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The Presence in Embryo Extract of a Myotrophic Protein That Affects Proliferation and Fusion of Chick Embryonic Myoblasts in Culture

Byung Je Yoo*, Chang Ho Lee, Kyu Bong Kwak, Chin Ha Chung, and Doo Bong Ha

*Dept. of Zoology, Seoul National University, Seoul 151-742; *Dept. of Biology, Taegu University, Taegu 705-030, Korea*

A myotrophic protein that seemed to be essential for the fusion of chick embryonic myoblasts in culture was isolated from chick embryo extract and was found to be identical or at least similar to the iron-transporting protein, transferrin. Embryo extract seemed to contain, in addition to this myotrophic protein, a heat stable protein that inhibits the fusion of myoblasts.

Iron seemed to be necessary for myoblasts to fuse and it was supposed that the role of the myotrophic protein in myoblast fusion is to supply iron to the cell.

The number of the myotrophic protein receptors on myoblast surface membrane decreased immediately after the start of myoblast fusion, supposedly due to the decreased need of iron after the fusion once commenced.

It was estimated that endocytosis of myotrophic protein took about 10 minutes and one recycling about 2 hours.

The accumulation of iron in myoblasts continued linearly with culture time and endocytosis of the myotrophic protein occured at a constant rate.

KEY WORDS: Myoblast fusion, Myotrophic protein, Embryo extract

In the course of differentiation of skeletal muscle cells of chick embryo, mononucleated myoblasts fuse one another to form multinucleated and striated muscle fibers. Prior to or shortly after the fusion, various muscle specific proteins such as myosin and actin are synthesized and accumulated with a distinctive pattern which is characteristic of each protein (Ha *et al.*, 1983).

Many substances have been known to enhance or inhibit the fusion when added to the culture medium (Linkhart *et al.*, 1981; Minty *et al.*, 1981; Kent, 1982; Bersten *et al.*, 1983; Ha *et al.*, 1983). Chick embryo extract which is an usual component of culture medium for chick embryonic myoblasts is also known to contain substances that regulate the myoblast fusion. It has long been observed that high concentration of the extract in culture medium or the frequent medium change delays or inhibits the fusion. A 10% concentration of the extract keeps the cell proliferation but inhibits the fusion, while 1% concentration shows a reverse effect. Therefore, it has been suggested that certain fusion regulatory myotrophic substances are present in embryo extract (Konigsberg, 1971; Slater, 1976; Linkhart *et al.*, 1981; Evinger-Hodges *et al.*, 1982).

The present paper deals with the effect of embryo extract on the fusion of chick embryonic myoblast cells in culture and the isolation of a myotrophic protein from the extract. It is suggested in this paper that the myotrophic protein isolated is identical to the iron-transporting protein, transferrin. The changes in the quantity of the

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myotrophic protein receptors on the cell membrane during myoblast differentiation and the flux of the myotrophic protein across the cell membrane are also analyzed.

Materials and Methods

Myoblast Culture

Myoblasts were cultured as described elsewhere (Ha, *et al.*, 1985). Different culture media were designated with numbers representing their compositions. Thus, the medium 811 refers one that consists of 10% horse serum and 10% embryo extract in the minimal essential medium(MEM), the medium 8102 refers one of 10% horse serum and 2% embryo extract, and the medium 910 refers one of 10% horse serum and no embryo extract. Embryo extract was prepared as described elsewhere (Ha, *et al.*, 1985).

Isolation of Myotrophic Protein

To isolate the proteins having myotrophic activity, embryo extract was precipitated in 60% saturated ammonium sulfate solution and the supernatant was analyzed by two CM-cellulose and DEAE- cellulose column. One CM-cellulose column was equilibrated with pH 4.0 buffer solution of 10 mM sodium citrate and the other CM-cellulose column with a buffer of pH 6.0. Each DEAE-cellulose column was equilibrated with Tris acetate buffer of pH 7.0, 8.0 or 9.0. After equilibration, the supernatant from ammonium sulfate precipitation was loaded on the columns and the same buffer solutions were eluted to remove unbound proteins. Then the bound proteins were eluted with the same buffer but containing 0.5M NaCl and the eluents were collected, dialyzed against Earle's balanced salt solution(EBSS), and added to myoblast culture to test its myotrophic activity. Eluents collected from columns of pH 7.0, 8.0 and 9.0 were found to have the activity, and hence the eluent from pH 8.0 column was used for the isolation of the myotrophic protein in this experiment.

The proteins contained in the resulting eluent was fractionated by successively eluting with buffers containig NaCl of various final concentrations ranging from 0.1 to 0.5M, and each fraction was again tested for their myotrophic activity. It was apparent that the fraction eluted with 0.1M NaCl contained the myotrophic activity.

Based on the above preliminary experiment, proteins in the embryo extract were first fractionated by DEAE-cellulose column with a linear gradient of 0-0.1M NaCl and the fractions revealing the myotrophic activity were pooled and subjected to the second DEAE-cellulose column with a linear gradient of 20-70 mM NaCl for the further purification. The myotrophic protein thus obtained were run on SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and isoelectric focusing to check its purity.

Removal and Addition of Iron

Effect of iron on the differentiation of cultured myoblast was examined by dialyzing embryo exctract, horse serum, and the isolated myotrophic protein against a buffer consisting of 10 mM EDTA, 0.1M acetate, and 0.1M sodium bicarbonate (pH 4.5).

Addition of iron to the iron-free sample was made by dialysis against the buffer consisting of 1 mM ferric chloride, 0.1M sodium citrate and 0.1M sodium bicarbonate (pH 8.6). After Fe-feeding, free irons which were not bound to proteins, was removed by dialyzing the sample against EBSS of pH7.0. At this pH, iron is known not to dissociate from transferrin (Aisen and Listowsky, 1980).

Radioactive Labeling of Myotrophic Protein

The isolated protein was labeled with ^{125}I using chloramine-T (Sigma): One mCi of $Na^{125}I$(0.1 ml) was added to the protein (5 mg in 250 mM phos phate buffer, pH 7.6), and to this mixture 0.1 ml of chloramine-T (12 mg/ml) was added three times with 2 minute intervals. The reaction was then stopped by adding 0.3 ml of sodium metabisulfite (6 mg/ml) and 2 ml of KI (160 mg/ml). The labeled protein was dialyzed against 10 mM Hepes Hank's balanced salt solution (HBSS) at 4°C.

The myotrophic protein was also labeled with ^{59}Fe according to the method of Renswoude *et al.* (1982).

Analysis of the Myotrophic Protein Receptor

Transferrin is known to bind with its receptor

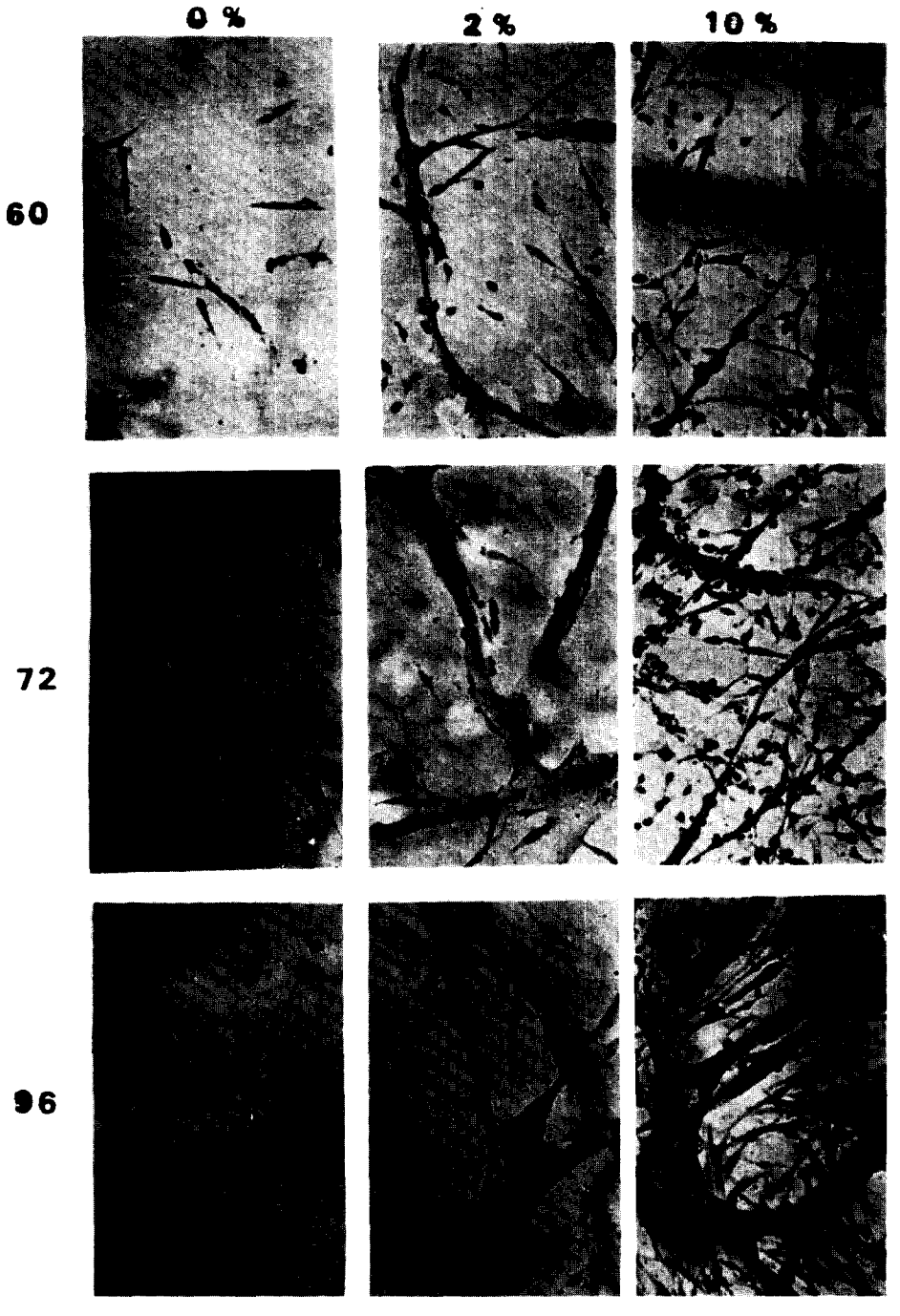

Fig. 1. Microphotographs of chick embryonic myoblasts cultured in the medium containing various concentrations of embryo extract. Percentages indicate the concentration of embryo extract and numbers on the left indicate the age of cells in hour.

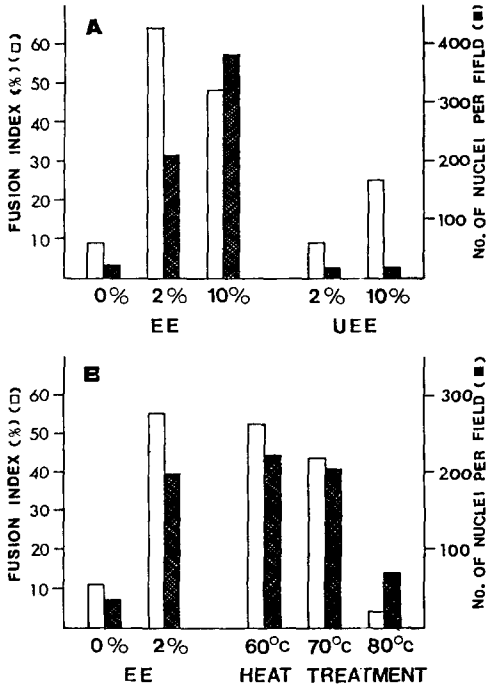

Fig. 2. Effects of ultrafiltration (A) and heat treatment (B) of embryo extract(EE) on the fusion and proliferation of cultured chick myoblasts. UEE indicates ultrafiltrate of EE(M.W. 10,000 cut-off). Embryo extract was heated at the indicated temperature for 10 minutes. After removing the denatured and precipitated proteins, the supernatant was added with 910 medium to the cells of 24 hour culture. At 91 hour of culture, the cells were stained and scored under a microscope.

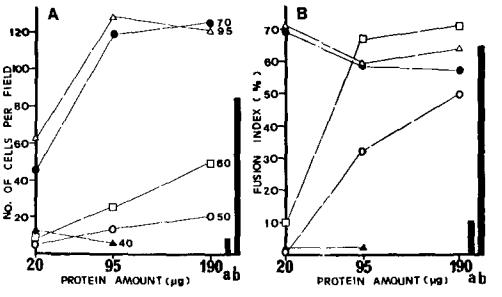

Fig. 3. Effects on the proliferation (A) and fusion (B) of cultured chick myoblasts by the fractions of embryo extract obtained from ammonium sulfate precipitation. Embryo extract was fractionated by adding solid ammonium sulfate to give a final concentration of 40% (▲), 40-50% (○), 50-60% (□), 60-70% (●), and 70-95% (△). Cells were initially plated at a density of 3.6 X 10^5 cells/ml in 811 medium. At 24 hours after the plating, the medium was changed to 910 (a) and 8102 (b).

but not endocytosed at temperatures at 0°C (Renswoude *et al.*, 1982). It has been reported that transferrin bound to the receptor releases its iron at pH below 5.5 and the resultant apo-transferrin dissociates from the receptor as raising pH near to 7 (Dautry-Varsat *et al.*, 1983).

Based on the above reports, the level of myotrophic protein receptor was estimated as follows. Cultured cells were washed with cold EBSS and left in 10 mM acetate Ringer solution (pH 5.0) for 15 min to release the protein bound to the receptor. The cells were then washed with 10 mM Hepes HBSS (pH 7.4) and incubated with the reaction buffer consisting of bovine serum albumin (1 mg/ml), the labeled myotrophic protein (3 μg/ml), and Hepes HBSS (pH 7.4) for 10 min. After the reaction, cells were washed again with 10 mM Hepes HBSS (pH 7.4) thoroughly, solubilized with 100% trichloroacetic acid (TCA) solution and counted for their radioactivity by a liquid scintillation counter.

The receptor of the myotrophic protein was also analyzed by microautoradiography on X-ray film: Cells of 24-hour culture were fed with the labeled myotrophic protein, cultured futher for a given time, washed with EBSS, fixed with ethanol-formalin-acetic acid (20:2:1), and microautoradiographed.

Transport of the Myotrophic Protein and Iron into Cell

To the cells cultured for 60 hours, the cells were added with labeled protein and were cultured further for a specfied time. They were then washed with EBSS five times and left in 10 mM acetate Ringer solution of pH 5.0 at 0°C for 15 min. The radioactivity, released into this solution was considered as the myotrophic protein bound to the receptor. On the other hand, cells adhered on the culture dish were washed with 10 mM Hepes HBSS (pH 7.4), dried, solubilized in 100% TCA, and used for quantitizing the protein and Fe transported into the cell by counting the radioactivity.

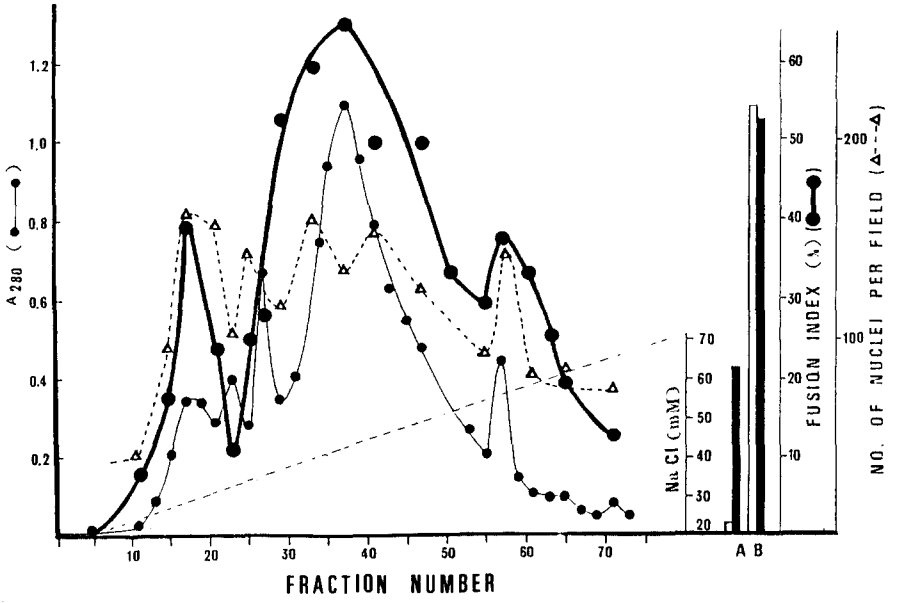

Fig. 4. Effects on the fusion and proliferation of cultured chick myoblasts by the fractions of embryo extract obtained from the 2nd DEAE-celloulose chromatography (see text). Closed bars on the right represent the degree of proliferation and open bars indicate the fusion index of myoblasts cultured in 910 medium(A) and in 8102 medium (B).

Results

Effect of Myotrophic Protein

Effect of embryo extract on the proliferation and fusion of chick embryonic myoblast cells in culture is shown in Fig. 1. As is generally known, the higher the concentration of extract, the higher was the rate of the cell proliferation and the lower was the fusion index (the ratio in % of the number of nuclei within myotubes to the total number of nuclei). This concentration effect had previously been observed in our laboratory (Ha *et al.*, 1985) but confirmed again in the present experiment

Since the presence of embryo extract in culture medium is essential for myoblast differentiation under the conditions used, the extract was ultrafiltrated and the fitrate was added to the culture to see if it contains any myotrophic protein. As shown in Fig. 2A, the filtrate (UEE), which lacked proteins of molecular weight higher than 10,000, had no effect on either proliferation or fusion, suggesting that the protein should have molecular weight above 10,000. It is found that this protein is relatively heat-stable (Fig. 2B).

When the extract was fractionated by ammonium sulfate precipitation, the myotrophic protein was found to present in the supernatant obtained with higher concentration of ammonium sulfate than 60% (Fig. 3).

In order to isolate the myotrophic protein, supernatant fraction of the embryo extract in 60% saturated ammonium sulfate was further fractionated by DEAE-cellulose ion exchange chromatography and each fraction was added to the culture in stead of unfractionated embryo extract.

As shown in Fig. 4, fractions of 17, 27—45, and 57 of the second DEAE-cellulose column chromatography gave cell proliferation and fusion as nearly normal as the control group, suggesting that the myotrophic proteins are contained in these fractions.

Therefore, proteins in these fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5) and isoelectric focusing (Fig. 6). As shown in Fig. 5, it was observed that a protein of molecular weight of approximately 76,000 occupied more than 90% of the total protein in every

fraction and, therefore, it seemed to be the candidate protein. The mobility of this protein was then found to be very similar to that of human transferrin.

This fraction was compared with untreated or heat-treated embryo extract for the myotrophic activity. The results are shown in Fig. 7A. The purified fraction revealed high fusion index from very low protein concentrations, and at above 1 μg/dish its effect reached a plateau. Untreated and heated embryo extracts under similar conditions showed a bell-shaped curve. On the other hand, the cell proliferation kept increasing with increasing concentrations of the myotrophic protein and of both extracts (Fig. 7B).

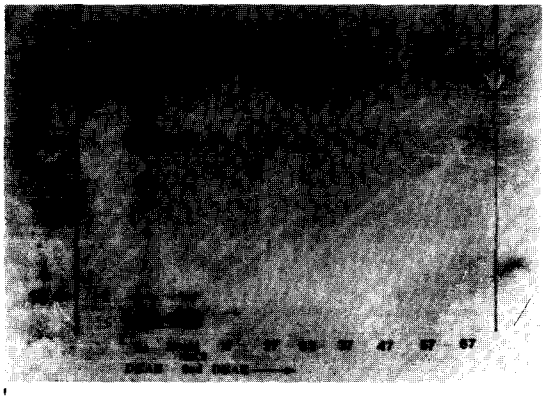

Fig. 5. Analysis of protein composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the fractions obtained from the 2nd DEAE-cellulose chromatography of the embryo extract. The 1st DEAE indicates the pooled fraction obtained from the 1st DEAE-cellulose column. 20 mM NaCl indictes the washing fraction of 2nd DEAE-cellulose column. Numbers under each lane are the fraction number of 2nd DEAE-cellulose chromatography.

Effect of Iron on the Activity of Myotrophic Protein

Since the electrophoretic patterns of the isolated myotrophic protein was very similar to that of transferrin and since transferrin has been reported to have myotrophic activity (Markelonis *et al*, 1980; Popiela and Ellis, 1981; Ii *et al*, 1982), the myotrophic effect of the protein was analyzed in the presence and in the absence of iron. Transferrin is well-known to transport iron into the cell. When the protein was added to the culture in the absence of Fe, both the cell proliferation and fusion index were very low, as low as those in the cells cultured without embryo extract (Fig. 8). When the iron was added, the proliferation and fusion resumed and reached the same level as the control system. Therefore, it is very likely that this protein is identical to transferrin.

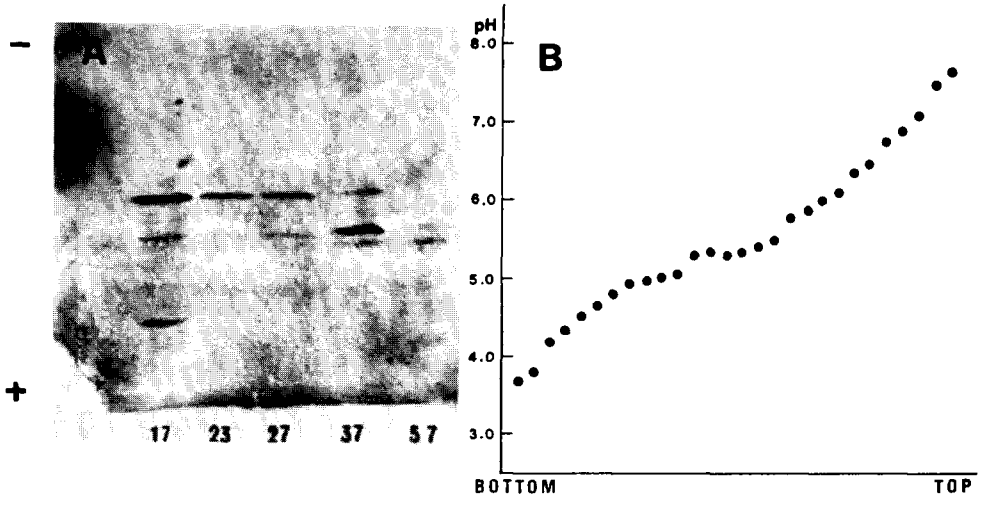

Fig. 6. Isoelectric focusing gel electrophoresis of the fractions of embryo extract obtained from 2nd DEAE-cellulose chromatography (A) and the profile of pH gradient (B). Numbers under each lane indicate the fraction number of 2nd DEAE-cellulose.

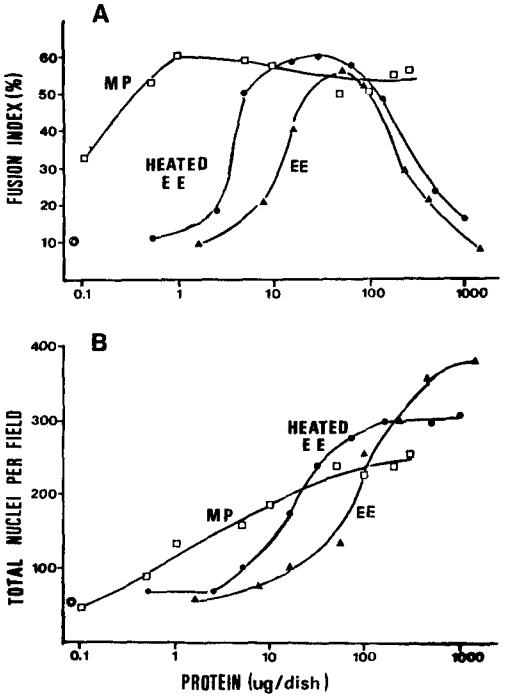

Fig. 7. Effects on the fusion (A) and proliferation (B) of cultured myoblasts by the purified myotrophic protein, the heated embryo extract and the untreated embryo extract at vrious concentrations. MP, the purified myotrophic protein; HEATED EE, the supernatant of heated embryo extract at 60°C for 10 minutes; EE, untreated embryo extract.

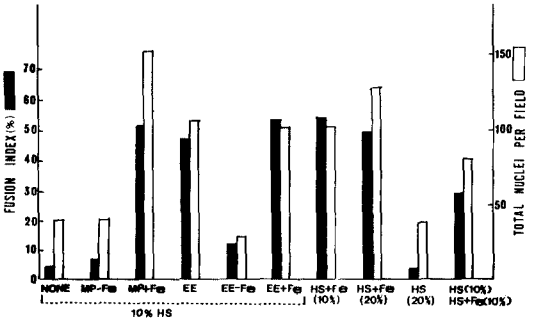

Fig. 8. Effects of iron on the fusion and proliferation of cultured myoblasts. At 24 hours of culture, 811 medium was changed with the media containing the substances indicated. At total 72 hours of culture, cells were stained and scored under a microscope. MP-Fe, the purified myotrophic protein deprived of Fe; MP+Fe, the purified myotrophic protein containing Fe; EE-Fe, EE deprived of Fe; EE+Fe, EE containing Fe; HS+Fe, horse serum containing Fe.

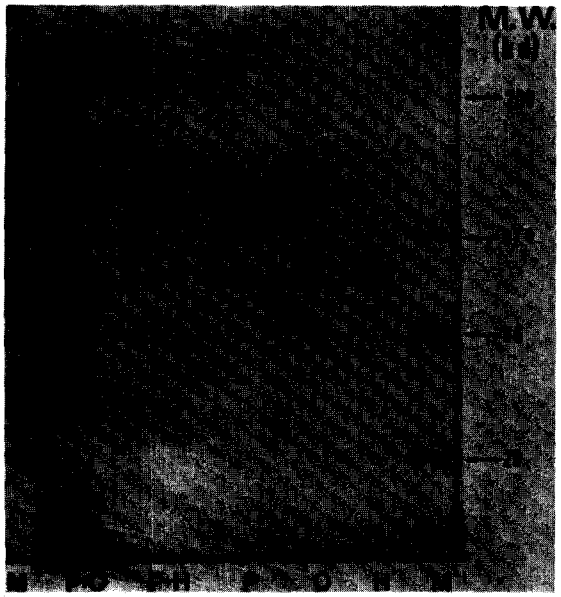

Fig. 9. Mobility of human transferrin, oval transferrin, and the purified myotrophic protein on sodium dodecyl sulfate gel electrophoresis. M, marker proteins; P+O, the purified myotrophic protein and oval transferrin; P+H, the purified myotrophic protein and human transferrin; P, the purified myotrophic protein; O, oval transferrin; H, human transferrin.

In order to confirm that the protein is identical to transferrin, it was electrophoresed together with human and egg transferrin. The myotrophic protein was inseparable from ovotransferrin (Fig. 9).

Receptor of Myotrophic Protein

Assuming that the isolated myotrophic protein is identical to transferrin, the quantitative change in the receptor of the myotrohpic protein in myoblast differentiation was analyzed using ^{59}Fe-labeled myotrophic protein. Fig. 10 reveals that the quantity of ^{59}Fe (as expressed cpm/mg protein) attached to the cell decreases abruptly during 48 to 72 hours of culture and gradually thereafter 72 hours. The period of such abrupt decrease coincides with the period of fusion.

The effect of embryo extract on the quantitative change in the receptor was analyzed and the results are shown in Fig. 11. Cells grown for 24 hours were transferred to the medium containing

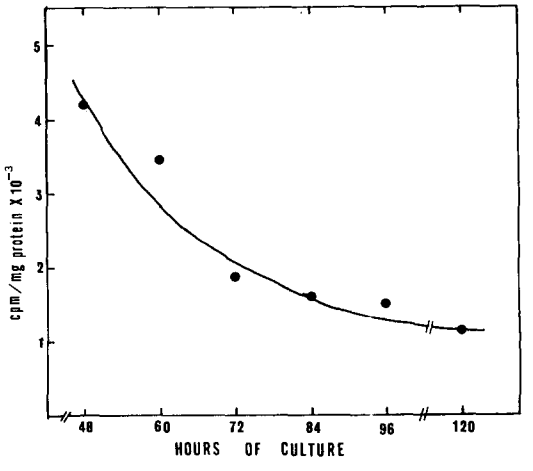

Fig. 10. Changes in the level of the myotrophic protein receptor in chick myoblasts cultured in 8102 medium for various times.

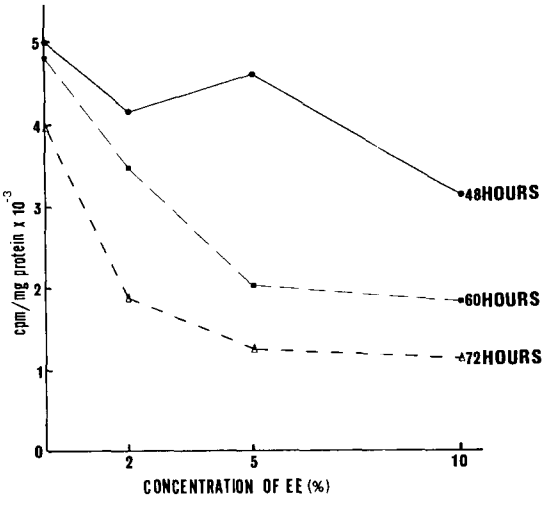

Fig. 11. Changes in the level of the myotrophic protein receptor in chick myoblasts cultured in the media containing various concentrations of embryo extract. The amount of the receptor is expressed as the radioactivity of ^{59}Fe.

embryo extract in the concentration of 0, 2%, 5% or 10%, and cultured further for specified times. The amount of the receptor, as expressed in radioactivity(cpm) per mg protein, was found to decrease as the concentration of embryo exctract increased and as the culture time prolonged.

Iron Transport and Internalization of Myotrophic Protein

Since transferrin is known to transport iron into the cell by binding with the receptor followed by endocytosis (Aisen and Listowcky, 1980; Renswoude *et al.*, 1982), the transport of the myotrophic protein across the cell membrane was examined by adding the labeled myotrophic protein with ^{59}Fe or ^{125}I to the cells cultured for 60 hours in normal medium and measuring each radioactivity. Fig. 12A shows that radioactivity of ^{59}Fe was detected in the cell after 10 min. and increased linearly with time. Fig. 12B shows that the amount of the bound myotrophic protein did not change after 10 min and that the amount of the myotrophic protein, which is internalized into the cell, increased constantly for 2 hours, after which it decreased.

Discussion

In the culture of chick embryonic myoblast cells, embryo extract is generally added in culture medium. When the concentration of embryo extract is higher, the fusion of myoblasts is known to be inhibited or retarded but the cells keep proliferating (Slater, 1976; Ha *et al.*, 1985). Without embryo extract in the culture medium myoblasts fail to proliferate and to fuse. Therefore, it is evident that embryo extract contains a myotrophic protein that is essential for the growth and differentiation of myoblasts.

The myotrophic protein isolated in the present experiment has obviously no inhibitory effect on the myoblast fusion, because the increase in its concentration in the culture medium did not cause any decrease in fusion index (Fig. 7A). On the other hand, the cell proliferation was increased as the concentration of the myotrophic protein increased (Fig. 7B), suggesting that it has an activity of stimulating cell growth. Since the fusion index is decreased by higher concentration of embryo extract or heat-treated embryo extract but not by excess myotrophic protein, it seems likely that embryo extract contains an inhibitor of myoblast

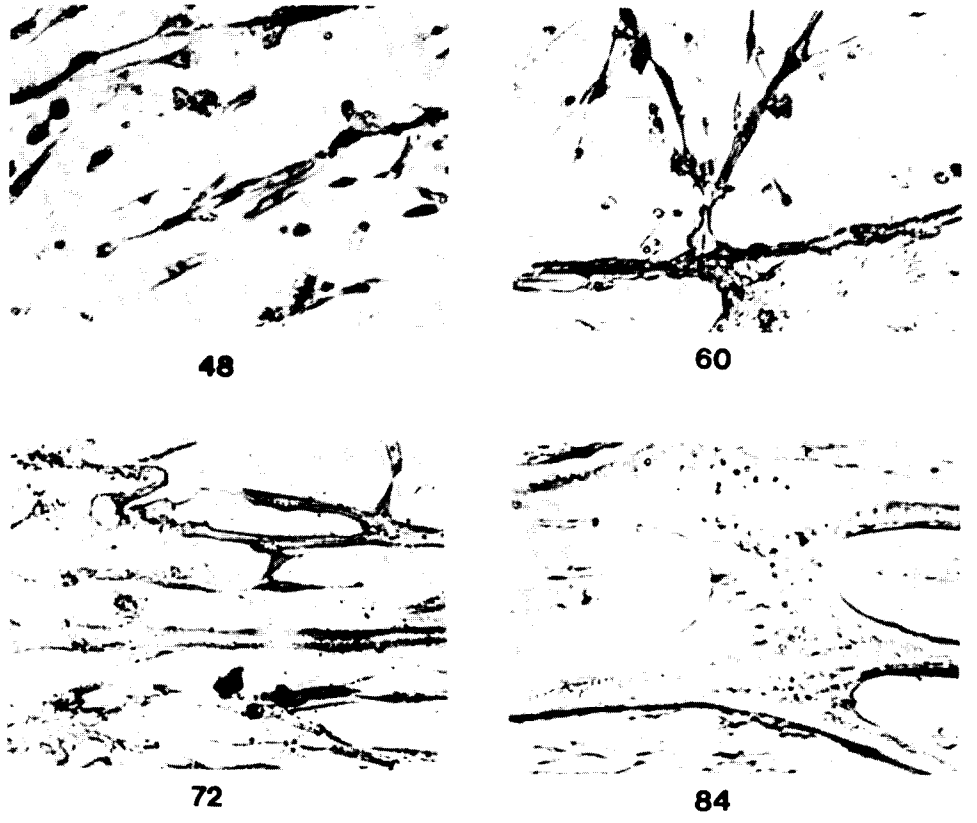

Fig. 12. Autoradiograms of chick myoblasts cultured for various times in 8102 medium containing the ^{59}Fe-labeled myotrophic protein. At 24 hours of culture, the protein labeled with ^{59}Fe was added to the medium and the culture was continued further for the indicated time.

fusion in addition to the myotrophic protein.

Since the isolated myotrophic protein behaved much like transferrin (Figs. 5 & 6), the effect of myotrophic protein on the myoblast fusion in the presence and absence of iron was studied (Fig. 8), and the results showed that the proliferation and fusion of myoblasts proceeded normally only when both myotrophic protein and iron were present. In this experiment the free iron in the myotrophic protein solution was thoroughly removed by dialysis (see Materials and Methods) before adding it to the culture. Therefore, all iron present was assumed to be bound to the myotrophic protein. Futhermore, horse serum, which is another component of the culture medium, obviously contain transferrin, but it is generally known that this transferrin is mostly in the form of apotransferrin. in fact, the apparent normal cell fusion was observed only when both horse serum and iron were present in this experiment.

Therefore, it is very probable that iron acts on the myoblast proliferation and fusion in the myotrophic protein-bound form, and such an effect appears to be mediated by the transport of iron into the cell.

In the culture where equal amounts of horse serum and iron-containing horse serum were added (Fig. 8; HS 10%; HS+Fe 10%), the degree of the proliferation and fusion fell in between that of 8102 medium (embryo extract in Fig. 8) and that of 910 medium (Fig. 2A, B). This result is interpreted as due to the competition of transferrin and apotransferrin for transferrin receptors. Since the myoblast proliferation and fusion increased as

the concentration of myotrophic protein increased (Fig. 7), the degree of the myoblast differentiation seems to depend on the concentration of myotrophic protein in the culture medium and, therefore, on the concentration of iron transported by myotrophic protein into the myoplasm.

The requirement of iron for myoblast differentiation has been indirectely suggested. For example, lysosomotrophic amines has been reported to inhibit myoblast differentiation (Kent, 1982). When the agents were added to culture medium, they accumulate in lysosomes and thereby raising the intralysosomal pH (Karin and Mintz, 1981; Renswoude et al., 1982). The rise in the pH of lysosomes could then inhibit the release of iron from transferrin which had been trapped in lysosome, and thus might inhibit myoblast differentiation.

Although, Shoji and Ozawa (1986) suggested that the iron might parcipitate in the regulation of RNA synthesis in chick myotubes, the precise role of iron in muscle differentiation is still not clear.

It has been widely observed that the cell fusion does not occur when the initial density of the cell in culture is below a certain limit (Yeoh and Holtzer, 1977), that the fusion is promoted when the density is high (Ha *et al.*, 1984), and that the frequent change of the culture medium retards the fusion (Konigsberg, 1971). These observations suggest that a fusion inhibitor is present in the culture medium and, when this inhibitor is depleted as the cells proliferate, the fusion is gradually induced (Evinger-Hodges *et al.*, 1982). Therefore, it is considered that embryo extract contains, in addition to the myotrophic protein, a substance that is inhibitory to the fusion. This view is supported by the present study (Fig. 7). For example, the excess embryo extract or heat-treated embryo extract caused a decrease in the fusion index while excess of the the isolated myotrophic protein did not. Furthermore, when embryo extract was added in excess, the fusion was inhibited in early period but resumed later (Ha *et al.*, 1985). The reason why both the cell proliferation and fusion increase as the content of embryo extract increases in spite of the possible coexistence of the myotrophic protein and fusion-inhibitor in the embryo extract might be that the fusion inhibitor is present in the embryo extract but in a low concentration and that when the content of embryo extract in the culture medium increases, the activity of myotrophic protein reaches the limit while the concentration of the inhibitor increases to a certain point where its activity becomes apparent.

The abrupt decrease in the number of receptors of the myotrophic protein during 48-72 hours of the culture (Fig. 10) suggests that iron is required prior to the fusion and therefore after the fusion the receptors are no longer needed. It is also possible that the ratio of membrane protein to the total protein will decrease as cells fuse one another and hence the number of receptors per unit amount of protein will correspondingly decrease as much as the receptors are located on the membrane. Hasegawa *et al.* (1982) and Sorokin *et al.* (1987) reported that the myotubes had more transferrin receptors on their surface than the myoblasts. However, there was no increase in the number of myotrophic protein receptor after the cell fusion was ceased.

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培養 鷄胚 筋原細胞의 分化에 미치는 鷄胚 抽出物內 Myotrophic Protein의 영향

兪炳濟* · 李昌鎬 · 郭圭鳳 · 鄭鎭河 · 河斗鳳 (서울大學校 自然大 動物學科,
*大邱大學校 理工大 生物學科)

培養 鷄胚 筋原細胞의 分化에 미치는 鷄胚抽出物의 영향을 조사하고, 이 抽出物로부터 筋原細胞의 分化에 필수적인 myotrophic protein(MP)를 순수분리하였다. 그리고 이 MP는 철 운반 단백질인 transferrin과 동일하거나 또는 대단히 유사한 단백질임을 알 수 있었다.

이 단백질은 철을 근원세포에 공급하고 이 철이 근원세포의 융합에 필수적인 역할을 하는 것으로 보인다. 또 鷄胚抽出物속에는 이 MP이외에 근원세포의 융합을 억제하는, 그리고 열에 비교적 安定한 단백질이 존재한다고 생각된다.

이 MP의 受容體(receptor)분석을 한 결과, 수용체의 수는 근원세포가 융합을 하고 나면 급속히 감소하는 것으로 나타났다. 그리고 근원세포 내로의 철과 MP의 수송에는 약 10분이 소요되는 것으로 나타났다.