Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells
(dissociated cell culture/serum-free medium/growth factor for motoneurons)

CHRISTOPHER E. HENDERSON, MONIQUE HUCHET, AND JEAN-PIERRE CHANGEUX
Neurobiologie Moléculaire et Laboratoire Associé au Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, Paris, France
Communicated by Rita Levi-Montalcini, January 8, 1981

ABSTRACT The effect of media conditioned by muscle cells on the development in vitro of chicken spinal neurons was studied. Neural tube cells of 4.5-day chicken embryos were dissociated after trypsinization and cultured in serum-free minimum essential medium conditioned for 4 days over cultures of fused chicken myotubes. After 20 hr in conditioned medium (protein concentration, 10–50 μg/ml), about 50% of surviving cells had extended neurites, whereas in cultures in nonconditioned medium this value was about 10%. The active factor(s) in conditioned medium is macromolecular and its activity was completely destroyed by incubation with trypsin. Concentrated samples of conditioned medium were analyzed by gel filtration on columns of Sepharose CL-6B. The activity was recovered in peaks with apparent molecular weights of 40,000 and 500,000 and at the exclusion volume of the column. Media conditioned by chicken liver and skin cells also contained neurite-promoting activity but at lower levels. No activity was detected in Nerve Growth Factor, insulin, fetal calf serum, or horse serum or in medium conditioned by chicken lung, chicken heart, or C6 glioma cells.

During the phase of embryogenesis leading up to the formation of a given synapse, a complex set of interactions between the nerve cell and its potential target is brought into play (1, 2). These interactions may be qualified as "anterograde," such as for example the effect of the nerve on the distribution and metabolism of receptors in the target cell membrane, or, conversely, as "retrograde" (3). It has been postulated for many systems (3–5) that the target cell secretes soluble retrograde factors upon which the neuron depends for its survival and morphological and biochemical differentiation.

The best candidate for such a role is the nerve growth factor (NGF) (6), but there is indirect evidence that, in the embryonic spinal cord, motoneurons destined to innervate skeletal muscle may be affected by comparable retrograde factors liberated by their target tissues. Results obtained in vitro, such as the enhancement of naturally occurring cell death by limb extirpation in the chick embryo (7) and the rescue of some motoneurons by the graft of a supernumerary limb (8), suggested that these factors might be necessary for cell survival. More recent experiments in vitro have pointed to roles for muscle-derived factors in enhancing neurite extension (9), transmitter synthesis (10, 11), and cell survival (12) of spinal neurons.

In this paper, we report the effects of media conditioned by skeletal muscle cells upon the development of dissociated cell cultures of embryonic chicken spinal cord. We have established a rapid quantitative assay for factors affecting one crucial aspect of the development of spinal neurons: neurite extension. Investigations using this assay have provided a preliminary characterization of the substance(s) responsible for the growth-promoting effect of muscle-conditioned medium.

MATERIALS AND METHODS

Spinal Cord Cultures. Neural tubes were dissected from 4.5-day White Leghorn chicken embryos (Hamburger–Hamilton stage 25) and freed of meninges and adhering tissue. Small pieces were treated with 0.03% trypsin in Tyrode's solution (without Ca²⁺ or Mg²⁺) for 45 min at 37°C. They were then washed twice with Tyrode's solution and dissociated by gentle trituration through a drawn-out Pasteur pipette in minimal essential medium (Eagle's) containing DNase (30 μg/ml) and 10 mM MgCl₂. Cell yields by this procedure were ca. 1.5 × 10⁶ cells per embryonic cord. Cells were plated at a density of 5 × 10⁵ cells per 35-mm untreated Corning plastic dish in 1 ml of medium or 10⁵ cells per 16-mm well in Costar cluster dishes. All media were supplemented with 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). All cultures were kept at 37°C in an atmosphere of 5% CO₂/95% air at 100% relative humidity.

Preparation of Conditioned Medium. Leg muscles from 11- to 12-day chicken embryos were dissected, and skin and bone were carefully removed. The muscles were mechanically dissociated in minimal essential medium and plated in gelatin-coated 100-mm Corning plastic dishes at a density of ca. 5 × 10⁶ myoblasts per dish in 10 ml of medium. The culture medium was as follows: 3 parts minimal essential medium (Eagle's), 1 part medium 199, 10% (vol/vol) heat-inactivated horse serum, 1% chicken embryo extract, 2 mM glutamine, penicillin at 100 units/ml and streptomycin at 100 μg/ml. Between days 2 and 4 of culture, 10 μM arabinocytosine was added to kill dividing cells. After 4 days, the medium was removed and the cells were washed twice with 3 ml of minimal essential medium. They were then fed minimal essential medium (Eagle's) containing 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (referred to as "nonconditioned medium"); after 4 days this medium (now referred to as "conditioned medium") was removed and replaced by fresh nonconditioned medium which in turn was conditioned for 4 days. Up to three batches of conditioned medium could be obtained in this fashion from a single culture of myotubes. Conditioned media was stored in a nitrogen atmosphere of 5% CO₂/95% air at 0°C.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: NGF, nerve growth factor.
Assay for Neurite Extension. A quantitative estimation of the response of spinal cord cultures to conditioned medium was obtained as follows. Starting at a point chosen at random on the perimeter of a dish and progressing towards the center, we counted the total number of cells and the number of these bearing neurites in 10 adjacent fields. Neurites were counted as fine processes that were more than one cell diameter long. About 400 cells were counted per dish; the results are expressed as the percentage of the cells counted that had one or more neurites.

Materials. Spectra/Por 2 dialysis tubing, molecular weight cutoff 12,000–14,000 (Spectrum Medical Industries, Los Angeles), was boiled three times for 30 min in sodium carbonate and then three times for 15 min in twice-distilled water before being sterilized at 105°C. All culture media, sera, and enzyme solutions were purchased from GIBCO Bio-cult (Paisley, Scotland).

RESULTS

Effects of Muscle-Conditioned Medium. When trypsin-dissociated neural tube cells from 4.5-day chicken embryos were plated in normal serum-free nonconditioned medium, only a few of them had extended neurite-like processes after the first day of culture (Fig. 1a). In the presence of medium conditioned by primary muscle cultures, a greater number of the cells present extended neurites within the same period (Fig. 1b). After 20 hr of culture in the experiment shown in Fig. 1c, 10 ± 1% (mean ± SEM; n = 4) of cells in nonconditioned medium had one or more neurites and in muscle-conditioned medium this value was 43 ± 3%.

Such differences in neurite extension (3-fold or greater) between cultures in conditioned and nonconditioned medium were observed in more than 100 experiments using 15 different preparations of conditioned medium. With a given preparation, successive batches of cultures sometimes differed in the absolute percentages of cells with neurites; thus, in different experiments, from 25% to 60% of total cells had neurites after 1 day in conditioned medium, whereas the percentage in nonconditioned medium varied from 2% to 20%. This variability probably arose from slight differences in culture conditions and embryo age from one experiment to another. Spinal cord cells plated in nonconditioned medium developed more neurites on collagen-coated dishes than on plastic dishes and showed a tendency to aggregate. Although conditioned medium increased the percentage of cells with neurite under these conditions as well, the quantitative assay was simplified by preincubation of the dissected spinal cords in Tyrode's solution for 90 min before trypsinization. This decreased the response in nonconditioned medium to the levels cited above.

In contrast to its effect on neurite extension, muscle-conditioned medium had no marked effect on either initial cell attachment or subsequent apparent cell survival in these spinal cord cultures. Cells that excluded 0.02% trypan blue were counted in 45 consecutive microscopic fields across the diameter of a dish. In one experiment, at 2 hr after plating there were 50.8 ± 1.3 (mean ± SEM; n = 45) living cells per field in nonconditioned medium and 47.4 ± 2.4 in conditioned medium. This represented a plating efficiency of 25% of cells present after dissociation. After 23 hr of culture, the numbers of surviving cells were 24.5 ± 1.5 per field in nonconditioned and 28.6 ± 1.0 in conditioned medium; at 66 hr after plating, these values were 23.4 ± 1.1 and 24.5 ± 1.9, respectively. It is unlikely that cell proliferation affected these results because [3H]thymidine included for the first 24 hr of culture labeled less than 5% of the nuclei as revealed by autoradiography of fixed cultures.

Definition of the Unit of Neurite-Promoting Activity. Serial dilutions of muscle-conditioned medium with nonconditioned medium were assayed for their effect upon neurite extension. The percentage of cells with neurite increased with increasing concentration of conditioned medium up to 40% (vol/vol) conditioned medium (Fig. 2). Above this concentration, the curve gave a plateau at 56 ± 3% cells with neurite. Defining 1 unit of activity as that amount of active factor in 1 ml required to produce 50% of the maximal effect upon neurite extension, we calculated that the conditioned medium shown contained 9 units/ml.

Characterization of the Active Factor(s). To estimate the size of the active factor in muscle-conditioned medium, portions of conditioned and nonconditioned medium were dialyzed thoroughly against nonconditioned medium. Their neurite extension activities had not changed after dialysis (Table 1, Exp. A). Another sample of conditioned medium was concentrated 10-fold by using an ultrafiltration membrane with an exclusion limit of molecular weight 10,000 and subsequently reconstituted 1:10 with nonconditioned medium and assayed. It too had retained its full activity (Table 1, Exp. B).

After being concentrated by ultrafiltration, samples of conditioned medium were applied to a column of Sepharose CL-6B equilibrated in nonconditioned culture medium. Fractions were analyzed directly for their effect on neurite extension in the spinal cord cultures (Fig. 3). Apparent recoveries of 42–104% of the applied activity were obtained in different experiments, associated with molecular species of apparent molecular weights approximately 40,000, 500,000, and >106 (i.e.,...

![Fig. 1. Effect of muscle-conditioned medium on neurite extension by spinal cord cells. (a and b) Phase-contrast micrographs (scale = 20 μm) of dissociated spinal cord cells after 22 hr in culture in nonconditioned (a) and conditioned (b) medium. (c) Quantitative assay of neurite extension after 20 hr in nonconditioned (NM) and conditioned (CM) medium. Vertical bars represent the SEM of results from four dishes for each medium. These cultures contained 23 ± 1 living cells per microscopic field in nonconditioned medium and 26 ± 2 per field in conditioned medium.](image)
excluded from the column), of which the first and last were predominant. Total protein concentration in the fractions was too low to be reliably estimated by the Folin assay.

In other experiments, the susceptibility of the active factor to trypsin was investigated. Portions of nonconditioned and conditioned medium were treated with trypsin (6 μg/ml) for 3 hr and then the reaction was stopped with trypsin inhibitor. The activity of the conditioned medium was completely destroyed by incubation with trypsin (Fig. 4b). The activity was retained when the trypsin inhibitor was added at the start of an otherwise identical incubation (Fig. 4c). Thus, the neurite extension factor is probably macromolecular and contains a polypeptide moiety.

Specificity of the Neurite-Promoting Activity. The protein concentrations of different batches of muscle-conditioned medium ranged from 10 to 50 μg/ml. When nonconditioned medium, which contains no protein, was supplemented with bovine serum albumin at 100 μg/ml, the percentage of cells that extended neurites did not increase (Table 1, Exp. C). The addition of 0.25% heat-inactivated horse serum (protein concentration ≈170 μg/ml) to nonconditioned medium resulted in a slight reduction of the number of neurites (Table 1, Exp. D).

Because the effect of conditioned medium on spinal cord cells was not mimicked by the addition of these concentrations of serum albumin or serum to nonconditioned medium, it is likely that neurite extension is promoted in response to a specific property of the factor(s) in muscle-conditioned medium.

To test higher serum concentrations we had to use polyornithine-coated dishes to ensure attachment of the spinal cord cells, and the results are therefore not comparable direct with those in Table 1. In the presence of 10% horse serum, the responses were 8.6 ± 1.5% cells with neurite in nonconditioned medium and 20.9 ± 1.8% in conditioned medium. In 10% fetal calf serum, these values were 10.1 ± 0.3% and 22.8 ± 0.7%, respectively. Thus, the neurite-promoting effect of muscle-con-
motivating activities comparable to the activity of muscle-conditioned medium (550 units/mg protein).

The same medium conditioned over muscle cells was applied to explants of 8-day chicken dorsal root ganglia cultured on a collagen substrate in the presence of 10% fetal calf serum. At concentrations at which its effect on spinal neurons was maximal, this medium only elicited slight neurite outgrowth from the ganglia compared to the dense halo observed with NGF at 1 ng/ml. It is known that muscle-conditioned media contain small amounts of an NGF-like substance (14).

**DISCUSSION**

In seeking to detect and study the factor(s) that may mediate retrograde effects of skeletal muscles upon the motoneurons of the spinal cord, we measured the extension of neurites from the whole population of cells resulting from dissociation of the spinal cord. This may be measured after a relatively short period (20 hr); however, it has the disadvantage that it is not restricted to the motoneurons. We chose 4.5-day chicken embryos (stage 25) as the source of neurons because, by this stage, most neuroblasts destined to be motoneurons have undergone their final mitosis but proliferation continues in other regions of the neural tube (15). It is known that cultures prepared from the spinal cord of stage 24 embryos contain neurons that have high-affinity uptake sites for choline (16) and that are capable of forming functional synapses with skeletal myotubes (17). We calculated that, in our hands, 6 × 10⁵ cells per stage 25 spinal cord survived the plating step and put out neurites. This is close to the value, 5 × 10⁶, estimated for the maximum number of α-motoneurons in the 4-day chicken embryo (18). However, we cannot be sure that the responsive cells in our cultures were α-motoneurons.

Conclusions about the properties and mode of action of the active factor(s) that can be drawn from studies on unconditioned conditioned media are necessarily limited. The results of Table 2, however, may be taken to indicate that not every conditioned medium or serum protein is capable of promoting neurite outgrowth from spinal neurons. Because we also showed that our muscle-conditioned medium had little effect on dorsal root ganglia explants, it seems reasonable to suppose that the neurite-promoting factor we have studied exhibits some specificity in regard to both its source and its target.

Finally, the muscle-conditioned medium we have studied may be compared with other media and factors that have been reported to affect the growth of cholinergic neurons. Dribin and Barrett (9) used as an assay the outgrowth of neurites from 15-day fetal rat spinal cord slices after 7 days in culture. The active factor in media conditioned by primary lung cultures was retained by membrane filters with a nominal molecular weight cutoff of 100,000. It was found to be stable at 58°C for 30 min and did not appear to be β NGF or fibronectin. The choline acetyltransferase activity of mouse spinal cord cultures was increased 11-fold after incubation with muscle-conditioned medium (10). The active material had a molecular weight in excess of 50,000 and resisted heating to 58°C. A molecule with an apparent molecular weight of 35,000–40,000 (19) capable of supporting the survival of dissociated ciliary neurons is found at high specific activity in extracts of the target muscles in the eye (5). Cultured ciliary ganglia, as well as spinal and sympathetic ganglia, respond to an extract prepared from lyophilized 18-day chicken hearts; this extract can be fractionated into two active components, the major one having an apparent molecular weight of 40,000 and the minor one, >100,000 (20). It is clear that studies on purified preparations are required before the real similarities and differences between these active factors can be established and before the existence of a spinal motoneuron growth factor may be considered proven.

**Table 2. Distribution of neurite-promoting activity**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Maximum protein concentration tested, μg/ml</th>
<th>Specific activity, units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplements:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Fetal calf serum*</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Horse serum*</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Medium N2†</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Conditioned media:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>16</td>
<td>550</td>
</tr>
<tr>
<td>C6 glioma</td>
<td>1200</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Lung‡</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>Lung (replaced)§</td>
<td>170</td>
<td>0</td>
</tr>
<tr>
<td>Skin‡</td>
<td>180</td>
<td>80</td>
</tr>
<tr>
<td>Skin (replaced)§</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>Liver‡</td>
<td>520</td>
<td>280</td>
</tr>
<tr>
<td>Heart‡</td>
<td>125</td>
<td>0</td>
</tr>
</tbody>
</table>

All supplements and conditioned media (except medium N2) were tested at a range of concentrations up to the maximum indicated. Specific activities were calculated from curves of the type shown in Fig. 2, as described in the text.

* Sera were not heat-inactivated.
† As in ref. 13.
‡ First plating.
§ Replaced three times.
We thank J. Thibault for a gift of NGF, D. Monard for samples of C6-conditioned medium, and Mrs. T. Sciuto for typing the manuscript. This work was supported by grants from the Muscular Dystrophy Association of America, the Collège de France, the Délégation Générale à la Recherche Scientifique et Technique, the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Commissariat à l’Energie Atomique. C. E. H. received a North Atlantic Treaty Organization/Science Research Council Postdoctoral Fellowship.