 and 100 μl of the concentrated solution was applied. Marker proteins (see *text*) were applied separately to all three columns. NGF concentrations were measured by radioimmunoassay. The peak elution positions of dextran blue, IgG, and $^3H_2O$ are shown.

where $V_e$, $V_0$, *and* $V_t$ are the macromolecule elution volume, void volume, and internal column volume, respectively. Muscle cell NGF elutes from the column in a position that is indistinguishable from that of IgG, as shown in the figure. From plots of ln (molecular weight) against $\sigma_w$ for the calibration proteins, molecular weights of 158,000 (extracellular secreted NGF) and 140,000 (intracellular NGF) were computed. Within experimental error for this method, these two numbers are not significantly different from each other and both are close to the value for L cell NGF (160,000), which was also estimated by gel filtration chromatography (16). It should be noted, however, that the elution profile of intracellular NGF (bottom) is somewhat asymmetric. It could be that there are minor components of lower molecular weight in the cell extract that have not been resolved.

## DISCUSSION

The information presented above reveals that primary rat muscle cells in culture, as well as a cloned myogenic cell line, synthesize and secrete a molecule that we are unable to distinguish from mouse submandibular gland by NGF immunological and biological criteria. This factor is present intra- and extracellularly, it is a potent stimulant of nerve growth when tested in the ganglion bioassay system, and its biological activity is blocked by antibody to mouse NGF. By radioimmunoassay, comparable amounts of NGF are secreted by cultures of primary rat muscle and by $L_8$ cells and the quantity of NGF per nucleus produced by proliferating myoblasts is similar to that secreted by fused myotubes. Although fusion accelerates the synthesis of proteins associated with muscle function (18), this event had no consistent effect on the production of NGF.

While NGF derived from muscle cells is biologically and immunologically closely similar to purified NGF from mouse submandibular gland, the gel filtration properties of the two factors differ significantly. For example, at concentrations that are biologically active, muscle NGF has a molecular weight

(140,000–160,000) considerably higher than mouse gland NGF (13,000). Although the molecular structure of the larger muscle cell factor is not known, immunological evidence indicates that it contains a peptide sequence similar to that of the 13,000 molecular weight gland factor. Mouse L cells also secrete a highly stable NGF whose mass is closely similar to that of muscle NGF (16). Upon treatment of L cell NGF with denaturing solvents, it dissociates to liberate a molecule that is electrophoretically and chromatographically identical to purified mouse gland NGF (16).

Depending upon the isolation procedure used, NGF can also be prepared from mouse submandibular glands as a high molecular weight complex which has been given the name 7S NGF (19). However, this protein, unlike muscle cell and L cell NGF, is a highly unstable macromolecule in solution and its properties are quite different (20) from those of the cell secreted growth factors. For example, 7S NGF is a protein complex of molecular weight near 140,000, and it is composed of three different kinds of protein molecules (19). Only one of these (called $\beta$-NGF) is the biologically active form of NGF; it is the 26,000 molecular weight dimer referred to above. However, at 7S NGF concentrations as high as 10 $\mu$g/ml, this protein dissociated to yield a mixture of its constituent chains, and, at 1 $\mu$g/ml, dissociation and liberation of the biologically active monomer of $\beta$-NGF is virtually complete (20). This behavior is strikingly different from that of either muscle NGF or L cell NGF, both of which are appreciably more stable in solution than 7S NGF. Consequently, it follows that the NGF secreted by both these cell types is not similar to the 7S NGF complex. At present, we know nothing about the molecular structure of these new molecules.

The properties of muscle NGF, which distinguish it from the proteins isolated from mouse submandibular gland, may be important for the biological function of the molecule as it is produced by muscle cells. For example, it is possible that muscle NGF has biological actions as yet unknown that differ from those of gland NGF. In this connection, it should be noted that muscle cells enhance the development of spinal cord neurons in culture, an effect that could be mediated by chemical factors (21).

The reason that skeletal muscle cells secrete NGF is not known, but there are several possible explanations. Since NGF stimulates the growth of sensory neurons and since muscle cells require a nerve supply for sensory receptors, it is possible that cells make this protein to attract or support sensory innervations. Further, it has been suggested that neurotrophic factors are produced by peripheral cells, including muscle, to enhance the growth of developing neurons (22). Since it is known that axons extend into a developing limb well before muscle fusion takes place (23) and that nerves contact single myoblasts (24), it is likely that if a muscle produces a neurotrophic factor, it does so before fusion takes place. This is consistent with the findings presented here that NGF is produced by myoblasts. Thus, it could be that NGF is an informational molecule that muscle cells produce to communicate with the nervous system.

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