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On the Possible Fusion-Promoting Factor Secreted from Cultured Myoblasts

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培養 鷄胚 筋原細胞로부터 분비된 細胞融合 촉진 물질에 관한 연구

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要 約

鷄胚 筋原細胞를 배양하면서 筋原細胞로부터 培養液으로 방출되는 물질을 분석함으로써 筋原細胞가 細胞融合 촉진물질을 분비하는지의 여부를 조사하였다.

근원세포의 배양에 한번 쓰인 배양액(muscle-conditioned medium, MCM)은 세포 융합 촉진효과를 가지고 있는 것으로 보아 근원세포로부터 세포융합 촉진물질이 배양액 내로 방출되는 것으로 보인다.

이 MCM의 단백질을 분석한 결과 분자량 약 175,000인 단백질이 배양액 내에서 분자량 약 145,000인 단백질로 분해되고, 이 분해된 단백질이 세포 융합 촉진 효과를 나타내는 것으로 생각된다.

INTRODUCTION

The term cell differentiation has been used to describe the process of diversity and specialization of the cell lineages descended from a developing zygote, and the mechanism of differentiation is one of the most interesting topics in the biological science.

The culture technique of myoblasts is useful for the study of the mechanism of differentiation since the characteristics of muscle differentiation observed in the intact embryo occur also in the monolayer culture of myogenic cells (Konigsberg, 1963; Yaffe, 1969; Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972; Emerson and Beckner, 1975; Turner *et al.*, 1976; Bonner and Adams, 1982).

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Numerous studies on the differentiation have confirmed that muscle development in culture takes place through two distinct phases (O'Neill and Stockdale, 1972; Konigsberg, 1982). Initially, cells rapidly proliferate without any indication of differentiation. Then, at predictable time, they come to face an abrupt and rapid transition from the proliferative to the differentiated state through cell fusion. These differentiating muscle cells show a variety of characteristics morphologically and biochemically, including cell fusion to form multinucleated myotubes, cessation of DNA synthesis with irreversible withdrawal from cell cycle, and synthesis of muscle specific proteins such as myosin, creatine phosphokinase, and acetylcholine receptor (Okazaki and Holtzer, 1966; Paterson and Prives, 1973; Buckley and Konigsberg, 1974; Nadal-Ginard, 1978; Devlin and Emerson, 1978; Ha *et al.*, 1981). It is a peculiarity of differentiation of striated skeletal muscle that proliferating myogenic cells fuse to form multinucleated myotubes, which has been the subject of a great deal of attention because it provides a helpful model to study the biomembrane fusion mechanism as well as differentiation mechanism (Dienstman and Holtzer, 1977; Devlin and Emerson, 1979; David *et al.*, 1981; Dahl *et al.*, 1978; Herman and Fernandez, 1982). Previously, Bischoff and Holtzer (1969) suggested that the myoblast had its own intrinsic program to regulate the rounds of the cell cycle and the commencement of fusion. However, recent studies have demonstrated that the onset of fusion is not rigidly determined, but can be easily and predictably controlled by some simple manipulation (Konigsberg, 1971; O'Neill and Stockdale, 1972; Slater, 1976; Yeoh and Holtzer, 1977; Linkhart *et al.*, 1981).

Konigsberg (1971) reported that the start of the myoblast fusion in the culture could be regulated by frequent changing of medium or by varying the cell density. They observed that the fusion could be delayed by decreasing the inoculum size, increasing the volume of medium, or circulating the medium continuously. Other studies (Buckley and Konigsberg, 1974; Slater, 1976; Yeoh and Holtzer, 1977; Nadal-Ginard, 1978; Linkhart *et al.*, 1981) also support the view that the myogenesis is modulated by environmental changes such as the depletion of certain mitogenic factors which would lead to an increase of the replication time of the cell and to a large protraction of G1 state of the cell cycle while the fusion takes place.

On the other hand, there are contrary studies suggesting that cells in culture release into the medium substances that are responsible for the myoblast fusion. Schubert *et al.* (1973) have demonstrated that both myoblast and myotube secreted at least twelve soluble proteins into the medium. Doering and Fishman (1977) have reported that myogenic cells secreted macromolecules that possessed significant fusion-promoting activity. These were retained by 10,000 dalton-cutoff ultrafiltration and could be destroyed by trypsin or by boiling. By using cells of the L6 line, Delain *et al.* (1981) have also shown that the rate of myoblast fusion could be increased significantly by proteins secreted from proliferating myogenic cells but not by proteins secreted from myotubes. Ha and Yoo (1984) reported that two proteins (65 KD and 45 KD) secreted from the cultured cells and accumulated in the medium during

the proliferating period seemed to be responsible for the promoting fusion.

In the present study, therefore, we attempted to find out the possible fusion-promoting substances which were released to the medium from the cultured chick embryonic myoblast cells by analyzing the proteins contained in the muscle-conditioned medium, a medium that had been exposed to cells for a given time.

MATERIALS AND METHODS

Materials: Chicken eggs were purchased frotm Yuil Farm near Seoul. Trypsin, antibio tic-antimycotic (a mixture of penicillin, fungizone and streptomycin), and minimal essential medium (MEM) were obtained from Gibco, horse serum from Hyclone, and ^{35}S-methionine (specific activity, 1109 Ci/mM) from New England Nuclear. Other chemicals were obtained from Sigma.

Cell culture: Cultures were prepared by a modified procedure of O'Neill and Stockdale (1972) as described by Ha *et al.* (1979). Breast muscle was isolated from 12-day chick embryo and dipped in sterile MEM. contaminated connective tissues were removed under a microscope and the mass of muscle was teared with 0.1% trypsin at 37°C under 5% CO_2 by incubating for 30 minutes, centrifuged and the supernatant was discarded to remove trypsin. The cells precipitated were resuspended in MEM containing 10% horse serum, 10% embryo extract, and 1% antibiotic-antimycotic solution (hereafter referred to as medium 811) and filtered through a Nitex HC-10 nylon mesh of 20 μm in a Swinny filter adaptor. The filtered cells were preplated for 10 minutes to remove fibroblasts which during the period settled down to attach to the bottom of the culture dish. The floating myoblasts were then counted by a hemocytometer, diluted with 811 to a density of 10×10^5 cells per ml.

The diluted cells were plated on collagen-precoated tissue culture dishes and incubated in a water saturated atmosphere of 95% air and 5% CO_2 at 37°C. At 24 hours after plating, the cells were transferred from medium 811 to the medium consisting of 10% horse serum, 2% chick embryo extract, and 1% antibiotic-antimycotic solution in MEM (hereafter referred to as 8102 medium) or to muscle-conditioned medium. All culture media were thereafter changed every 48 hours.

Embryo extract was prepared from 12-day chick embryos. Embryos were washed in MEM and eye-balls, beaks and feet were removed from the body. The embryos were then chopped and homogenized in MEM by passing through a syringe. The extract was kept frozen at −70°C. Before use, the clarified extract was obtained by centrifugation at 18,000×g for 60 minutes.

Preparation of Muscle-Conditioned Medium: Muscle-conditioned medium was the medium which had once been exposed to cells for a definite period. After 24 hours of culture in 811 medium, the cells were transferred to control 8102, 8102 containing 3 mM butyric acid or 8102 containing 4% dimethylsulfoxide (DMSO).

Then the medium (muscle-conditioned medium, MCM) was collected and used for another culture to observe whether a fusion-promoting substance had been secreted from cells of the first culture. The control MCM (8102) that had been exposed to cells between 24 hours and 48 hours of culture was termed as C:24-48, that between 48 and 72 hours as C:48-72, and so on. In the same way, the MCM which contained butyric acid was termed as B:24-48, B:48-72, and so on, and the MCM containing DMSO was termed as D:24-48, D:48-72, and so on.

In order to label the protein synthesized in the cell and secreted from the cell into the MCM, ^{35}S-methionine was added to the culture to a final concentration of 20 μCi/ml medium.

To determine the radioactivity of labeled proteins, the MCM was treated with TCA (final 10%) to precipitate the proteins and the precipitate was resolubilized in 100% (w/v) TCA and was precipitated again by addition of distilled water to make the TCA concentration 10%. The above procedure was repeated three times and the radioactivity of the final protein solution in 100% TCA was measured by a liquid scintillation counter.

Microscopic Analysis: At the appropriate time, the cultured cells were washed 3 times with phosphate buffered saline and were fixed twice in a mixture of 95% ethanol, 40% formaldehyde, and acetic acid (20:2:1, v/v) for 5 minutes. After rinse with distilled water, the cells were stained with hematoxylin for 5 minutes and washed in tap water thoroughly, observed under a microscope (×400) and the number of nuclei in randomly chosen 10 fields were scored. Growth index was expressed as the average number of nuclei per field. Fusion index was defined as the ratio of the number of nuclei that were found in fused myotubes to the number of total nuclei.

Absorption and Degradation of the Secreted Proteins by the Myoblast: Cells were cultivated in the MCM that contained proteins labeled with ^{35}S-methionine and released from previous culture and, at the indicated times, the media and cells were separated.

The cells were washed three times with cold MEM and treated with MEM containing 5% SDS. The radioactivity of the cell content was then measured. At the same time, the medium was treated with TCA to precipitate proteins, which were then electrophoresed and fluorographed. The acid soluble fraction was measured for its radioactivity.

Radioactivity detected from the cell content was regarded as the protein that was absorbed by the myoblast or tight-bound to the cell membrane and the radioactivity detected from the acid soluble fraction of the medium was regarded as the protein that was degraded by the cell.

Electrophoresis and Flourography: The proteins precipitated from MCM were dissolved in 2% SDS solution containing 5% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue, were incubated in boiling water for 3 minutes and electrophoresed. The electrophoresis was performed on polyacrylamide gel essentially according to Laemmli (1970) using 8~14% gradient gel at 30 mA. The gel was then stained with Coomassie blue and destained. Fluorography of the dried gel was done according to Bonner and Laskey (1974).

RESULTS

After 24 hours of culture, the cells were transferred from medium 811 to control 8102 or to MCMs, cultured for further 24 hours, and stained to measure the fusion index and growth index by microscopic examination. As shown in Fig. 1, the fusion indices were increased by about 30% in all MCMs tested, but the growth indices were not practically changed. DMSO and butyric acid, that were known to inhibit the myoblast fusion seemed not to prevent the myoblast from releasing fusion-promoting factor(s) into the medium because the MCM prepared in the presence of these inhibitors showed essentially the same effect as the control MCM. Thus, the fusion inhibitors seemed not to prevent the synthesis and secretion of the fusion-promoting proteins though they prevented the fusion itself.

Since it is clear that MCM enhances the myoblast fusion possibly through some substance that was released from cells, the proteins contained in the MCM were analyzed. To detect the proteins that had been secreted from cells into the MCM, cells were cultured in the presence of ^{35}S-methionine for a specified period and the medium (now MCM) was collected. Then the proteins in the MCM were precipitated by TCA (10%) and the precipitate was collected by centrifugation and resolubilized in 100% (w/v) TCA solution. This procedure was repeated three times to remove free ^{35}S-methionine completely. The final protein solution in 100% TCA was then measured for its radioactivity.

The data shown in Fig. 2 reveal that in the case of 8102 the amount of protein that was presumed to be released from the cell into the culture medium was greatly increased

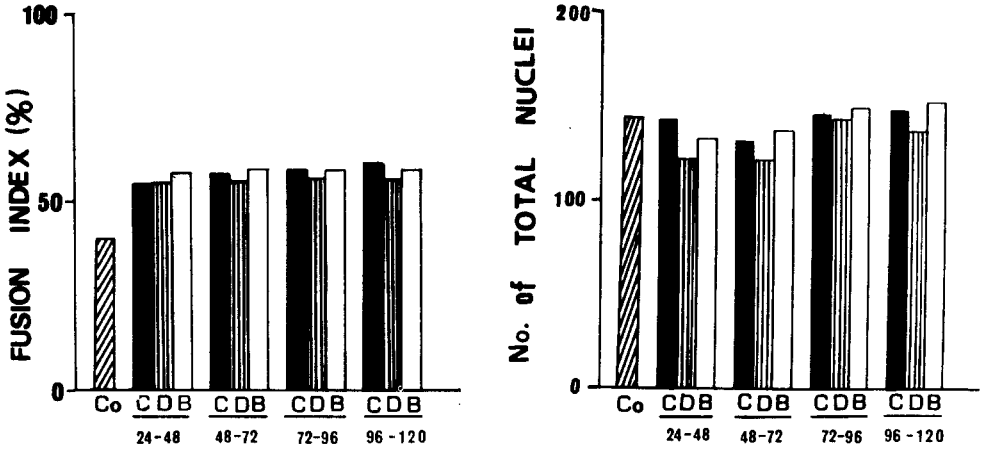

Fig. 1. The effect of MCM on myoblast fusion and proliferation. Cells were cultured in medium 811 for the first 24 hours and then in 8102 or in various MCMs for further 24 hours, fixed and stained.

Co, control 8102; C, MCM prepared from 8102; D, MCM prepared from 8102 containing 4% DMSO; B, MCM prepared from 8102 containing 3mM butyric acid. The conditioned periods (in hours) of MCMs are shown under lanes.

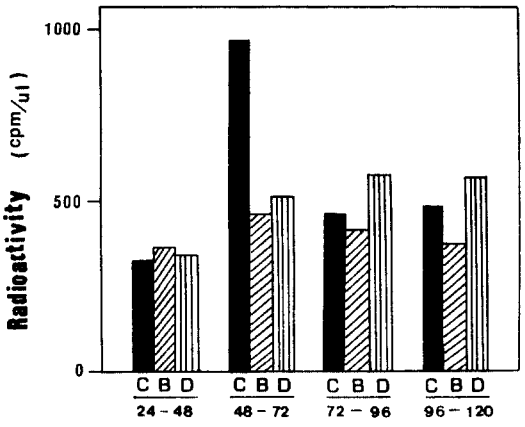

Fig. 2. The amount of proteins secreted into the medium from cultured myoblasts.
Cells were cultured in the presence of ^{35}S-methionine for the specified period and the medium (MCM) was collected.
The proteins in the MCM were then precipitated by TCA and the radioactivity of the precipitate was measured. C, MCM prepared from 8102; B, MCM prepared from 8102 containing 3mM butyric acid; D, MCM prepared from 8102 containing 4% DMSO.
The conditioned periods (in hours) of MCMs are shown under lanes.

during the period of 48~72hr. At that period, the majority of myoblasts commences fusion. On the other hand, the cells cultured in the presence of butyric acid or DMSO seemed not to release any proteins into the medium because the amount of radioactive

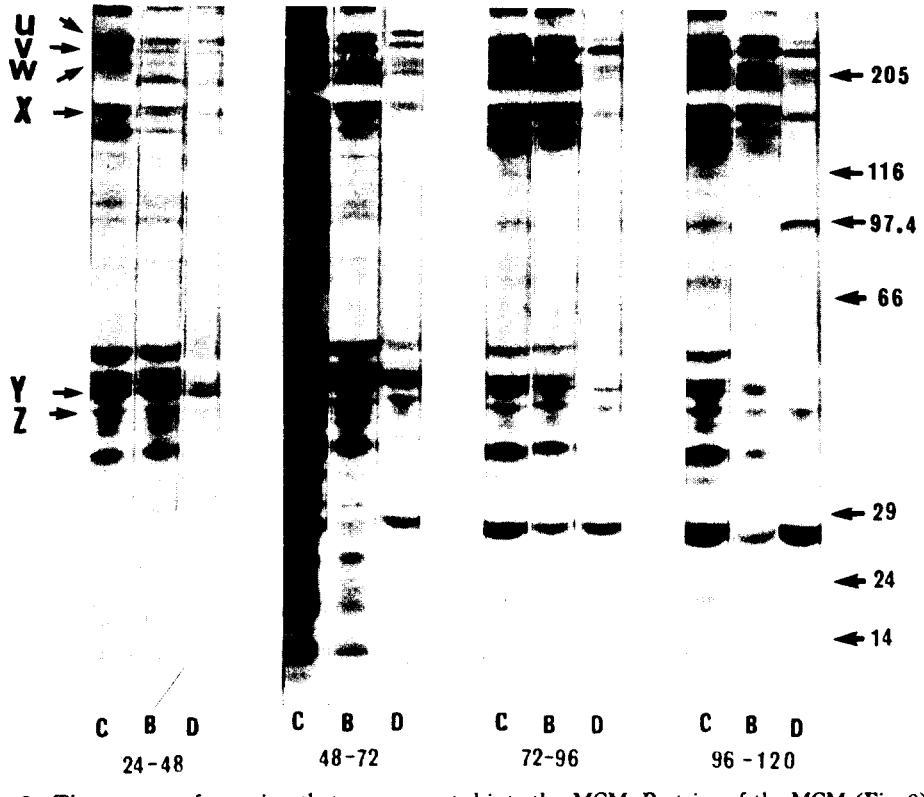

Fig. 3. The pattern of proteins that were secreted into the MCM. Proteins of the MCM (Fig. 2) were analyzed by SDS-PAGE and fluorographed. C, MCM obtained from 8102; B, MCM obtained from 8102 containing 3mM butyric acid; D, MCM obtained from 8102 containing 4% DMSO. The conditioning periods of the MCM are shown below the lane. Six proteins marked as U-Z are common proteins found in all MCMs. Numerals on the right are molecular wirghts estimated from marker proteins.

protein detected in the MCM was not increased through the entire period of culture (24~120 hours). From these data it was obvious that there was a close correlation between the protein secretion and the myoblast fusion, possibly implying that the increased protein secretion from the cell is required for the myoblast fusion or resulted from the cell fusion.

The proteins thus secreted into the medium in various conditions were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. As shown in Fig. 3, the protein pattern was varied by conditions of preparing the MCM; that is, the presence or absence of fusion inhibitor and the age of the culture from which the conditioned medium was obtained. However, six proteins seemed to be present in all MCMs. They are indicated as U, V, W, X, Y, and Z in the Figures and their molecular weights estimated are 290, 250, 220, 175, 45 and 42K dalton, respectively. Since these proteins exist in all 12 different MCMs which showed fusion-promoting activity (Fig. 1), it can be suggested that one or more of these proteins may be involved in the fusion-promoting activity of the MCM.

To examine if the secreted proteins are absorbed or internalized into new younger myoblasts, three MCMs, C:24~48, B:24~48, and D:24~48 containing ^{35}S-methionine were dialyzed against control 8102 medium to remove free ^{35}S-methionine, butyric acid and DMSO and to supplement depleted nutrients. The dialyzed MCMs were then added to the new myoblast culture at 24 hours of culture. After the culture, the medium was collected at 48, 52, 62 and 72 hours of culturing periods and cells were rinsed with cold MEM to remove the absorbed radioactivity and treated with 5% SDS-MEM to extract cell proteins. We have then measured the radioactivity of total cell proteins which should have come from the labeled proteins that had been synthesized and secreted into the MCM by the

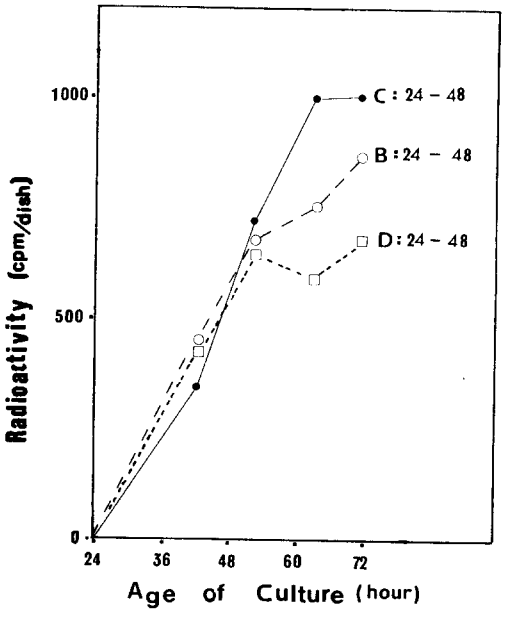

Fig. 4. The absorption by myoblasts of proteins that were released from myoblasts of previous culture. Three MCMs (C:24~48, B:24~48, D:24~48) that contained radioactively labeled proteins were refed to new myoblasts. At the indicated time, the cells were treated with 5% SDS in MEM to rupture and the radioactivity of the total protein was measured.

cells from which the MCM was obtained. As shown in Fig. 4, the amount of secreted proteins that were absorbed by new myoblasts or tightly bound to their surface membrane was increased sharply as the culture time was prolonging and then slowly after about 60 hours when the cells complete the fusion.

Since the released proteins in the MCM seemed to be absorbed or internalized by the newly cultured myoblasts through a unknown process (possibly by endocytosis), protein patterns of the MCM obtained after the last culture were analyzed (Figs. 5 and 6).

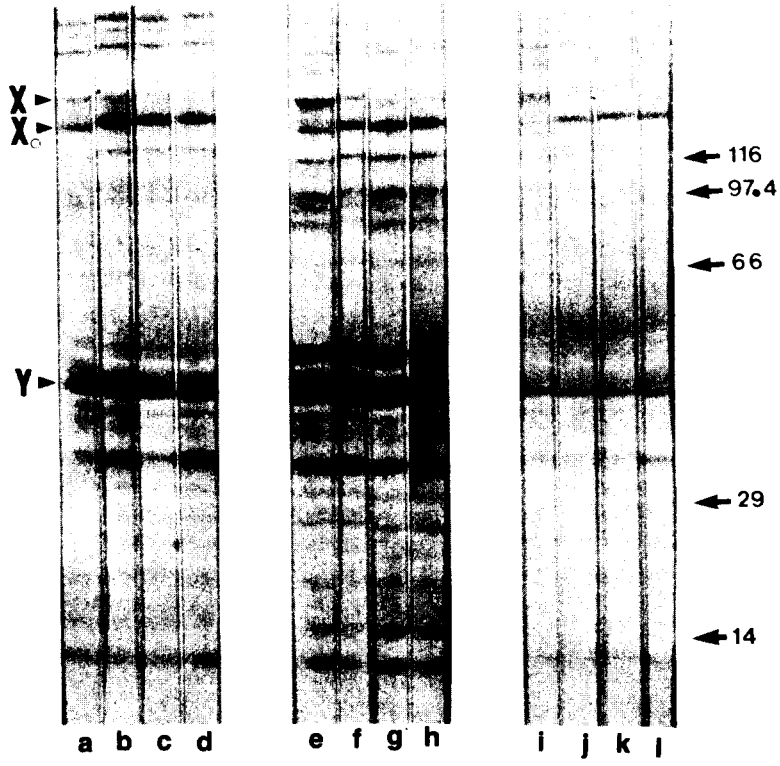

Fig. 5. The degradation of released proteins. The MCMs that contained labeled proteins released from the previous culture were fed to the 24 hour culture for further 24, 36 or 48 hours. At the indicated time, the medium was collected, electrophoresed and fluorographed.

a-d: The MCM prepared from 8102	e-h: The MCM prepared from 8102 containing 3 mM butyric acid	i-l: The MCM prepared from 8102 containing 4% DMSO.
a. 0 hour	e. 0 hour	i. 0 hour
b. 24 hours	f. 24 hours	j. 24 hours
c. 36 hours	g. 36 hours	k. 36 hours
d. 48 hours	h. 48 hours	l. 48 hours

The fresh MCM that was not used for second culture has a distinct band of protein which is marked X in Fig. 5 (lanes a, e and i). The molecular weight of this protein was estimated to be 175K dalton. However, when the MCM was used to feed new myoblats (myoblasts cultured for first 24 hours in 811) for 24, 36 or 48 hours, this protein band gradualy disappeared as the culture time was prolonged, and at the same time, another protein of approximately 145K dalton (band Xo) became denser and denser (lanes a, b, c and d, respectively). Essentially the same phenomena were also observed in MCMs that had been prepared in the presence of butyric acid or DMSO (lanes e-h and i-l, respectively). It seemed to be likely that the protein X was degraded to the protein Xo by the action of a cellular protease of the differentiating myoblasts. No other proteins (U,V,W and Z) showed similar changes except protein Y, which was reduced in amount temporarily during the culture period of 48~60 hours when the cells fused each other rapidly.

Because the protein X seemed to be degraded to yield a smaller protein (Xo), it was supposed that the fragment severed from the protein X should appear in soluble fraction of the MCMs. Thus, the MCMs obtained after feeding new myoblasts for a given time (24~72 hours) were treated with TCA to precipitate proteins and supernatant was collected to count its radioactivity. Any radioactivity detected in this soluble fraction is likely come from the severed fragment of the protein X. Fig. 6 shows that the radioactivity of the acid soluble fraction increases linearly as increasing the period exposing the MCMs to the newly cultured myoblasts.

DISCUSSION

The muscle-conditioned medium (MCM) has been known to promote the process of fusion

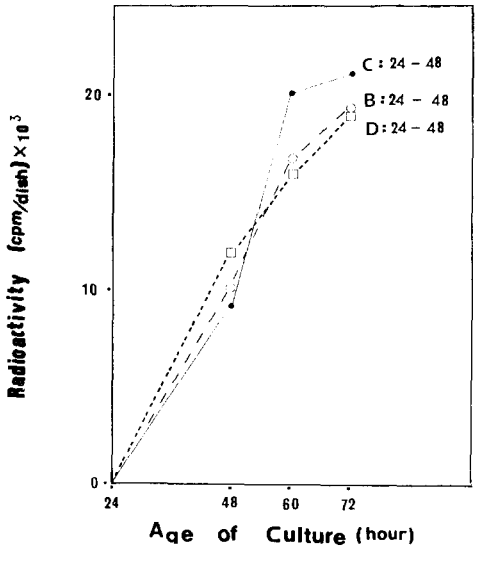

Fig. 6. The degradation of released proteins. The MCMs that contained labeled proteins released from the previous culture were fed to the 24 hour culture for further 24, 36, or 48 hours. At the indicated time, the medium was collected, treated with TCA (final 10%), and centrifuged to remove the protein precipitate. The radioactivity of the acid soluble fraction was regarded as the products of the cellular protease action the proteins in the MCM.

of the proliferating myoblast cells. Doering and Fishman (1977), for example, have shown that the fusion of muscle cells grown in the MCM occurred about 10 hours earlier than the control culture. However, it is still uncertain how the MCM can promote the fusion process. By analyzing the components in the MCM, Doering and Fishman (1977) have suggested that MCM may contain a fusion promoting factor, which is cell-specific and is secreted into medium during incubation period. Ha and Yoo (1984) have reported that two different proteins, 65K and 45K dalton, were accumulated in the medium during the period when cells prepared for fusion. They suggested that these proteins might participate in and promote the myoblast fusion. It may be that, when the level of the secreted protein(s) in the medium reaches a critical point, the myoblasts commence fusion.

The present study confirmed that the muscle cells secrete proteins that promote the fusion process irrespective of the age of the cells. The proteins were secreted from cells whose fusion was arrested by butyric acid or DMSO. This finding is contrary to the result of Delain *et al.* (1981) who reported that the fully developed myotubes did not secrete any fusion promoting factor.

Furthermore, it is suggested, from the present study, that at least one of the proteins U, V, W, X, Y and Z in Fig. 3 which is secreted and is accumulated in all kinds of MCMs may be involved in the promoting the fusion of myoblasts. If indeed the proteins U, V, W, X, Y and Z are responsible for the fusion-promoting effect of the MCM, then the main question is that how or by what mechanism these proteins can accomplish it. One simple explanation could be that these substances have some kind of enzymatic activity such as protease activity to affect the membrane proteins (suggested by Kang *et al.*, 1983) or to affect the fusion inhibitor that is normally contained in the medium (proposed by Evinger-Hodge *et al.*, 1982; Ha and Yoo, 1984). The significance of the membrane protein for the progression of cell fusion has been suggested by Knudsen and Horwitz (1978) who considers that a membrane protein or myoblast adhesion molecule makes myoblasts recognize and fuse each other. While no proteolytic activity against globin, insulin, or casein was detected in any MCM (data not shown), an endogenous metalloprotease activity has been reported in the cultured myoblast which might be associated with the cell fusion (Couch *et al.*, 1983; 1984).

In this study, it seems probable that there are some kind of interaction between the secreted protein and the myoblast which results in the degradation of the secreted protein (Figs. 5 & 6). The protein X (Fig. 5) contained in the MCM seems to be degraded to Xo, which may be an active form of X. This behavior of protein X is similar to the F glycoprotein of Sendai virus which is cleaved by a cellular protease of chick embryo to become fusogenic (Scheid and Choppin, 1974). In addition, Fig. 5 suggests that a part of protein Y is internalized temporarilly into the rapidly fusing cell. From these results, one hypothesis on myoblast fusion can be suggested: The muscle cell secretes proteins X and Y, and their action is controlled by cellular substances such as enzymes or receptors. That is,

protein X is not able to regulate myoblast fusion process until it is degraded to Xo by a cellular protease, while protein Y may participate in cell fusion by a receptor mediated endocytosis.

Numerous studies were undertaken to investigate the lipid metabolism linked to biomembrane fusion and suggested that Ca^{+} may act to trigger a phospholipase so that the enzyme removes polar groups, and hence water, from the phospholipid bilayer of the membrane (Parsegian, 1977). Since lysophosphatidylcholine is fusogenic (Poole *et al.*, 1970), it is also probable that Ca^{+} stimulates its production by activating phospholipase. An interesting paper has recently appeared, reporting that phosphatidylinositol 4-phosphate and phosphatidylinositol 4, 5-bisphosphate are degraded and diacylglycerol and phosphatidic acid are formed when fusion competent chick embryonic myoblasts are stimulated to fuse by increasing the concentration of calcium ion in the medium to 1.4 mM (Wakelam and Pette, 1983). Considering this report together with the result of the present experiment, it may be suggested that fusion-promoting factor (such as proteins X, Y or others) may be a lipase that stimulates the biomembrane fusion. Therefore, it seems to be necessary to study the characteristics and the function of proteins X and Y to clarify the precise process of biomembrane fusion.

SUMMARY

In order to find out whether myoblast cells release into the culture medium any substances that induce or promote the fusion of myoblasts, chick embryonic myoblasts were cultured and the cultured medium (muscle-conditioned medium, MCM) was collected.

The MCM was then added to the newly cultured myoblasts to examine if it has fusion-promoting activity. The MCM was also analyzed for its protein content before and after its addition to the second culture.

The MCM apparently showed fusion-promoting activity when applied to unfused young myoblasts, suggesting that it contained substances that promote the fusion and that had been released from cells of the previous culture.

Analysis of proteins in the myoblasts and in the MCM suggested that the released protein was absorbed by or tightly bound to myoblasts of the second culture. One of the released proteins of about 175 kilodalton was degraded to a polypeptide of approximately 145 kilodalton, which appeared to act upon the membrane proteins of unfused myoblasts so as to stimulate their membrane to fuse with neighboring cells.

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