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The Effects of Fractions of Chick Embryo Extract on the Fusion of Cultured Chick Embryonic Myoblasts

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培養 鷄胚 筋原細胞의 融合에 미치는 鷄胚 抽出液 分劃의 영향

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要 約

鷄胚의 筋原細胞의 증식과 融合에 미치는 계배 抽出液의 영향을 조사하였다.

1. 배양액내의 계배 추출액의 농도가 높을수록 근원세포의 증식은 촉진되었으며, 반면에 근원세포 융합은 지연되었다.

2. 계배 추출액의 단백질을 Sephadex G-75로 分劃하고, 각 분획을 근원세포의 배양액에 첨가한 결과 分子量 40,000과 22,000 dalton 사이의 분획이 근원세포의 증식과 융합을 촉진시켰다.

3. 계배 추출액의 단백질을 ammonium sulfate로 분획시켜 각 분획을 근원세포의 배양액에 첨가한 결과 70% 이상 포화 용액에서 침전하는 분획이 근원세포의 증식과 융합을 현저히 증가시켰다. 이 유효 분획을 Sephadex G-75로 재차 분획하여 각 분획의 효과를 조사한 결과 근원세포의 증식과 융합을 촉진시키는 분획이 계배 추출액을 Sephadex G-75로 분획하여 얻은 유효 분획과 거의 동일한 효과를 나타내었다.

INTRODUCTION

In the *in vitro* culture of presumptive skeletal muscle cells from chick embryo, the undifferentiated myoblasts continue proliferating during the first 2 to 3 days and then the

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terminal differentiation of myoblasts to striated muscle fibers commences that generally lasts in another 2 to 3 days. The differentiation process involves the fusion of cell membrane to form the postmitotic multinucleate myotubes and the concomitant synthesis of contractile proteins, the acetylcholine receptor, and the muscle specific form of creatine phosphokinase (Okazaki and Holtzer, 1966; Nadal-Ginard, 1978; Devlin and Emerson, 1978; Ha *et al.*, 1979).

A central interest related to the muscle differentiation is therefore the nature of the control mechanism that regulates the transition of the proliferating single cells to the differentiated myotubes. Muscle culture provides an easier way to study the control mechanism because one can manipulate culture conditions and get a high degree of synchrony of differentiation (O'Neill and Stockdale, 1972).

Numerous studies have shown that the initiation time of the muscle cell differentiation in culture is not rigidly determined by intrinsic program, but can be easily and predicably shifted in time by changing, for example, the volume of initial media, concentration of components of media or the time of media change; (O'Neill and Stockdale, 1972; Doering and Fischman, 1974; Slater, 1976), suggesting that the time of initiation of the myoblast fusion in culture is controlled by some alterations in the media. However, it is not certain whether the change of the time of differentiation resulted from the depletion of certain medium constituents or alternatively from the addition to the medium of specific components released from the cells. Doering and Fischman (1977) reported by analyzing the muscle-conditioned medium that proteins having molecular weight between 30,000 and 100,000 daltons which were thought to be secreted by the muscle cells caused precocious myogenic cell fusion. On the contrary, Konigsberg (1982) showed using perfusion chamber that the cell fusion initiated earlier when the concentration of medium components such as horse serum and embryo extract was decreased, suggesting that the effect was due to the depletion of some growth stimulatory components in the medium.

Myogenesis of muscle cells *in vitro* requires the presence of embryo extract (EE) in the culture medium (Konigsberg, 1979), and this suggests that EE contains a substance(s) necessary for the growth and differentiation of myoblasts. Many attempts have been made to define the effect of EE on the myoblast differentiation and isolate effective substance(s) from it.

It was shown that EE stimulated the proliferation of myoblasts and delayed the differentiation (Slater, 1976). The initiation of differentiation may be caused by the inactivation of a specific fusion inhibitor or/and by the consumption of a growth-stimulating substance in EE. It was also reported that the activity of EE to stimulate the proliferation and to depress the differentiation was heat labile (Slater, 1976), resided in the protein above 300,000 daltons (Konigsberg, 1982), and was similar to the fibroblast growth factor (Linkhart *et al.*, 1981).

De la Haba and Amundsen(1972) showed that EE contributed at least two factors; one promoting the fusion of myoblasts and the other promoting the development of myotubes.

Recently, transferrin was purified from EE and shown to be one of myotrophic substances (Ii *et al.*, 1982). This protein promoted myoblasts to proliferate and myotubes to grow when added to the chick muscle cell culture. Oh and Markelonis (1980) showed that sciatin present in the chick EE was the compound required for myogenesis. Transferrin and sciatin seem to be very similar molecules, if not identical. However, there must exist some other unknown factor(s) in EE that promotes the fusion and proliferation of myoblasts, since the activity of purified transferrin was lower than that of natural EE that contained transferrin.

The identification of the specific EE-derived factors necessary for cell growth and for cell differentiation would be beneficial to the study of biochemical events that regulate the cell differentiation. Therefore, the present study was undertaken to isolate the possible factor(s) from EE, employing ammonium sulfate precipitation and chromatography on Sephadex G-75. It was found that effective factor(s) appeared to exist in the fraction having molecular weight between 22,000 and 40,000 daltons and were precipitable at the ammonium sulfate saturation higher than 60%.

MATERIALS AND METHODS

Cell Culture

Cultures were prepared according to O'Neill and Stockdale (1972) with some modification, using presumptive myoblasts isolated from the breast of 12-day old chick embryos. Breast muscle was isolated from embryo and washed 3 times with Earle's balanced salt solution (EBSS). The tissue was then cleaned off connective tissue and fat, and minced in EBSS. Cells were then dissociated mechanically by passing through the orifice of a pasteur pipette. The cell suspension was treated with 0.1% trypsin in a humidified 5% CO_2 incubator at 37°C for 30 minutes, trypsin was removed by centrifugation and the remained was suspended in 811 medium. The medium 811 is the "growing medium" which consisted of Eagle's minimal essential medium (MEM) containing 10% horse serum, 10% EE and 1% antibiotic-antimycotic. The cell suspension was dispensed by mechanical agitation and filtered through a 20 μm of Nitex HC-10 nylon mesh in a Swinny adaptor to remove undissociated cells. The cells were then preplated on collagen-coated Petri dishes for 10 minutes to remove fibroblasts. Collagen solution was prepared by autoclaving 50 mg of collagen per 100 ml of distilled water and filtrating through 0.45 μm Millipore membrane.

After preplating, the number of cells in suspension was counted in a hemacytometer and after diluting the cells to a desired density with 811 medium, cells were plated on 35 mm tissue culture dishes (Falcon) or multi-well plates (Limbro) that had been coated

with collagen. Culture was performed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

For the first 24 or 28 hours, cells were grown in 811 medium in order to allow viable cells to attach to the substratum and proliferate. At 24 or 28 hour, the medium was removed and the cells were washed twice with warm MEM. Cells were then cultured with medium 8102 or with experimental media for a specified time. The medium 8102 is the "differentiating medium" and has the same composition with the "growing medium", medium 811, except that the concentration of EE is 2%. Otherwise noted, the medium 8102 or the experimental media were changed routinely every 48 hours.

Preparation of Embryo Extract

The embryo extract was prepared from 12 day-old chick embryos. The whole embryos were washed 3 times with EBSS, and eyes, beaks, and the distal parts of legs were dissected out. Embryos were then chopped and minced by pressing through a syringe without needle. An equal volume of EBSS was added to the minced embryo, and the mixture was stirred intermittently for 30 minutes at room temperature and kept frozen at -60°C. Before use, the minced was thawed and centrifuged at 18,000 g for 60 minutes at 4°C. The supernatant was spun again at 100,000 g for 90 minutes at 4°C and the resulting supernatant (EE) was kept frozen at -20°C.

Fractionation of Embryo Extract

Ammonium Sulfate Fractionation: To EE (15 mg protein/ml), solid ammonium sulfate was slowly added with constant stirring at 0°C until a given concentration was reached. The suspension was centrifuged at 20,000 g for 30 minutes and the supernatant was again precipitated by the addition of ammonium sulfate to acquire desired fraction.

The pellets were dissolved in distilled water and dialysed at 4°C against 5 changes of 40 volumes of EBSS in a capped flask for at least 6 hours each time.

Sephadex G-75 Chromatography: Embryo extract was loaded on a Sephadex G-75 (mesh size 40-120 μm, Pharmacia) column (1.7×38 cm) equilibrated with 7-fold diluted phosphate buffered saline (PBS, pH 7.0). Proteins were eluted with the same buffer at a flow rate of 2.4 ml/cm^2/hour at 4°C. Before elution, samples were filtered through a Millipore membrane (nominal pore size 0.45 μm) to remove any insoluble material. The resulting samples were freeze-dried and then dissolved with distilled water to their original PBS concentration. Each fraction was sterilized through a Millipore membrane.

Fractionation of EE by Ultrafiltration: EE was fractionated by filtration on a PM 10 Diaflo membrane (molecular weight cutoff of 10,000, Amicon) under nitrogen pressure at 0°C. To facilitate the fractionation, EE was diluted with distilled water. After the fractionation, the filtrate was concentrated to original volume.

Microscopic Observation

At the indicated time during the culture, cells were washed twice with PBS and fixed

for 10 minutes in a mixture of 95% ethanol, 40% formalin, and acetic acid in ratio of 20:2:1 (v/v). After rinsing with distilled water, the cells were stained with Azure II.

Cell proliferation was estimated under a microscope by scoring the number of nuclei in randomly chosen several fields. Fusion index was expressed as the percentage of the number of nuclei within myotubes to the total number of nuclei. The nuclei were assumed to be in myotubes if no evidence of a cell membrane could be seen betweeen them and if there were more than 3 nuclei in a myotube.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To prepare samples for electrophoresis, the fractionated EE that contained 2% SDS, 2% glycerol, 2% 2-mercaptoethanol, and 0.01% bromophenol blue was heated at 100°C for 3 minutes. Electrophoresis was performed essentially according to Laemmli (1970) by using 12% polyacrylamide slab gel. After the electrophoresis, gels were stained with 0.2% Coomassie blue solution containing 50% methanol and 10% acetic acid, and were destained in a mixture of 35% methanol and 10% acetic acid.

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

The Effects of Embryo Extract on the Proliferation and Fusion of Myoblasts

To examine the effects of the concentration of EE on the proliferation and fusion of myoblasts, cells were grown in 811 medium for 24 hours and the medium was then changed to media containing different concentrations of EE.

Fig. 1A shows that the fusion of myoblasts cultured in the medium with higher concentration of EE commenced later than cells grown in lower concentration of EE. Cells in the medium containing 2% EE (medium 8102) initiated fusion at about 44 hours, and the fusion reached stationary phase within 12 hours. On the other hand, cells in the medium containing 10% EE (medium 811) initiated fusion about 15 hours later than cells in 8102, and abrupt fusion occurred within short time thereafter.

Fig. 1B shows the effect of the EE concentration on the proliferation of cultured myoblasts. As increasing the concentration of EE in the culture medium, all proliferation was enhanced in a dose-dependent fashion. Comparing Fig. 1A and 1B, it is evident that cells in 8102 medium give a similar pattern of proliferation and fusion profile. Before reaching maximum level of fusion, the cells kept proliferating. After fusion reached the maximum value, there was no further increase in the number of cells.

The present observation that higher concentration of EE enhances cell proliferation and delays fusion is consistent with those reported by others (Konigsberg, 1971; Slater, 1976). These results suggest that EE contains some factor(s) responsible for promoting proliferation and/or delaying fusion of myoblasts.

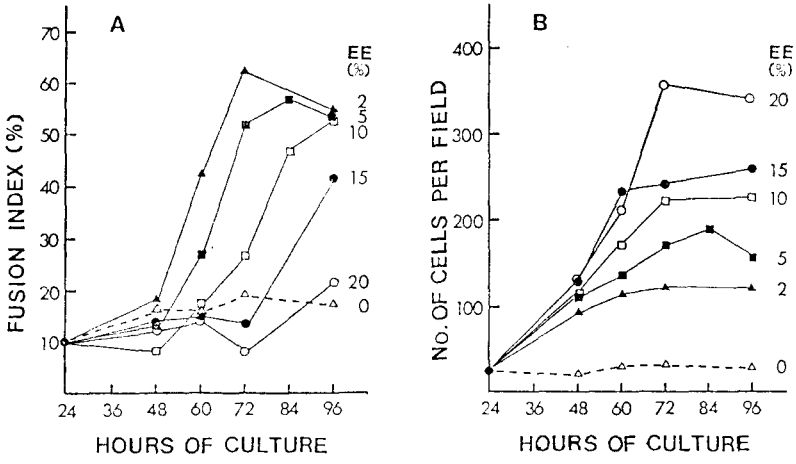

Fig. 1. The effects of concentration of the embryo extract on proliferation and fusion of chick embryonic myoblasts in culture. Cells were plated at a density of approximately 7.9×10^4 cells/cm^2. At 24 hours after plating, the medium 811 was changed to medium with different concentration of EE (2~20%). At the time indicated, cells were fixed, stained, and observed under a microscope. A, fusion index; B, average number of cells per field.

The Effects of Low Molecular Weight Fraction (MW<10,000) of Embryo Extract on the Proliferation and Fusion of Myoblasts

The embryo extract was ultrafiltered through a Diaflo membrane filter(molecular weight cutoff 10,000). The filtrate containing proteins of molecular weight below 10,000 was added at final concentration of 2 or 10% instead of the unfiltered EE.

Addition of this low molecular weight fraction to the culture media in the concentration of 2% had no detectable effects on both proliferation and fusion of myoblasts, although at higher concentration (10%) fusion was slightly promoted (Table 1). Thus, it is evident that factor(s) in EE having cell-proliferating and/or fusion-inducing activity has molecular weight higher than 10,000 daltons.

Table 1. The effect of low molecular weight fraction of embryo extract on the proliferation and fusion of chick embryonic myoblasts in culture.

	Control (no embryo extract added)	Unfiltered embryo extract		Ultrafiltered embryo extract	
		2%	10%	2%	10%
No. of total nuclei per field	62	839	1,522	66	75
Fusion index(%)	8.0	64.4	48.2	9.0	25.3

Cells at the initial density of 3.6×10^4 cells/cm^2 were grown in the medium 811 for first 28 hours and then in the same medium but contained the size-fractionated EE obtained from the ultrafiltration (see text). After total 91 hours of culture, the cells were stained and scored.

The Effects of Fractions of Embryo Extract Separated by Sephadex G-75 on the Proliferation and Fusion of Myoblasts

To identify factor(s) which is possibly implicated in the myoblast differentiation, EE was fractionated by Sephadex G-75 and each fraction was added to the culture media instead of whole EE.

Protein profile of the eluent showed two major peaks; one in void volume and the other in bed volume(Fig. 2). As shown in the right side of Fig. 2, the culture in the medium 811 (c) gave higher number of cells and lower fusion index than those in the medium 8102 (b). The fraction 15 showed high activity of stimulating the cell proliferation but little activity of promoting cell fusion. Fractions from 18 to 21 increased evidently both the proliferation and the fusion of myoblasts. As the number of fraction increased (i.e. toward fractions having lower molecular weights), the fusion-promoting activity was higher than the mitogenic activity, and fraction 21 showed much lower number of cells and the highest fusion index, a tendency similar to those in the medium 8102. Therefore,

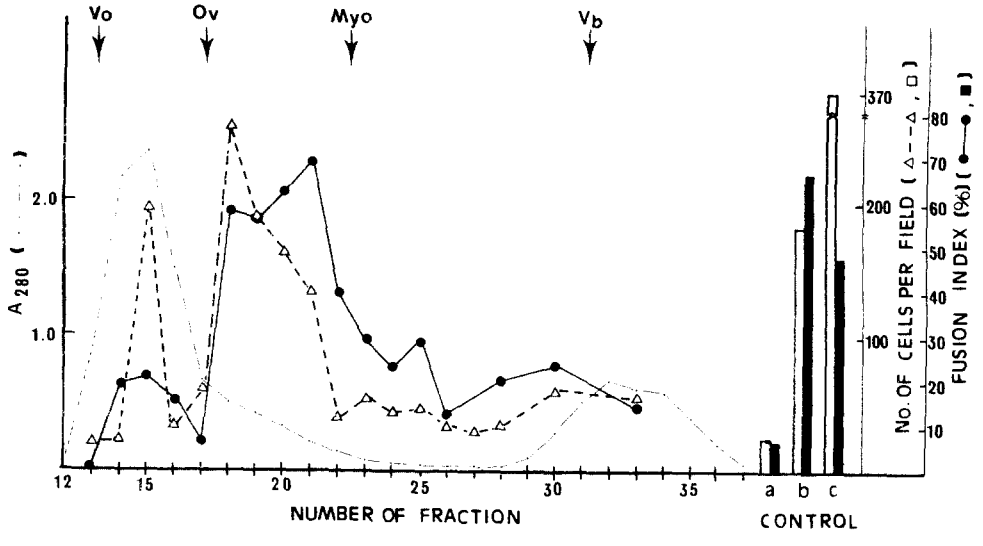

Fig. 2. The effects of fractions of embryo extract on the proliferation and fusion of chick embryonic myoblasts in culture. 1.4 ml of EE (22 mg protein) was loaded onto a Sephadex G-75 column (1.7×38 cm), and the proteins were eluted with 7-fold diluted PBS. Each fraction (2.9 ml) obtained was concentrated to the original concentration of PBS and added to the culture medium. Protein profile of the eluent was estimated by measuring the absorbance at 280 nm.

Cells were plated at a density of 3.6×10^4 cells per cm^2 in 811 medium. At 28 hour after the plating, the medium was changed to MEM containing 10% horse serum, 1% antibiotic-antimycotic and 17% (because the fraction was much diluted during the filtration) fractionated EE. To the control culture the same amount of PBS was added. After total culture of 91 hours, cells were stained. The molecular weight markers (ov, ovalbumin 45 Kd; myo, myoglobin 17.8 Kd) used in calibrating the column are indicated by arrows. V_o, void volumn; V_b, bed volumn. a, cells in the medium containing no embryo extract; b, cells in medium 8102; c, cells in medium 811.

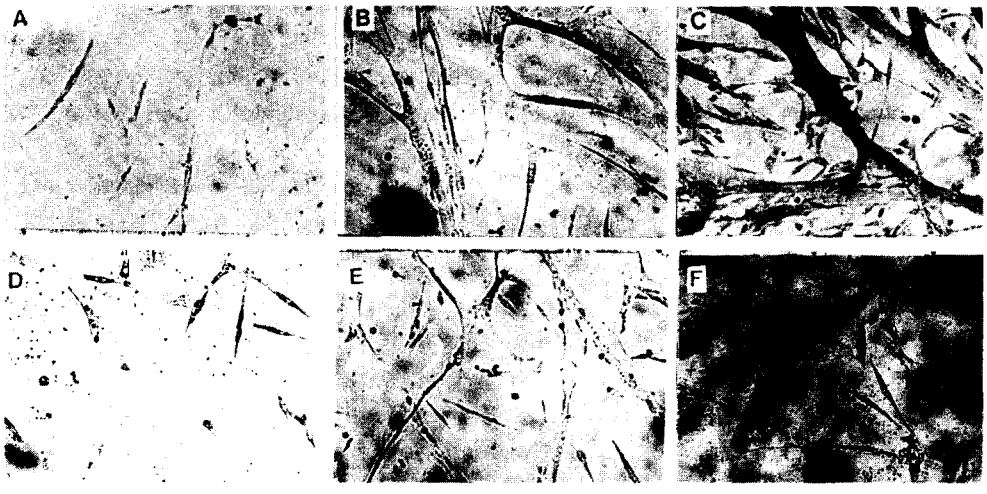

Fig. 3. The effects of fraction of embryo extract obtained from Sephadex G-75 on the proliferation and fusion of chick embryonic myoblasts in culture. For the details see the legend to Fig. 2. A, medium containing no embryo extract; B, medium 8102; C, medium 811; D, E, F, media containing fraction 14, 19 and 27, respectively, instead of unfractionated EE.

it is likely that any factor(s) implicated in the differentiation of the myoblasts is contained in the fractions between 17 and 22. This suggests that cell-proliferating factor and fusion-inducing factor in EE may be proteins having similar molecular weight, if not the same molecule. These proteins were found to have molecular weights in the range of 22,000 to 40,000 daltons, as judged by elution profile of standard proteins.

Since most of the proteins in EE were eluted in the void volume of the sephadex G-75 column, it is of noteworth that this step provides a good purification step to isolate fusogenic and mitogenic factors.

Fig. 3 shows more clearly the effects of different concentrations of EE and of fractions obtained from Sephadex G-75.

In the present experiment, EE was fractionated on Sephadex G-75 column by eluting

Fig. 4. The effects of tonicity of media on the viability and fusion of chick embryonic myoblasts in culture. Cells were plated in a density of 3.6×10^4 cells/cm^2 in 811 medium. At 28 hour after plating, the medium was changed to normal medium (8102, A), 5% hypomedium (B), and 10% hypomedium (C). Microphotographs were taken after total 96 hours of culture.

the proteins with 7-fold diluted PBS, and the eluted fraction was concentrated to the original concentration of PBS. However, the final tonicity of each fraction might have changed during these and following lyophilization processes. Therefore, the effect of tonicity on the viability and fusion of myoblasts was examined by adding distilled water to normal medium to a final concentration of 5 or 10%.

As shown in Fig. 4, there was no morphological difference between these diluted media and control media. Therefore, the results mentioned so far seem not due to erroneous tonicity.

The protein pattern of each fraction obtained by Sephadex G-75 chromatography was analyzed by SDS-PAGE. Significance of the data will be discussed later.

The Effects of Fractions of Embryo Extract Separated by Ammonium Sulfate Precipitation.

To identify factor(s) in EE which is responsible for the myoblast differentiation, EE was fractionated by ammonium sulfate, as described in "Materials and Methods."

The fraction precipitated in ammonium sulfate solution of higher than 60% saturation enhanced both the proliferation and fusion of myoblasts (Fig. 6). Furthermore, the degree

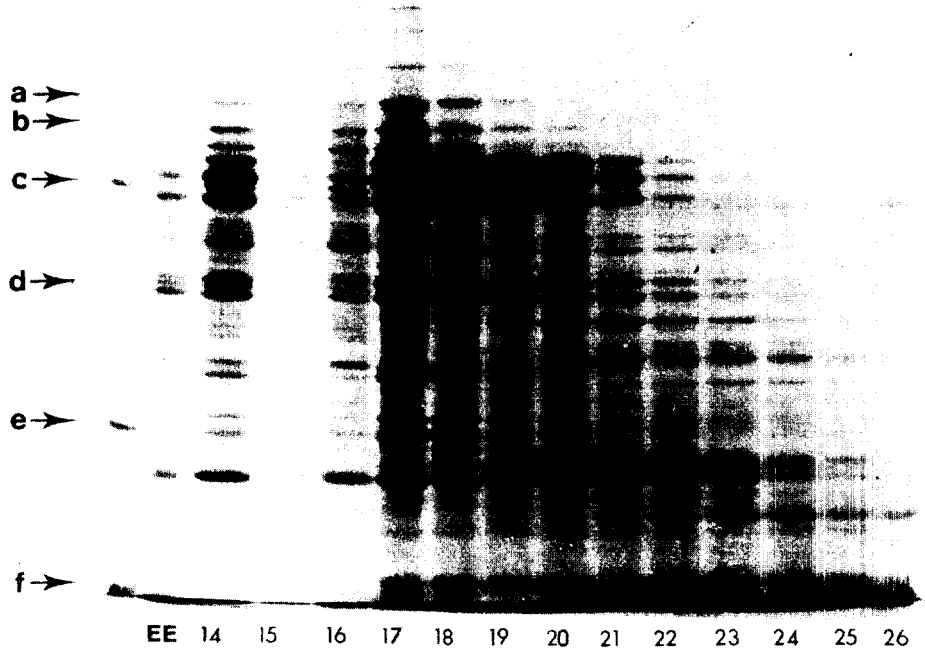

Fig. 5. The protein pattern of fractions of chick embryo extract separated by Sephadex G-75. Each fraction was electrophoresed on 12% polyacrylamide gel. Numbers indicated under each lane represent the fraction numbers eluted from Sephadex G-75 column. EE, embryo extract; a, β-galactosidase (116 K); b, phosphorylase b (97.4 K); c, albumin (66 K); d, ovalbumin (45 K); e, carbonic anhydrase (29 K); f, β-lactoglobulin (18.4 K).

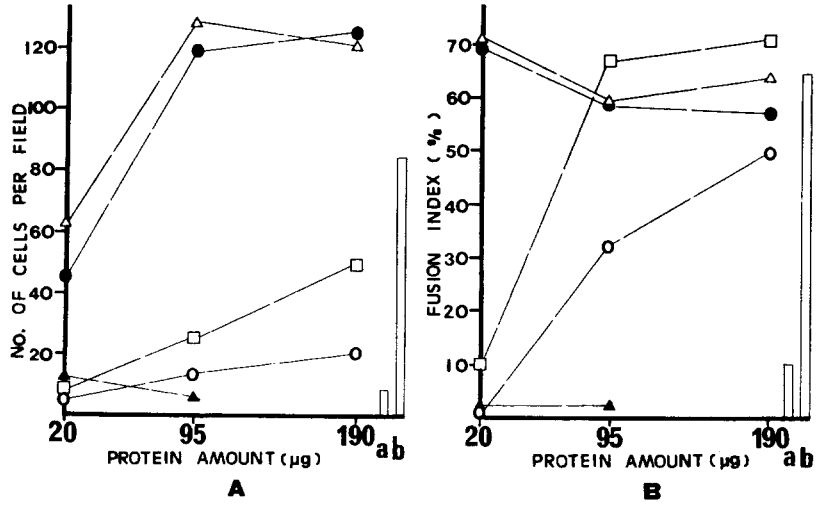

Fig. 6. The effects of fractions of embryo extract obtained by ammonium sulfate precipitation on the fusion and proliferation of chick embryonic myoblasts in culture. EE was fractioned by adding solid ammonium sulfate to final concentration of 40% (▲), 40~50% (○), 50~60% (□), 60~70% (●), and 70 ~95% (△). Cells were plated at a density of 3.6×10^4 cells per cm^2 in 811 medium. After 28 hours, the medium was changed to 0.3 ml MEM containing 10% horse serum, 1% antibiotic-antimycotic, and one of the fractions of EE. The fractions of EE contained 2, 95, or 190 μg proteins. The protein amount of EE in 8102 was 95 μg/0.3 ml. After total 91 hours of culture cells were stained and scored. A, cell proliferation; B, fusion index. a, medium containing no embryo extract; b, 8102 medium.

of enhancement of this fraction was higher than that of unfractionated whole EE. Comparing the effects of fractions with those of control on the basis of protein concentration which was 95 μg in the control medium (medium 8102) per well, cells cultured in media with fraction of above 60% saturation proliferated more but fused less than those in control 8102 media. Thus, the ammonium sulfate precipitation is useful step to enrich factor(s) implicated in the myoblast proliferation and fusion.

Proteins precipitated in 40~50% and 50~60% saturated ammonium sulfate solution also induced the fusion of myoblasts when they were added to the culture at the concentration of 95 or 190 μg. This effect could be due to the contamination of the same factor (s) that existed in 60~95% fraction, or alternatively due to the presence of another factor(s) which induces the myoblast fusion.

To test if the fraction obtained in 60~70% saturated ammonium sulfate solution has a possible factor having molecular weight between 22,000 and 40,000 daltons, as obtained by direct chromatography of EE on Sephadex G-75, the ammonium sulfate fraction was further fractionated on the same Sephadex G-75 column. The elution pattern(Fig. 7) was similar to that shown in Fig. 2. Effective fractions appeared just behind the protein-rich void volume, although the width of peak was broader than that of Fig. 2. When the

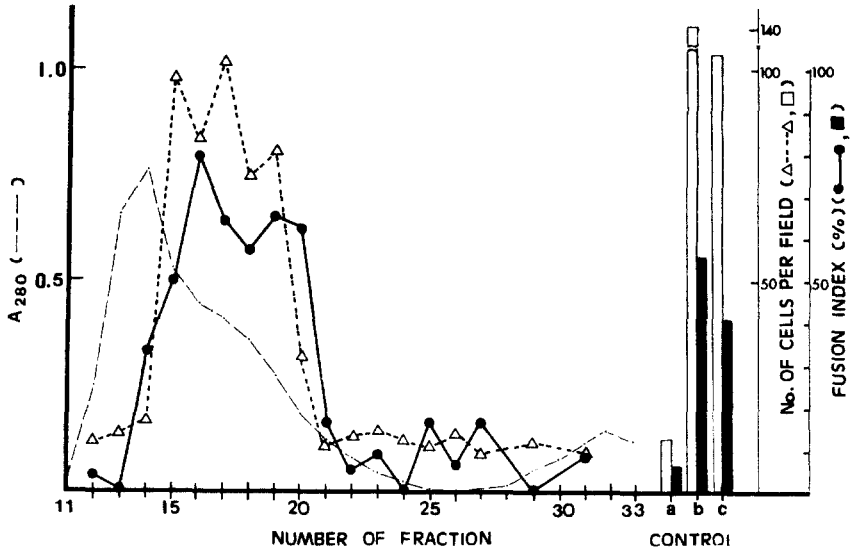

Fig. 7. The effects of fractions of embryo extract, obtained by combining ammonium sulfate precipitation and Sephadex G-75 chromatography, on the proliferation and fusion of chick embryonic myoblasts in culture. The fraction obtained in 60~70% saturated ammonium sulfate solution was further fractionated on Sephadex G-75. Assay was performed as described in the legend to Fig. 2. a, medium containing no embryo extract; b, medium 8102; c, medium 811.

eluent (fraction number 15~20) was added to the culture medium, both proliferation and fusion of myoblasts occurred concurrently.

These observations indicate that the factor(s) in EE responsible for the differentiation of myoblasts is precipitated in the ammonium sulfate solution of 60% saturation.

Fractions obtained from the Sephadex G-75 after the 60% ammonium sulfate saturation was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). The band pattern of Fig. 8 is very similar to that of Fig. 5, and there was no characteristic band which appeared in the fractions effective to the myoblast differentiation. Therefore, it seems that the effective factor(s) in EE, if present, should be very small in the amount to be dectected by the gel.

DISCUSSION

Numerous studies on myogenesis *in vitro* have shown that the cell proliferation preceding myotube formation is not rigidly fixed but influenced by the composition of the culture medium and the initial cell density (Okazaki and Holtzer, 1966; Yaffe, 1971; O'Neill and Stockdale, 1972), and that the intrinsic change in the composition of the medium occurred during the time of these cultures (Konigsberg, 1971; Hauschka, 1974). The molecule(s) implicated in the cell differentiation must be present in either of horse

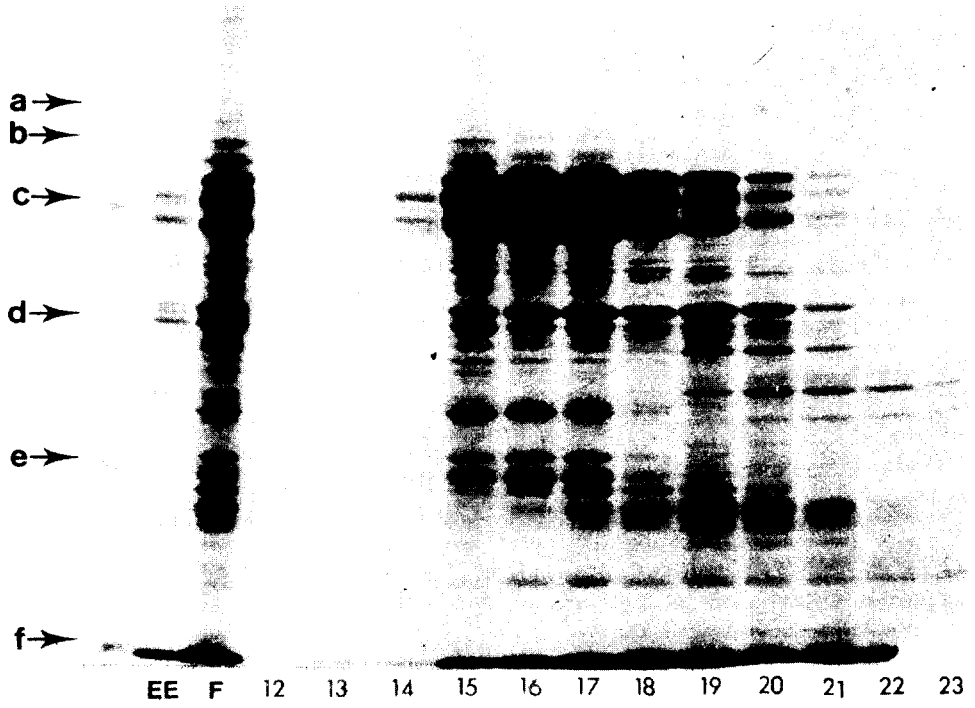

Fig. 8. The protein composition of fraction of embryo extract separated by 60~70% saturated ammonium sulfate solution followed by chromatography on Sephadex G-75. The numbers under each lane indicate the fraction number eluted. EE, embryo extract; F, fraction by ammonium sulfate before chromatography. a, β-galactosidase (116 K); b, phosphorylase b (97.4 K); c, albumin (66 K); d, ovalbumin (45 K); e, carbonic anhydrase (29 K); f, β-lactoglobulin (18.4 K).

serum or embryo extract, because only these materials caused the alterations in the time of cell differentiation when added to the culture medium (Slater, 1976; Linkhart *et al.*, 1981).

On the other hand, Doering and Fischman (1977) reported that muscle cells in culture secreted substances that could induce the fusion of myoblasts. Even in this case, some factor(s) must be present in the horse serum and in EE that is implicated in the control of the myoblast differentiation because adding EE to muscle-conditioned medium also delayed the fusion of myoblasts (Linkhart *et al.*, 1981).

Embryo extract may have two classes of factors; one class acting to promote proliferation and the other acting to prevent fusion (Slater, 1976). Recently, the later factor was discovered from the materials secreted into the medium by the rat liver cell line by Evinger-Hodges *et al.* (1982). They suggested that this material is identical to the fusion inhibitor in EE, but the suggestion has not been ascertained.

Linkhart *et al.* (1981) showed that EE had specific mitogenic activity which stimulated the proliferation of mouse myoblasts and prevented them from fusion, and that the myo-

blasts eliminated this specific mitogenic activity from the culture medium before fusion. They suggested that the depletion of this specific mitogenic activity from the medium triggers extensive myotube formation and synthesis of muscle-specific gene products. However, it is not certain whether the depletion of the mitogenic factor(s) was due to the materials secreted by myoblasts. The depletion of the mitogenic factors from the culture medium would lead to a prolonged replication time of the cells and to a large protraction of G_1 stage (Konigsberg *et al.*, 1978). Because myoblasts fuse only at G_1 stage, it was suggested that below some threshold level of mitogenic factors, cells cycled more slowly and the probability to fuse each other increased (Konigsberg, 1982).

However, EE may also contain another factor(s) inducing fusion of myoblasts. Fusion of myoblasts does occur in the absence of EE, although the extent of the myotube formation varies with different batch of horse serum used. If mitogen by itself is implicated in muscle cell differentiation, fusion of cells cultured in the presence of serum without EE must occur more massively than that of culture in the presence of both EE and serum since serum contains less mitogen(s) than EE. However, it is obviously not the case. Therefore, in addition to mitogenic factor(s) there must exist myoblast fusion-allowing molecule(s) in EE. The fusion of myoblasts might then be delayed by the mitogenic factor(s) in EE even under sufficient fusion-competent conditions, because the mitogenic factor enhances the proliferation of cells. Linkhart *et al.* (1981) reported that when purified fibroblast growth factor having mitogenic activity was added to muscle-conditioned media, the cell proliferation was stimulated and fusion was delayed.

The fractionation of EE by Sephadex G-75 revealed that the fractions effective in inducing myoblast fusion nearly coincided with the fractions that were enhancing myoblast proliferation (Fig. 2). If fusion inducing factor(s) is present in EE in addition to mitogenic factor, this factor seems to have similar molecular weight as mitogenic factor.

When EE was fractionated by ammonium sulfate precipitation, it was shown that fractions obtained in ammonium sulfate solutions of 40～50% and 50～60% saturation increased the rate of fusion of myoblasts, provided that the protein content was high enough (Fig. 6). This observation leads to an assumption that a fusion-allowing protein(s) may be present in these fractions.

Many attempts have been made to isolate from EE substances effective to the cell differentiation. De la Haba and Amundsen (1972) showed that retendate of 50,000 dalton cutoff ultrafiltration had activity that promoted fusion of myoblasts while the filtrate had activity that promoted the formation of myotubes. But they did not analyze the mitogenic activity in their report.

Recently, Ii *et al.* (1982) purified transferrin (80 K daltons) from chick EE and showed that this molecule when added in place of EE promoted the proliferation and fusion of chick myoblasts. On the other hand, Markelonis *et al.* (1980) isolated a myotrophic protein of 84 K daltons from chick sciatic nerve and reported that this protein, sciatin as they

named, was an essential component of EE for chick myogenesis. Popiela and Ellis (1981) isolated a protein of 80 K daltons from chick ischiatic-peroneal nerves which promoted myoblast proliferation. These two proteins resemble transferrin in many aspects. The molecular weights and isoelectric points are close to those of transferrin. The fractions of EE obtained by Sephadex G-75 chromatography (Fig. 2) which were effective to differentiation of myoblast in the present study seem to contain proteins different from these proteins, because of the discrepancy in their molecular weights.

The present experiment showed a possibility of purifying the factor(s) in EE which is implicated in controlling the time of myoblast differentiation. To compare transferrin with the effective proteins in our fractions and to elucidate the nature of control mechanism that proliferating myoblasts differentiate into muscle fiber, this factor(s) in EE must be purified further.

ABSTRACT

In order to find factors which are essential for the differentiation of chick embryonic myoblasts in culture, chick embryo extract was fractionated by ammonium sulfate or/and Sephadex G-75, and the effects of each fraction on the proliferation and fusion of the myoblasts were examined. The results obtained were as follows:

(1) High concentration of embryo extract in the culture medium enhanced the cell proliferation and delayed the fusion of myoblasts.

(2) The Sephadex G-75 fractions of embryo extract having proteins of molecular weight between 40,000 and 22,000 enhanced the proliferation and fusion of myoblasts when added to culture media.

(3) The fraction of embryo extract precipitated in 60~95% saturated ammonium sulfate solution enhanced evidently both the proliferation and fusion of myoblasts. Elution of this effective fraction by Sephadex G-75 showed similar elution profile and effects on the myoblast differentiation as those observed by Sephadex G-75 chromatography of the whole embryo extract, suggesting that the Sephadex fractions and ammonium sulfate fractions contain the same factors that enhance the proliferation and fusion of myoblasts.

REFERENCES

Doering, J.L. and D.A. Fishman, 1974. The *in vitro* cell fusion of embryonic chick muscle without DNA synthesis. *Devel. Biol.* **36**:225-235.

Doering, J.L. and D.A. Fishman, 1977. A fusion-promoting macromolecular factor in muscle conditioned medium. *Exp. Cell Res.* **105**:437-443.

De la Haba, G. and R. Amundsen, 1972. The contribution of embryo extract to myogenesis of avian striated muscle *in vitro*. *Proc. Natl. Acad. Sci.*, USA **69**:1131-1135.

Devlin, R.B. and C.P. Emerson, 1978. Coordinate regulation of contractile protein synthesis during

myoblast differentiation. *Cell* **13**:599-611.

Evinger-Hodges, M.J., D.Z. Ewton, S.C. Seifert, and J.R. Florini, 1982. Inhibition of myoblast differentiation *in vitro* by a protein isolated from liver cell medium. *J. Cell Biol.* **93**:395-401.

Ha, D.B., R. Boland, and A. Martonosi, 1979. Synthesis of the calcium transport ATPase of sarcoplasmic reticulum and other muscle proteins during development of muscle cells *in vivo* and *in vitro*. *Biochim. Biophys. Acta* **585**:165-187.

Hauschka, S.D., 1974. Clonal analysis of vertebrate myogenesis. II. Environmental influence upon human muscle differentiation. *Devel. Biol.* **37**:329-344.

Ii, I., I. Kimura, and E. Ozawa, 1982. A myotrophic protein from chick embryo extract: its purification, identity to transferrin, and indispensability for avian myogenesis. *Devel. Biol.* **94**: 366-377.

Konigsberg, I.R., 1971. Diffusion-mediated control of myoblast fusion. *Devel. Biol.* **26**:133-152.

Konigsberg, I.R., 1979. Skeletal myoblasts in culture. *Methods in Enzymol.* **58**:511-527.

Konigsberg, I.R., 1982. Progression through G_1 and myogenic differentiation *in vitro*. In: *Differentiation in vitro* (ed. by M.M. Yeoman and D.E.S. Truman) Cambridge University Press, pp. 141-182.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**:680-685.

Linkhart, T.A., C.H. Clegg, and S.D. Hauschka, 1981. Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromalecular growth factors in the culture medium. *Devel. Biol.* **86**:19-30.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and H.J. Randell, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.

Markelonis, G.J., V.F. Kemerer, and T.H. Oh, 1980. Sciatin: purification and characterization of a myotrophic protein from chicken sciatic nerves. *J. Biol. Chem.* **255**:8967-8970.

Nadal-Ginard, B., 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15**:855-864.

Oh, T.H. and G.J. Markelonis, 1980. Dependence of *in vitro* myogenesis on a trophic protein present in chicken embryo extract. *Proc. Natl. Acad. Sci., USA* **77**:6922-6925.

Okazaki, K. and H. Holtzer, 1966. Myogenesis: fusion, myosin synthesis, and the mitotic cycle. *Proc. Natl. Acad. Sci.*, USA **56**:1484-1490.

O'Neill, M.C. and F.E. Stockdale, 1972. A kinetic analysis of myogenesis *in vitro*. *J. Cell Biol.* **52**:52-65.

Popiela, H. and S. Ellis, 1981. Neurotrophic factor: characterization and partial purification. *Devel. Biol.* **83**:266-277.

Slater, C.R., 1976. Control of myogenesis *in vitro* by chick embryo extract. *Devel. Biol.* **50**: 264-284.

Yaffe, D., 1971. Developmental changes preceding cell fusion during muscle differentiation *in vitro*. *Exp. Cell Res.* **66**:33-48.