Partial Purification of a Factor Promoting Chicken Myoblast Multiplication in vitro

By Eijiro Ozawa* and Kazuhiro Kohama**

(Comm. by Shigeo Okinaka, M. J. A., Dec. 12, 1973)

Carrel (1924) found that embryo extract added to culture medium was very effective in promoting the growth of fibroblasts; the plasma or extracts of adult tissues was also shown to have some effect, but their activities were much less than that of embryo extract. Embryo extract is now widely used as an important constituent of media for embryonal primary culture of various kinds of cells including muscle cells. Some properties of a factor in embryo extract concerning the growth of muscle cells were described by De La Haba and Amundsen (1972), but no pronounced success in purifying the factor has been reported.

We found that adult chicken serum could exert an effect comparable to that of chick embryo extract on the growth of muscle cells in vitro. An attempt was then made to purify the factor in adult chick serum and, as a result, about 80 fold purification was attained.

This communication is mainly concerned with the method of purification of the factor. Its properties related to its function will be described in the next paper (Kohama and Ozawa, 1973).

Experimental. Hens and roosters were purchased from a local farm and were sacrificed by cutting the carotid arteries. Arterial blood was collected and left standing until blood clot retracted. It was then centrifuged to remove the clot. The supernatant, the serum, was stored in a deep freezer (−80°C).

The activity of the serum and the purified factor was assayed by the method of which the details will be published elsewhere. Briefly, 12 day chick myogenic cells, which had been purified by differential adhesion technique (Kaighn, Ebert and Stott, 1966), were cultured in 2.4 ml of the medium composed of 85% Eagle's minimum essential medium (MEM, Nissui Co.) and 15% horse serum. To each plate, 0.1 ml of appropriately diluted factor was added, unless specifically mentioned. The number of cells plated was $3 \times 10^4$ per 35 mm Falcon plastic plate. After 3 or 4 days' incubation, the cells were fixed and

---

* Department of Pharmacology, Faculty of Medicine, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, 113.

** Department of Pharmacology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113.
the number of the nuclei of myoblasts and myotubes were counted as
described before (Ozawa, 1972). Using these values, the activities
were then calculated using 5- or 3-point assay of slope ratio assay
method (Burn, 1950).

The concentration of protein was determined by biuret reaction
and/or by optical density at 280 nm standardized by bovine serum
albumin.

Results. The factor which promotes the growth of myogenic
cells was purified as follows:

Step 1. Frozen serum was thawed and diluted with twice
volumes of 0.9% NaCl solution. To 100 ml of the diluted serum, 27.7 g
of solid ammonium sulfate was added and dissolved. After several
tens of minutes, the resulting precipitates were removed by centrifu-
gation. To the supernatant, further 13.4 g of solid ammonium sulfate
per 100 ml of the original diluted serum was added. The resulting
flocculated material was collected by centrifugation. Usually, the
material was found to be packed on the top layer of the content of
the centrifugation tube. The clear solution underneath the packed
material was removed with a siphon. The packed material remaining
in the tube was dissolved in a small amount of distilled water, and the
resulting solution was then dialysed against 5 mM phosphate buffer
(pH 7.0).

Step 2. Solid urea was carefully added to the dialysed solution
described above, the final concentration being 1 M. To 100 ml of the
resulting solution 44 g of solid ammonium sulfate was added and
dissolved. The solution was then allowed to stand for about 30
minutes. The resulting flocculated material was removed by centri-
fugation. If the resulting solution had reddish tone, 0.5 or 1 g of
ammonium sulfate per 100 ml was further added. Then the solution
was centrifuged again to remove the flocculated material. The result-
ing clear solution was dialysed against 5 mM phosphate buffer (pH
7.0) containing 60 mM NaCl.

Step 3. DEAE-Sephadex resin, swollen in 5 mM phosphate
buffer (pH 7.0) containing 60 mM NaCl, was poured into a column
and washed with the same solution. The dialysed solution of the Step
2 was centrifuged to remove the denatured materials, if necessary,
and the supernatant was applied to the column. The column was then
washed with a large volume of the same buffer containing 60 mM
NaCl. The factor was eluted with the same buffer containing 155 mM
NaCl.

Step 4. The eluate in the Step 3 was concentrated with collodion
bag SM 132 00 of Sartorius Membrane Filter Gmbh. A column of
Sephadex G 200, swollen in 5 mM phosphate buffer (pH 7.0) contain-
ing 155 mM NaCl, was prepared. The concentrated solution was applied to the column, which was then eluted with the same buffer containing 155 mM NaCl. Active principle appeared in the eluate of which the apparent partition coefficient (Kv) ranged from 0.33 to 0.67.

Table I. Comparison of activities of original serum and purified factor

|                  | Protein concentration of the factor in culture medium | Mean of myogenic cell number of four plates (25 fields were counted per each plate) | Relative specific activity |
|------------------|------------------------------------------------------|----------------------------------------------------------------------------------|---------------------------|
| Control          | 0 μg/ml                                               | 41.8                                                                             | —                         |
| Original serum   | 195                                                   | 143.5                                                                             | (1.0)                     |
| Purified factor  | 1.0                                                   | 83.5                                                                              | 80.5                      |

4×10⁴ cells were cultured in a Falcon plastic 35 mm plate containing 2.2 ml of 85% MEM and 15% horse serum and 0.3 ml of purified factor, 24 times diluted serum or 0.9% NaCl. In each case, four plates were incubated for 4 days at 37.5°C. After fixation and staining, nuclei of myogenic cells were counted.

Fig. 1. Photograph of the chick myogenic cells cultured in the medium containing 85% MEM, 15% horse serum, and 1 μg/ml of the purified factor. The cell number plated was 4×10⁴ per 35 mm Falcon plastic plate. The volume of the culture medium was 2.5 ml per plate. The cells were incubated for 53 hours. The inserted bar indicates 100 μm.
The specific activity of an eluted fraction which showed the highest activity was about 80 times higher than that of the original serum on the protein basis (Table I). Cultured cells in the media with the purified factor (1 μg of protein per ml) were shown in Fig. 1. The condition of culture is described in the legend to the figure.

The factor was destroyed on boiling for 1 minute, whereas its activity was well retained at 38°C for at least 30 minutes. The factor lost its activity irreversibly at pH 2.5 or lower. The activity was reduced intensely when the factor was shaken with chloroform. Treatment with 1% sodium dodecyl sulfate or 6 M urea eliminated the activity irreversibly. The factor was resistant to a fairly high concentration of trypsin or neuraminidase so far examined.

The molecular weight of the factor was estimated by the method of Martin and Ames (1961) modified by Kato et al. (1971). According to a preliminary experiment, it was about 1.1×10^5, if we assume that the molecule would be spherical.

The human, horse, or mouse serum scarcely replaced the chicken serum in promoting the growth of chicken muscle cells under the condition of the present assay system.

Discussion. In spite of the presence of abundant horse serum in the assay system, a distinct difference in the degree of growth of chicken myogenic cells was noticed between the plates with or without chicken serum. Not only the horse serum, but also the human or mouse serum was scarcely effective in promoting chicken myogenic cell growth. These findings indicate that the factor promoting myogenic cell growth has species specificity.

Although the fraction containing the factor is purified 80 fold on the protein basis, it is far from being homogeneous. If this factor is a kind of protein hormone in its nature, like insulin or growth hormone, the concentration of the factor in the serum should be very low such as 10^-9 g per ml (Williams, 1968; Daughaday, 1968). If this is true, we must accomplish further several 1,000 fold purification. Thus the purification of the factor is still in a preliminary stage and will require further painstaking effort.

Summary. The method of purification of the factor promoting myoblast multiplication was described. The highest specific activity of a fraction thus purified was about 80 times higher than that of the original serum on the protein basis.

Acknowledgement. The authors wish to express their cordial thanks to Profs. S. Ebashi and M. Otsuka for their interest in this work and valuable suggestions. A very preliminary part of this work was performed while one of the authors (E. O.) was staying at the Carnegie Institution of Washington, Baltimore, U.S.A.; his
sincere thanks are due to Prof. J. D. Ebert, who permitted him to work on this problem. He is also very much obliged to Prof. emeritus S. Okinaka who has kindly provided him a chance to make this investigation. This work was supported in part by the research grants of the Ministry of Welfare, No. 216.

References

Burn, J. H. (1950): Biological Standardization (2nd ed.). Oxford Univ. Press, London.
Carrel, A. (1924): Physiol. Rev., 4, 1.
Daughaday, W. H. (1968): In “Textbook of Endocrinology”, ed. R. H. Williams; Saunders, Philadelphia.
De La Haba, G., and Amundsen, R. (1972): Proc. Nat. Acad. Sci. USA, 69, 1131.
Kaighn, M. E., Ebert, J. D., and Stott, P. M. (1966): Proc. Nat. Acad. Sci. USA, 56, 133.
Kato, J., Atsumi, Y., and Inaba, M. (1971): J. Biochem., 70, 1051.
Kohama, K., and Ozawa, E. (1973): Proc. Japan Acad., 49, 857.
Martin, R., and Ames, B. (1961): J. Biol. Chem., 236, 1372.
Ozawa, E. (1972): Biol. Bull., 143, 431.
Williams, R. H. (1968): In “Textbook of Endocrinology”, ed. R. H. Williams; Saunders, Philadelphia.