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The Effect of Muscle-Conditioned Medium on the Fusion of Chick Embryonic Myoblast Cells in Culture*

Doo Bong Ha and Yung Joon Yoo
(Department of Zoology, College of Natural Sciences, Seoul National University)

배양 계배 근원세포의 융합에 미치는 Muscle-Conditioned Medium의 영향*

河 斗 鳳·兪 英 濬
(서울大學校 自然大 動物學科)

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요 약

근원세포의 융합에 관여하는 융합유도물질의 존재를 규명하기 위하여 계배의 근세포를 배양하면서, muscle-conditioned medium (MCM) 이 근원세포의 융합에 미치는 영향을 조사하고, 근원세포로부터 배양액 내로 방출되는 단백질을 분석하여 다음과 같은 결과를 얻었다.

(1) MCM은 뚜렷한 융합촉진 효과를 나타냈으며, 이러한 효과는 첨가된 MCM의 농도가 증가함에 따라 증가하였다.

(2) MCM의 융합촉진 효과는 주로 근원세포로부터 방출되는 융합유도물질에 의하여 일어나는 것으로 판단된다.

(3) 배양근원세포로부터 분비되는 45,000달톤과 65,000달톤의 단백질이 융합유도물질일 가능성이 높다.

INTRODUCTION

The term cellular differentiation has been used to describe the process of diversification and specialization of the cell lineages descended from the developing zygote. One of the most helpful model system to examine this process is the cultured skeletal muscle cells (Konigsberg, 1963; Yaffe, 1969; Bischoff and Holtzer, 1669; O'Neill and Stockdale, 1972; Emerson and Beckner, 1975; Turner *et al.*, 1976; Ha *et al.*, 1979; Daubas *et al.*, 1981; Bonner *et al.*, 1982). Muscle development in culture regularly occurs in two distinct

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phases (O'Neill and Stockdale, 1972; Konigsberg, 1982); initially, cells rapidly proliferate with no indication of any differentiation. Then, at a predicable time, an abrupt and rapid transition occurs to the cell population to differentiate. This differentiating muscle cell shows a variety of characteristics; cessation of DNA synthesis with irreversible withdrawal from cell cycle, and production of muscle specific proteins such as myosin, creatine phosphokinase and acetylcholine receptor (Okazaki and Holtzer, 1966; Paterson and Prives, 1973; Turner *et al.*, 1974; Buckley and Konigsberg, 1974; Doering and Fishman, 1974; Bischoff, 1978; Nadal-Ginard, 1978; Devlin and Emerson, 1978; Ha *et al.*, 1981). Among these characteristics, the fusion of myoblast is the most dramatic event which has been the subject of a great deal of attention, since it is an important developmental process (Dienstman and Holtzer, 1977; Devlin and Emerson, 1979; David *et al.*, 1981) and provides a good model system to study the mechanism of membrane fusion (Herman and Fernandez, 1982).

Two main hypotheses have been proposed to explain the transition of myoblast cells from the proliferative to the differentiated state through fusion. Previously, some kinds of intrinsic program to the cell were thought to regulate the number of mitotic division and timing of the last cell division to initiate fusion (Bischoff and Holtzer, 1969). However, recent experiments demonstrated that the time of initiation of myoblast fusion is not rigidly determined but can be easily and predictably shifted by simply manipulating cell density and medium composition (Konigsberg, 1971; O'Neill and Stockdale, 1972; Slater, 1976; Yeoh and Holtzer, 1977; Linkhart *et al.*, 1981).

Konigsberg (1971) has demonstrated that the time of initiation of fusion could be regulated by changing medium or varying initial cell density. Fusion can be delayed by decreasing the inoculum size, increasing the volume of medium, or continuously circulating the medium. In addition, O'Neill and Stockdale (1972) have showed that the onset of fusion occurred earlier in the unfed control cultures than in the cultures whose medium was replaced by fresh medium after plating. According to them as well as to others (Buckley and Konigsberg, 1974; Slater, 1976; Yeoh and Holtzer, 1977; Nadal-Ginard, 1978; Linkhart *et al.*, 1981), myogenesis seems to be modulated by environmental changes such as the depletion of hypothetic mitogenic factor(s) in the culture medium. This depletion would lead to an increase of the replication time of the cells and to a large protraction of G1 stage in the cell cycle during which fusion takes place.

On the other hand, there are reports that suggest that some substances having fusion-promoting activity are secreted from myoblast cells into the medium during early period of the culture. Schubert *et al.* (1973) have demonstrated that both myoblasts and myotubes secreted a minimum of 12 soluble proteins. Doering and Fishman (1977) have demonstrated that myogenic cells secreted macromolecules that may possess significant fusion-promoting activity. These molecules were retained by 10,000 dalton cut-off ultrafilter and could be destroyed by boiling or by trypsin treatment. By using cells of the L6 line,

Delain *et al.* (1981) have also showed that the fusion rate of myoblasts could be significantly increased by proteins that seemed to be secreted by proliferating myogenic cells, but not by those secreted by myotubes.

The present study was, therefore, undertaken to isolate any fusion promoting factors that are involved in the control of myoblast fusion in chick embryonic muscle cell culture.

MATERIALS AND METHODS

1. *Cell Culture*

Materials: Fertilized hen's eggs were purchased from Purina Korea egg farm near Seoul. Horse serum, amino acids, vitamins, trypsin and antibiotic-antimycotic (mixture of penicillin, fungizone and streptomycin) were obtained from Grand Island Biological Co. ^{35}S-Methionine was purchased from New England Nuclear (specific activity, 1244.5 Ci/mmol).

Muscle cell culture: Cultures were prepared by a modification of the procedure of O'Neill and Stockdale (1972) by using presumptive myogenic cells from 12 day embryonic chick breast. The breast tissue was washed by sterile Eagle's balanced salt solution (EBSS), teared and pipetted repeatedly with a pasteur pipette. The tissue was then digested with 0.1% trypsin at 37.5°C under 5% CO_2 by incubating for 30 minutes, and was dispersed by repeated pipetting. The dispersed cells were collected by centrifugation and were resuspended in Eagle's minimum essential medium (MEM) containing 10% horse serum, 10% chick embryo extract and 1% antibiotic-antimycotic solution (hereafter referred to as 811). The suspension was dispersed by gentle mechanical agitation and filtered through a 20 μm of Nitex HC-10 nylon mesh in a Swinny adaptor. After the filtration step, the cells were preplated three times serially to remove contaminating fibroblasts. The unadsorbed, viable cells were counted in a hemocytometer, and were diluted to a population of 5×10^5 cells per ml with 811. Cell viability was estimated by trypan blue exclusion. The cells were plated on 100 mm or 35 mm Pyrex tissue culture dishes which had been precoated with autoclaved and Millipore-filtered collagen solution (0.5 mg/ml). Cells were incubated at 37.5°C in a water-saturated atmosphere of 95% air and 5% CO_2. At 20 hours after the initial cell plating, medium was changed to MEM containing 10% horse serum, 2% chick embryo extract and 1% antibiotic-antimycotic solution (hereafter referred to as 8102) or to muscle-conditioned medium described later. After this first medium change with 8102, the medium was routinely changed with 8102 every 48 hours or with specified medium at specified time.

Chick embryo extract: Twelve-day old chick embryos were washed in EBSS and the front part of head including eyes and beak was removed. The embryos were then minced and homogenized by passing through a syringe, and were diluted with equal volume of EBSS. The resulting extract was kept frozen. Before use, the clarified extract was obtained by centrifugation at 18,000 $\times$g for 60 minutes.

Muscle-conditioned medium: From the cell culture, the 8102 medium was withdrawn from the plates at 48 hours after the start of the culture. This medium in which cells had been grown for 28 hours was filtered through a sterile 0.45 μm Millipore filter to remove cell debris. Medium prepared in this way was referred to as first muscle-conditioned medium (lst MCM). Second MCM was obtained in the same way by using this lst MCM once more for the culture of 20 hour-age for another 28 hours. When the MCM was used as culture medium, amino acids were supplemented.

In the following description, the age of culture is indicated as the sum of the initial 20 hours in the 811 medium and additional hours of culture in the given medium. Therefore, 48 hours of culture for example means that cells were cultured in 811 for 20 hours and then in the specified medium for additional 28 hours.

2. *Microscopic Analysis*

At specified time, the culture plates were taken out and washed three times with phosphate buffer solution and fixed twice for 5 minutes in a mixture of 95% ethanol, 40% formaldehyde and acetic acid (20:2:1, v/v). After rinsing these plates with distilled water, the cells were then stained with hematoxylin for 5 minutes, washed briefly in tap water, treated in 1% hydrochloric acid in 70% ethanol until the nuclei became clearly visible under microscope, washed thoroughly in tap water, and then dehydrated in ethanol. Cells were observed under microscope (X400), and nuclei in 10 randomly chosen fields were counted. Average number of nuclei per field was used as the growth index. Fusion index was obtained from the ratio of the number of nuclei within myotubes to the number of total nuclei.

3. *Analysis of Proteins in Muscle-Conditioned Medium*

After 20 hours of culture in the medium 811, the medium was changed with 8102 containing ^{35}S-methionine (20 μCi/ml) and cells were grown in this radioactive medium for further 30, 40 or 48 hours, after which the media (MCM) were collected, filtered through a 0.45 μm Millipore filter and used for electrophoretic analysis of protein.

Proteins in this MCM were dissolved in 2% SDS solution containing 5% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue and were heated at 100°C for 3 minutes and the electrophoresis was performed essentially according to Laemmli (1970) by using 8~12% gradient polyacrylamide slab gel. After electrophoresis, gels were stained with 0.2% Coomassie blue solution containing 50% methanol and 10% acetic acid, and was destained in a mixture of 35% methanol and 10% acetic acid. Usually a volume of sample containing 50 or 100 μg of protein was loaded on each lane of the electrophoretic gel.

The destained gel was dried immediately under vacuum. Autoradiogram was made by exposing the dried gel to an X-ray film (Fuji) at −70°C. Autoradiograms were scanned with a Transdyne densitometer.

Protein content in the electrophoresis sample was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

RESULTS

Fusion Index and Cell Growth

Microscopic observation of cultures in the present study showed that myoblast cells exhibited clear and typical pattern of growth and fusion. During the first 20 hours in 811, most cells in the initial suspension proliferated without differentiation. Elongation occurred usually after the first change of medium with 8102 at 20 hours and was followed by extensive alignment of cells during 30~40 hours after the plating. Fusion was initiated at about 36 hours and the number of nuclei within myotube increased while the population of mononucleated single cells decreased. By 72 hours about 70% of the nuclei in the culture were found in myotubes. Although a small number of cells kept dividing continuously, the rate of division decreased as fusion progressed and the fusion index reached a maximum at 72 hours (Figs.1 and 2).

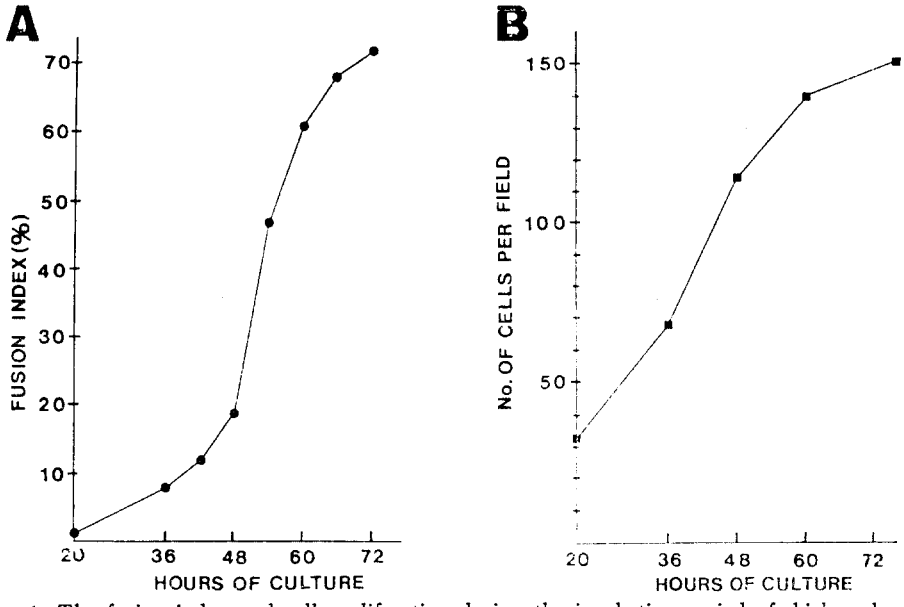

Fig. 1. The fusion index and cell proliferation during the incubation period of chick embryonic myoblast cells in culture. At the time indicated, the cultured cells were stained and the nuclei in 10 randomly chosen fields were counted under microscope (*X*400). A, Fusion index (the ratio of the number of nuclei within myotubes to the number of total nuclei). B, Growth index (average number of nuclei per field).

Effect of Muscle-Conditioned Medium

In order to observe the effects of MCM on the fusion of myoblast, cells were grown in 811 for initial 20 hours and then the medium was changed with either fresh 8102, 1st MCM or 2nd MCM. Cells grown in these media were stained for microscopic observation

at 28, 40 or 52 hours after the change of the medium (total hours of growth were 48, 60 or 72 hours, respectively) and the fusion index and cell number were counted. Since cells grown in 2nd MCM were degenerated rapidly after 48 hours the data at 60 and 72 hours in 2nd MCM were excluded.

As shown in Table 1, the fusion index increased remarkably whereas the cell number decreased when the cells were grown in 2nd MCM. Microscopic examination of cells in 2nd MCM exhibited much fewer myoblasts among the branched myotubes than that of control cultures in which abundance of mononucleated cells were observed (Fig. 2). These data indicate that MCM has a fusion promoting activity since the cells in MCM were accelerated to fuse at an earlier stage. Thus, it is likely that myoblasts produce and secrete into the media a factor that promotes the fusion of myoblasts, and this factor must have been accumulated in the medium during the period of culture.

To examine whether or not the fusion promoting activity of MCM was dose-dependent,

Table 1. The effects of muscle-conditioned medium on the fusion and growth of chick embryonic myoblast cells in culture.

	Fusion Index			Growth Index		
	48h	60h	72h	48h	60h	72h
Control	19	61	72	115	140	152
1st MCM	21	67	65	112	120	118
2nd MCM	51	—	—	78	—	—

Table 2. The fusion and growth indices of chick embryonic myoblasts in culture in different compositions of the medium. The cell age was 48 hours (the first 20 hours in 811 and then 28 additional hours in the medium given).

	Medium composition (%)			Fusion index	Growth index
	8102	2nd MCM	MEM		
A	100	—	—	19	115
	75	25	—	20	103
	50	50	—	17	105
	25	75	—	33	16
	—	100	—	51	78
B	75	—	25	18	111
	50	—	50	25	112
	25	—	75	28	84
	—	—	100	6	40
C	—	25	75	27	62
	—	50	50	41	61
	—	75	25	44	73

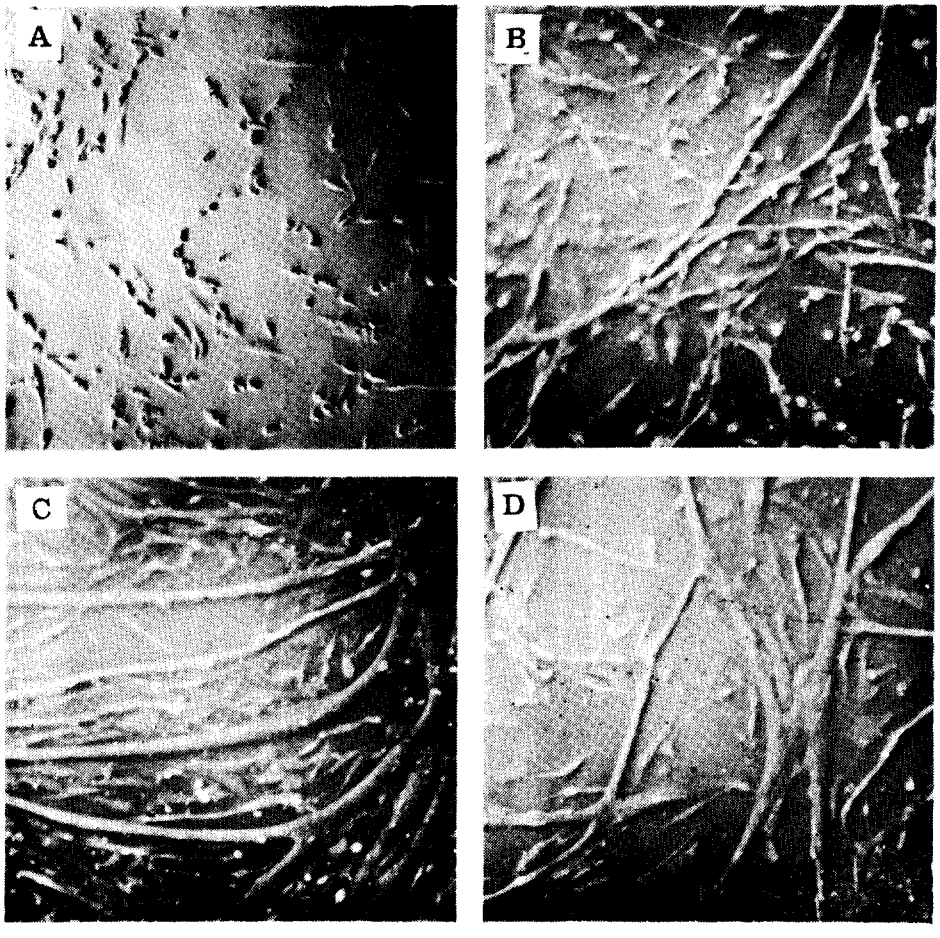

Fig. 2. Photomicrographs of chick embryonic myoblast cells in culture. Cells were photographed under a phase contrast microscope (*X*100). A, 20 hours (20 hours in 811). B, 48 hours (20 hours in 811 and 28 hours in 8102). C, 72 hours (20 hours in 811 and 52 hours in 8102). D, 48 hours (20 hours in 811 and 28 hours in 2nd MCM).

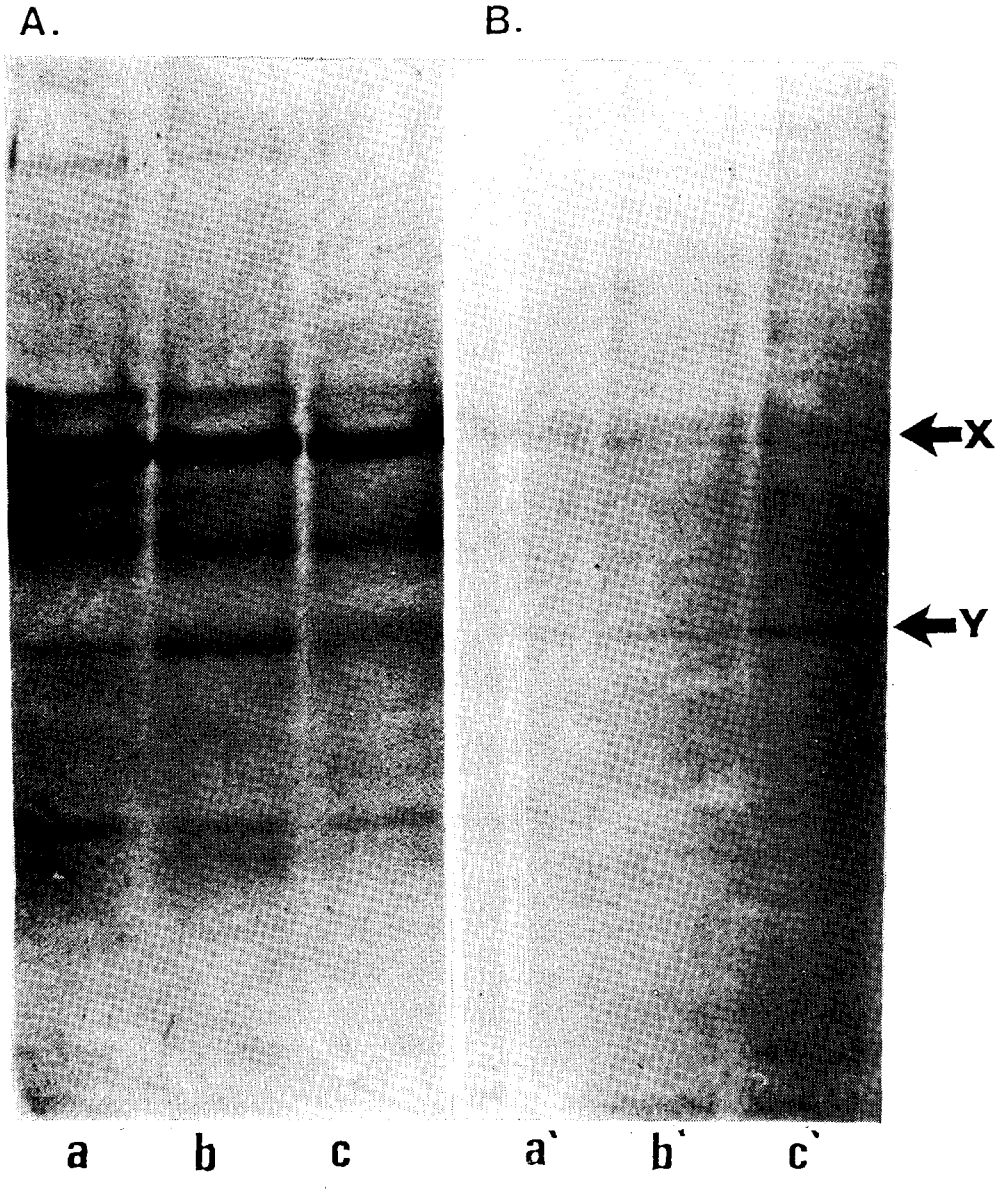

Fig. 3. The protein composition of cultured medium. The medium (8102) was used for culture of 20 hour cells for 10(a), 20(b) and 28 hours(c) and subjected to SDS-polyacrylamide slab gel electrophoresis. A, Coomassie-blue stained pattern. B, Autoradiogram of A.

cells were cultured in 3/4-, 2/4-, and 1/4-diluted MCM for 28 hours (total age of the culture was 48 hours). Both the fusion index and cell number decreased by reducing the amount of MCM added to fresh 8102 medium (Table 2, column A). These data suggest that fusion is initiated earlier than control as the concentration of the fusion promoting factor present in MCM is increased.

However, decreasing the concentration of horse serum and embryo extract in 8102 also promoted fusion (Table 2, column B) and decreased the cell number. These data may imply that the increase in the rate of fusion is due to the depletion of a growth factor that may exist either in serum or in embryo extract. But the fusion of myoblasts seemed to be promoted mainly by the accumulation of fusion promoting factor, because the fusion promoting effect of MCM was much greater (Table 2, column A) than those of low concentrations of serum and embryo extract (Table 2, columns B and C).

Accumulation of Secreted Proteins in the Medium

If any protein is released from cells into the culture medium, the amount must be very small and hard to be detected in the electrophoresis since the medium itself contains a bulk amount of proteins. However, when cells are grown in the medium containing radioactive amino acids for a while, any protein released from cells must be radioactive and hence it may be easy to detect the protein by electrophoresis and autoradiography.

Therefore, in the present experiment, cells were first grown in 811 for 20 hours, the medium was changed with 8102 containing ^{35}S-methionine to label proteins synthesized in the cell, and at 10, 20 or 28 hours later the medium was collected to analyze the labeled proteins.

Fig. 3 A is the Coomassie blue-stained electrophoresis gel of these media. The concentration of most proteins in these three media were almost the same, except one protein of approximately 45,000 daltons, whose concentration was markedly increased in 20 hour medium. However, this protein appeared not to be responsible for the fusion promoting activity since its relative concentration was too high and did not further increase in 28 hour medium, which showed the maximum fusion promoting activity. At present, it is uncertain why the level of this protein increased in the media and remains to be further investigated. All other major proteins appeared to be the components of horse serum and embryo extract, such as serum albumin.

Fig. 3 B is the autoradiogram of the gel presented in Fig. 3 A. Two proteins indicated as X and Y in the figure increased in their radioactivity as the exposed time of the medium to cells increased from 10 hours to 28 hours. The molecular weights of these proteins were estimated to be approximately 65,000 and 45,000 daltons. To quantitate their concentration change more precisely, densitometric scanning was performed (Fig. 4). The contents of proteins X and Y were 2.0% and 2.5% in 10 hour medium, 3.7% and 3.7% in 20 hour medium, and 4.5% and 5.6% in 28 hour medium respectively, of total radioactive proteins.

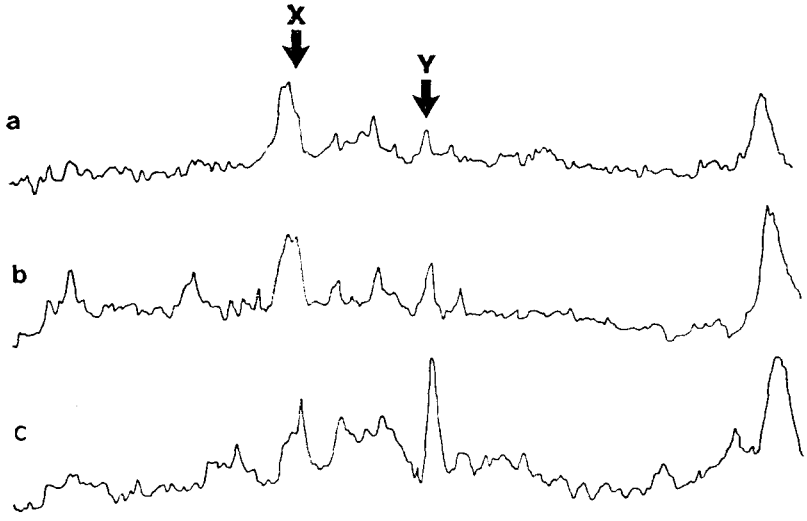

Fig. 4. Densitometric scanning of autoradiogram of electrophoresis gel (Fig. 3, B). a, 10 hour medium; b, 20 hour medium; and c, 28 hour medium.

Since MCM showed higher fusion promoting activity when the conditioned time was longer, increase in the contents of these two proteins (X and Y) in the medium as the conditioned time is increased is thought to be suggestive that these released proteins are responsible for the enhancement of myoblast fusion.

DISCUSSION

Muscle-conditioned medium (MCM) has been known to promote the process of fusion of cultured myogenic cells. Konigsberg(1971) has initially reported that myoblast fusion was controlled by some metabolic processing of the medium and depended on cell population size. Doering and Fishman (1974) have shown that the fusion of myoblast cells grown in MCM occured about 10 hours earlier than the control culture did.

However, it is still uncertain how the MCM promotes the fusion process. By analyzing the component in MCM, Doering and Fishman (1977) have suggested that MCM may contain a fusion promoting factor, which is cell specific and is secreted from the cell to the medium during incubation period. Therefore, it may be considered that, when the level of the secreted protein(s) in the medium reaches a critical point, the myoblasts then commence fusion. Ha *et al.* (1983) have also detected several secreted proteins in MCM, which are considered to act on the cell membrane to initiate the fusion. However, these workers used a protein-free medium (MEM) to assay the secretion of the protein from the cells. The use of MEM is certainly easier way to isolate any proteins released from cells into the medium(MEM) than that of normal medium that contains

huge amount of proteins of horse serum and embryo extract, but gives a harsh condition to the cell that may lead to surface alteration and extentive shedding of macromolecules of the cell in the culture.

Therefore, in the present experiment, MCM was obtained by growing the cell in the normal 8102 medium instead of MEM in order to keep the cell in more physiological conditions, and any proteins released from the cell were distinguished from the medium proteins by radioactively labeling the proteins in the cell. In this way, the present study confirmed that MCM not only had a fusion promoting activity, but also contained proteins secreted from myoblasts during the conditioning period of the medium. Since MCM promoted the fusion of myoblast cells and contained at least two proteins (indicated as X and Y in Result) that are released from the cells, it may be assumed that these proteins are responsible for the fusion promoting activity of MCM.

Fusion promoting activity may be interpreted in another way. Many investigators have suggested that this effect is due to the depletion of some growth factor in the medium. For example, Linkhart *et al.* (1981) and Konigsberg(1982) reported that depletion of cell-specific mitogenic activity in the medium triggered the myoblast fusion. In the present study, it was also confirmed that fusion could be promoted by reducing the concentration in the culture medium of horse serum and embryo extract in which it is known to contain growth factor (Table 2, column B). However, this effect was much less than that of MCM (Table 2, column A). Furthermore, fusion promoting activity of MCM was dose-dependent. Thus, it is more likely that the promotion of fusion is mainly due to some fusion promoting factors released from cell into the medium. Proteins X and Y detected in the present study may be strong candidates for the factor.

If indeed the proteins X and Y of the present study are responsible for the fusion promoting activity in MCM, the main question to be asked is how and by what mechanism these proteins can promote the fusion of myoblast. One simplest explanation could be that these fusion promoting factors have some kinds of enzymatic activity which catalyzes or promotes the fusion by altering the components of the cell membrane. For example, the factors may have a proteolytic activity, so that the altered membrane protein now can expose its crucial portion to the medium to trigger the fusion between neighboring myoblasts. Similarly, Kang *et al.* (1983) have proposed a model in which MCM has a scissor-like activity (i.e. proteolytic activity) against certain membrane proteins, cuts these proteins and therefore induces the fusion. If any fusion inhibitor exists in the medium as suggested by Evinger-Hodges *et al.* (1982), the fusion promoting factors could be considered to degrade and inactivate this inhibitor and thus facilitate the fusion.

Bersten *et al.* (1983) have reported that transglutaminase is present in cell-free lysates of chick myoblasts. If this enzyme is secreted from myoblasts and cross-links membrane proteins of the neighboring cell, myoblast fusion can be promoted. It has also been reported that extracellular Ca^{2+} concentration, that is essential for this enzyme activity, regu-

lates the process of fusion.

To test the possibilities listed above, the most urgent thing to do is the isolation and characterization of proteins X and Y. These studies are being undertaken to evaluate the present study and subsequently to clarify the mechanism of cell-to-cell fusion.

SUMMARY

In order to investigate the mechanism of myoblast fusion during muscle differentiation in culture, the effect of muscle-conditioned medium on the fusion was studied and possible release from cultured myoblast cells of proteins which may be responsible for the promotion of myoblast fusion was analyzed.

The muscle-conditioned medium showed a marked fusion-promoting activity in a dose-dependent fashion. This fusion-promoting activity of the muscle-conditioned medium appeared to be due to the accumulation of at least two proteins which were released from the myoblast into the culture medium. These released proteins were analyzed by electrophoresis and autoradiography and found to have molecular weights of 45,000 and 65,000.

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