Extracts of Skeletal Muscle Increase Neurite Outgrowth and Cholinergic Activity of Fetal Rat Spinal Motor Neurons

Abstract. *A soluble extract of rat skeletal muscle increased neurite outgrowth and cholinergic activity of dissociated ventral spinal neurons in culture. The effects were concentration-dependent, saturable, and labile in the presence of heat or trypsin. The morphological enhancement was produced only by skeletal muscle extract and decreased with developmental age, whereas the cholinergic enhancement was produced by extracts of cerebral cortex and cardiac and skeletal muscle and did not change with age. These changes were specific for ventral cord neurons, but no species specificity was observed with respect to the muscle source or the neuronal target.*

Survival, neurite outgrowth, and cholinergic activity of spinal motoneurons in vitro are influenced by the presence of muscle target tissue (*1–3*). Coculture of skeletal myotubes with spinal cord cells enhances neuronal survival, promotes outgrowth of neurites, and increases the activity of choline acetyltransferase (*4*). Media conditioned with myotubes can reproduce some of the effects of coculturing (*5–8*), which indicates the existence of diffusible trophic factors in vitro but which may be of limited value for the complete characterization of these factors since only small quantities can be obtained from monolayers of cultured cells (*9*). Direct extraction of such factors from skeletal muscle tissue would substantially increase the amount of material available for purification and provide a direct method for assaying the role of muscle-derived factors in the survival of motoneurons during development and the sprouting of motoneurons in the denervated adult. Extraction of intraocular tissue has served as a method for obtaining parasympathetic neuronotrophic substances (*10*). We report that proteins from an extract of rat skeletal muscle (*11*) promote neuritic outgrowth and increase neuronal cholinergic activity when added to cultures of dissociated ventral spinal cord (*12*) from fetal rats.

Skeletal muscle produced morphological changes in dissociated ventral spinal cord (Fig. 1). Treatment of spinal cord cells, plated at low density, with muscle extract for 3 days increased the percentage of cells extending processes and the density and length of the processes. These changes in neurite outgrowth were quantitated by the method described in the legend to Table 1. The technique revealed a twofold to fivefold increase in the number of processes per cell (Table 1). This increment in neurite outgrowth was linear with increasing muscle extract concentration up to 600 μg of protein per milliliter of medium. The average length of neuronal processes increased an average of 3.5 times (Table 1). The concentration of muscle extract required to produce a half-maximal increase in process density was equivalent to that necessary to cause an equal increase in process length. Many of the processes were shown to be neuronal by the specific binding of tetanus toxin (*13*), which we monitored with an indirect immunofluorescent staining technique (Fig. 1, C and D) (*14*).

Since cultures of dissociated spinal cord contain many other cell types in addition to motoneurons, it was important to determine whether muscle extract also enhanced acetylcholine synthesis, an activity specific in ventral cord for motoneurons. Four days after the addition of muscle extract (200 μg of protein per milliliter of medium) to spinal neuron cultures, acetylcholine synthesis and accumulation (*15*) increased more than threefold (Table 1). The increase was dependent on both time and concentration and was accompanied by a comparable increase in choline acetyltransferase activity (Table 1).

There was no change in total cell survival, as measured by number of cells or amount of DNA per dish. However, since motoneurons comprise only a small percentage of ventral cord cells, it is possible that their selective loss occurs in untreated cultures. Bennet *et al.* (*16*) demonstrated that the addition of medium conditioned with muscle extract enhances the survival of identified motoneurons in dissociated cell cultures. However, selective survival of cholinergic neurons cannot fully explain our results, since acetylcholine synthesis increased twofold in control cultures during the first week. Furthermore, early cholinergic development can proceed in the absence of muscle. (*17*).

Process length and density increased in the presence of skeletal muscle extracts only (Table 2). Cholinergic activity, however, was stimulated by extracts of other tissues innervated by cholinergic neurons (cardiac muscle and cerebral cortex) in addition to skeletal muscle extract. This supports the suggestion that the neurite-promoting and cholinergic activities are separable (*18*).

Further evidence for such a separation was obtained by preparing extracts of limb muscle from rats of different postnatal ages. When added to dissociated ventral spinal cord, the activity of the neurite-promoting substance decreased with the age of the muscle donor, with an 80 percent decrease occurring in the first

Table 1. Effect of fetal rat muscle extract on neurite morphology, acetylcholine synthesis, and choline acetyltransferase activity in cultured ventral and dorsal spinal cord cells. Two-day-old cultures of dissociated dorsal or ventral spinal cord were incubated for 3 days in the presence or absence of muscle extract (400 to 500 μg of protein per milliliter of medium). The ratio of the number of processes per cell was determined by counting processes with lengths greater than one cell diameter in random microscopic fields and dividing by the number of cells in the same field. One to 3 percent of the total surface area was counted for each plate. Cultures grown with or without muscle extract (200 μg of protein per milliliter) for 4 days were assayed for total acetylcholine synthesis and accumulation or for choline acetyltransferase activity. Values are means ± standard errors. Numbers in parentheses represent the number of measurements per group, as derived from multiple experiments.

Culture	Morphology		Acetylcholine synthesis		Choline acetyltransferase activity	
	Ratio of processes per cell	Process length (μm)	Picomoles per dish per hour	Picomoles per microgram of DNA per hour	Picomoles per dish per hour	Picomoles per milligram of protein per hour
Ventral cord						
Control	0.36 ± 0.01 (60)	50 ± 1 (539)	1.53 ± 0.22 (35)	2.52 ± 0.24 (32)	19.8 ± 2.4 (10)	994 ± 116 (10)
Experimental	1.51 ± 0.05 (39)	163 ± 5 (363)	5.66 ± 0.44 (71)	7.98 ± 0.34 (50)	82.9 ± 7.3 (16)	3841 ± 315 (16)
Dorsal cord						
Control	0.53 ± 0.01 (24)	45 ± 2 (245)	0.27 ± 0.18 (20)	0.34 ± 0.21 (20)	4.2 ± 2.1 (4)	215 ± 107 (4)
Experimental	0.60 ± 0.02 (24)	63 ± 3 (332)	0.28 ± 0.14 (20)	0.36 ± 0.19 (20)	5.9 ± 1.7 (8)	218 ± 88 (8)

3 weeks of life. Cholinergic activity, however, was not altered during this period, suggesting that it was not under the same developmental regulation.

Not all spinal cord cells were affected by the muscle extract. No increases in process density and length, cholinergic activity, or survival were seen in cultures of dorsal spinal cord. On the other hand, no species specificity was observed for the muscle source or the ventral cord target. Comparable morphological changes and cholinergic activities were observed with muscle extracts from 9-month fetal calves and newborn sheep, rats, and chicks, and greater activity was seen in extracts of 4.5- to 6.5-month fetal calves, 18-day fetal rats, and 12-day embryonic chickens. Furthermore, muscle extracts from these species exerted comparable effects on ventral spinal cord cultures from 4- to 5-day embryonic chickens and 14- to 15-day embryonic rats.

Table 2. Effect of extracts of various tissues from newborn rats on neurite morphology (ratio of the number of processes per cell) and acetylcholine synthesis in cultured ventral spinal cord cells. Portions of each extract containing 200 μg of protein were added to separate cultures of dissociated spinal cord. Each extract was tested in triplicate. Neurites were counted after 3 days and acetylcholine synthesis was assayed after 4 days. Data are mean percentages (± standard deviations) of the control values. The morphological data are the means for three experiments and the data on acetylcholine synthesis are the means for two experiments. Numbers in parentheses represent number of individual determinations per experimental group; for the morphological analysis they refer to the number of microscopic fields counted and for acetylcholine synthesis they refer to the number of cultures assayed.

Tissue	Ratio of processes per cell	Acetylcholine synthesis	
		Picomoles per dish per hour	Picomoles per microgram of DNA per hour
Control	100 ± 21 (81)	100 ± 6 (6)	100 ± 8 (6)
Spleen	107 ± 23 (48)	93 ± 16 (6)	100 ± 2 (6)
Liver	82 ± 25 (42)	98 ± 4 (6)	102 ± 4 (6)
Lung	104 ± 20 (48)	107 ± 2 (6)	118 ± 12 (6)
Skin	115 ± 12 (49)	104 ± 30 (6)	112 ± 39 (6)
Kidney	121 ± 31 (52)	86 ± 9 (6)	91 ± 15 (6)
Cerebral cortex	108 ± 20 (60)	149 ± 2 (6)	151 ± 17 (6)
Cardiac muscle	124 ± 27 (40)	162 ± 14 (6)	194 ± 27 (6)
Skeletal muscle	200 ± 21 (50)	171 ± 15 (6)	225 ± 29 (6)

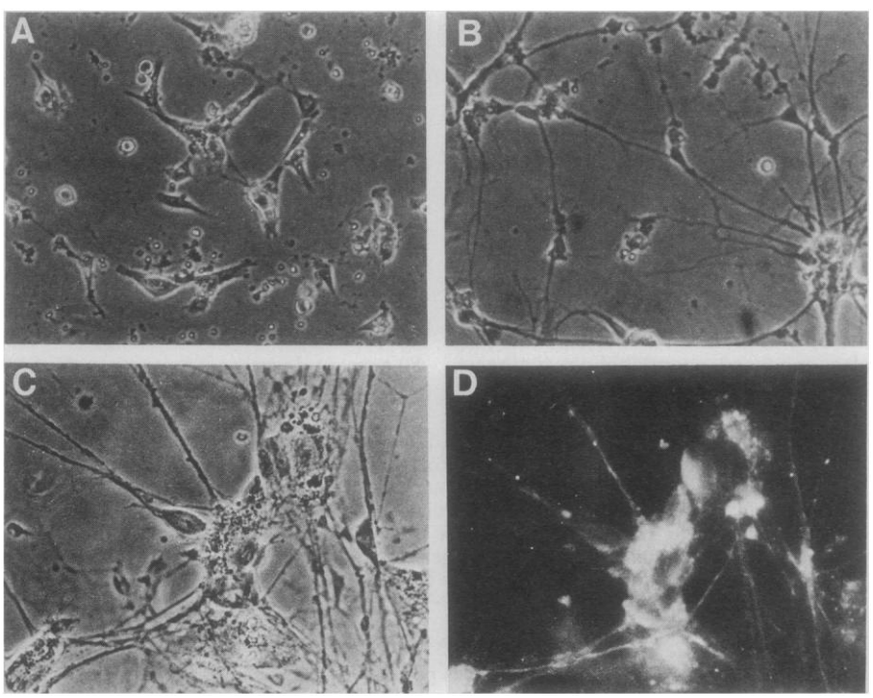

Fig. 1. Effects of rat skeletal muscle extract on the morphology of rat ventral spinal cord cells. Two days after plating and 12 to 24 hours after the addition of $1 \times 10^{-5} M$ cytosine arabinoside, cultures of dissociated ventral cord were supplemented with Dulbecco's modified Eagle's medium containing 10 percent horse serum and either 7 percent (by volume) PBS (A) or 7 percent (by volume) muscle extract (450 μg of protein per milliliter of culture medium) in PBS (B). Cultures were maintained in these media for 3 days and then photographed (×125). (C and D) Specific binding of tetanus toxin to cell processes in muscle extract–treated cultures. Such binding identifies neuronal cells. (C) Phase contrast photomicrograph (×252) of cells treated with muscle extract for 3 days; (D) ultraviolet fluorescence micrograph of the same field.

Preliminary characterization of muscle extract suggests that the neurotrophic factor is a protein. The cholinergic and neurite-promoting activities of the extract were destroyed by heating for 1 hour at 60°C or by boiling for 15 minutes. They were also destroyed by exposure to trypsin (final concentration, 0.1 mg/ml) for 30 minutes at 37°C. Soybean trypsin inhibitor (1 mg/ml) blocked the effects of trypsin without altering the morphological or cholinergic effects.

The factor is neither nerve growth factor (NGF) nor a muscle protein cross-reactive to NGF (*19*). NGF had no effect on ventral cord culture morphology or cholinergic activity, nor did prior incubation of the extract with antiserum to NGF diminish the effects of the extract.

The extract was not affected by freezing and lyophilization and could be kept for several hours at 37°C and for several days at 4°C. It was also stable in 1 m*M* β-mercaptoethanol, iodoacetic acid, and dithiothreitol, but was destroyed in 6*M* urea, 10 percent ethanol, and by a basic *p*H. On Sephacryl S-200 the factor migrated with a molecular weight of greater than 35,000.

These findings suggest that muscle extract from several species provides an excellent source for motoneurotrophic factors. The morphological effect appears to be highly specific for skeletal muscle extract. The cholinergic activity shows less tissue specificity, being increased with extracts of tissues that receive dense cholinergic innervation. Differences between our results on tissue specificity and those of a previous study (*7*) might be explained by differences between the production and storage of motoneurotrophic factors derived from skeletal muscle in vivo and those of motoneurotrophic factors derived from myotubes in vitro (*20*). Direct tissue extraction indicates that factors which regulate the differentiation of a class of neurons are made by the particular target tissues innervated (*21, 22*) and may provide a way to accurately assess the role of innervation, muscle activity, and developmental age in the regulation of motoneurotrophic factors.

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11. Extract was prepared from the limb muscles of newborn Sprague-Dawley rats. After homogenization in three volumes of 10 m*M* Dulbecco's phosphate-buffered saline (PBS) (*p*H 7.4) containing 1 m*M* EDTA and 0.5 m*M* EGTA, the solution was centrifuged at 32,000*g* for 1 hour. The supernatant was then centrifuged for 2 hours at 100,000*g*. The final supernatant was dialyzed against PBS for 12 hours and filter-sterilized before use.
12. Cultures were obtained from dissociated, arachnoid-free ventral spinal cord tissue prepared from rat embryos in the 14th to 15th day of development (stages 24 to 27) [G. A. Christie, *J. Morphol.* **114**, 263 (1964)]. The cells were plated onto polylysine-coated, 35-mm-diameter culture dishes at a concentration of 5×10^5 viable cells per dish in Dulbecco's modified Eagle's medium supplemented with 10 percent horse serum (inactivated by heating at 50°C for 30 minutes), 2 m*M* glutamine, 33 m*M* glucose, and 0.15 m*M* Garamycin. Cytosine arabinoside (10^{-5} *M*) was added to this medium for 12 to 24 hours 1 day after plating to retard the proliferation of nonneuronal cells and thereby reduce the supportive effect of glia [T. Meyer, W. Burkart, H. Jockusch, *Neurosci. Lett.* **11**, 59 (1979); R. L. Schnaar and A. E. Schaffner, *J. Neurosci.* **1**, 204 (1981)].
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Human c-Ki-*ras*2 Proto-Oncogene on Chromosome 12

Abstract. *A human colonic adenocarcinoma transforming gene, recently identified as a cellular homolog of the Kirsten sarcoma gene (v-*ras*), was used to assign the human cellular Kirsten* ras*2 gene to chromosome 12 by the Southern hybridization method. A single 640 base-pair Eco RI–Hind III fragment of the transforming gene, isolated by DNA transfection and molecular cloning, can detect a single Eco RI fragment (2.9 kilobase pairs) of DNA from phenotypically normal cells. The data suggest a constant chromosomal location of c-Ki-*ras*2.*

The use of DNA-mediated gene transfer techniques (*1*) to identify the origin of transforming sequences in malignant cells has provided evidence that changes in genes in the normal cell can give rise to activated forms with oncogenic potency [(*2–5*); see (*6*) for review]. This emerging class of cellular transforming sequences has been detected by a biological assay in which mouse NIH 3T3 cells incubated with DNA from tumor cell lines of different tissue origins acquire an altered morphology and the ability to grow in soft agar. These traits are often associated with malignant cells (*2–5*). NIH 3T3 cells treated with DNA from human tumor cell lines acquire human DNA that can be identified by means of a species-specific molecular probe (Alu) (*7*) that detects repetitive sequences interspersed in the human genome (*4, 5*). DNA from such mouse cell transformants can be used to construct a recombinant genomic library from which phage clones containing specific human transforming sequences can be isolated (*6, 8, 9*).

Combining human-rodent somatic cell hybrids and Southern (*10*) hybridization techniques [see (*11*) for review], we have chromosomally assigned the c-Ki-*ras*2 gene (*12*). The probe was a transforming gene isolated from the SW480 human colon carcinoma cell line by DNA-mediated transfection and molecular cloning (*5, 6*). A pBR322 plasmid clone (p640) containing a unique 640 bp Eco RI–Hind III fragment of the colon transforming gene was used as a probe for filter hybridization studies.

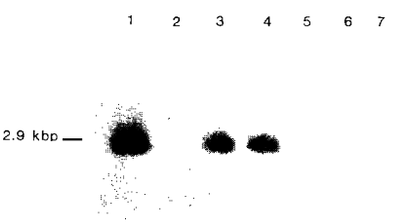

Fig. 1. The p640 colon transforming gene probe was hybridized to DNA's from human (lane 1), mouse (lane 2), and hybrid cells (lanes 3 to 7). The probe detects a single 2.9-kbp Eco RI fragment of human DNA. Under these hybridization conditions (*21*), mouse DNA does not anneal with the probe. Hybrid cell DNA's in lanes 3 and 4 are positive for c-Ki-*ras*2.

DNA's from human and mouse cell lines were cleaved with Eco RI, electrophoresed through agarose, and transferred onto nitrocellulose. The p640 plasmid was labeled with ^{32}P by nick translation (*13*) and hybridized to filters (Fig. 1). A single 2.9-kbp Eco RI human DNA fragment hybridized with the probe, whereas no signal was obtained from mouse DNA under these hybridization conditions. We then screened the DNA's from 38 man-mouse hybrid cell lines for the presence of c-Ki-*ras*2. At the same time, correlated homogenates of hybrids were tested for genetic markers for the 22 human autosomes and the X chromosome to determine their human chromosome complement. The detection of a 2.9-kbp fragment by the probe indicated the presence of the c-Ki-*ras*2 gene in the DNA of a hybrid cell line (Fig. 1, lanes 3 and 4). This 2.9-kbp DNA fragment was coordinately present in cell hybrids only with the human chromosome 12 markers, lactate dehydrogenase B and peptidase B (Fig. 2). The data have been summarized so that only the percent discordancy for each human chromosome is given in Fig. 2 (percent of hybrids in which c-Ki-*ras*2 and a given human chromosome marker did not cosegregate). In 13 karyotyped hybrids, c-Ki-*ras*2 segregated only with a normal human chromosome 12 (Fig. 2). The c-Ki-*ras*2 gene is therefore asyntenic with at least eight other human proto-oncogenes (*14*).

The hybrids used for assignment studies were derived from 12 unrelated individuals, indicating a constant chromosome location for c-Ki-*ras*2 in normal human cells. A survey of more than 20 DNA's from unrelated individuals revealed no Eco RI polymorphism in the region of the gene detected by the probe. Intensities of signals on x-ray film after hybridization of the p640 probe to these DNA's were similar, suggesting that the copy number of the gene is invariant (less than twofold), at least in the normal human fibroblasts and leukocytes examined. In addition, DNA's from HeLa, HEL (human embryonic lung), and RD (human rhabdomyosarcoma) cell lines yielded hybridization signals comparable to those obtained with normal cell DNA.

Several transforming genes detected by the NIH 3T3 transfection system bear