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The Effects of Ovoinhibitor on Myoblast Differentiation

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근세포 분화에 미치는 ovoinhibitor의 영향

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요 약

단백질 분해효소와 ovoinhibitor가 계배근세포 융합에 미치는 영향을 알아보기 위하여 세포 융합지수의 측정, Western blot 분석법에 의한 ovoinhibitor의 세포 내농도 측정 및 단백질 분해효소의 활성도를 조사하였다.

세포 배양 30시간과 72시간에 처리된 ovoinhibitor는 상당히 근세포 융합을 억제시켰다. 근세포 내 ovoinhibitor의 농도는 배양 48시간에서 가장 높았고, 배양이 진행됨에 따라 계속 떨어져서 대체로 융합지수가 올라갈수록 그 농도가 낮아지는 경향을 보였다. 근세포 분화가 진행됨에 따라 총 단백질 분해효소의 활성도는 계속 증가하였는데 특히 배양 48시간에서 72시간 사이의 급격한 증가를 나타내는 시기는 세포 융합이 가장 활발히 일어나는 시기와 일치했다.

이상의 결과는 단백질 분해효소 저해제가 근세포 분화에 상당한 영향을 끼침을 보여 주며, 단백질 분해효소와 단백질 분해효소 저해제는 하나의 조절단위로써 근세포 분화에 어떤 작용을 할 것으로 사료된다.

INTRODUCTION

During the differentiation of skeletal muscle, mononucleated myoblasts fuse to form multinucleated syncytia or myotubes. Myoblasts, briven from a vertebrate embryo, provide the helpful models system to examine this process (Konigsberg, 1963; Yaffe, 1969; O'Naill and Stockdale, 1972; Turner *et al.*, 1974; Ha *et al.*, 1979). *In vitro* myoblast differentiation

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occures in distinct phases regularly (O'Neill and Stockdale, 1972; Konigsberg, 1982). Initially, cells rapidly proliferate without indication of any differentiation. Then, at a predicable time, an abrupt and rapid transition occurs, thereafter, they become multinucleated myotubes through the rapid fusion of plasma membrane. These differentiating cells show varing characteristics; cessation of the DNA synthesis with the irreversible withdrawal from the cell cycle, and the production of muscle specific proteins such as myosin, creatin phosphorylases, and acetylcholine receptors (Okazaki and Holtzer, 1966; Paterson and Prives, 1973; Turner *et al.*, 1974; Nadal-Ginard, 1978; Davin and Emerson, 1978; Ha *et al.*, 1981).

The inhibitors of proteases are ubiquitous. They have been described in wide variety of animals and plants as well as in microorganisms (Chung *et al.*, 1983; Kucera *et al.*, 1984). Also the widespread distribution of a trypsin inhibitor in vertebrate muscle tisses has been known (Noguchi and Kandatsu, 1967; Waxman and Krebs, 1978; Toyohara *et al.*, 1983; Hjelmeland, 1983). Even though the prevention of the unwanted proteolysis has been supposed for their general physiological function, the detailed function has been rarely elucidated.

In chicken, there have been known several protease inhibitors such as ovoinhibitor, ovomucoid, and plasma proteinase inhibitor (Tomimatsue *et al.*, 1966; Sugimoto *et al.*, 1984). Especially Chicken ovoinhibitor, an egg-white glycoprotein, is one of the most complex naturally occuring polyfunctional inhibitor of proteases. It is much larger in size (Mr 47,600) than most other known trypsin inhibitors from plants and other animals, and it is capable of inhibiting simultaneously 2 molecules of trypsin and 2 molecules of chymotrypsin (Tomimatsu *et al.*, 1966).

However, there is no report about the relationship between fusion processes and the action of trypsin inhibitor during myoblast differentiation. This study was undertaken to establish the effects of proteases and protease inhibitor on the myoblast differentiation *in vitro*.

MATERIALS AND METHODS

Cell culture

Cultures were prepared by the method of O'Neill and Stockdale (1972) with minor modifications. Pectoral muscle was dissected out from 12-day old chick embryos. After removing connective tissues, the muscle was minced and incubated for 30 min at 37°C in 0.1% trypsin with gentle agitation. 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 (8:1) and filtered through four layers of lens paper. The cell suspension was preplated on collagen-coated dishes for 10 min to remove fibroblasts. The cells were then plated at 5×10^5 cells/ml on tissure culture dishes which had been precoated with autoclaved and Millipore-filtered collagen

solution (1.5 mg/ml). Cells were incubated at 37.5°C in water-saturated atmosphere of 95 % air and 5% CO_2. At 24 hours after the initial cell plating, the medium was changed to MEM containing 10% horse serum, 2% embryo extract and 1% antibiotic-antimycotic solution (9102). After this first medium change with 8102, the medium was routinely changed with 8102 every 48 hours. Cultured cells were harvested in ice-cold PBS(pH 7.0) and microfuged for 6 min.

Treatment of ovoinhibitor

Ovoinhibitor (Sigma), a kind of trypsin inhibitor, was dissolved in PBS and used at a final concentration of 0.1 and 1 μg/ml. Inhibitor was added at 34hr and 72hr after plating.

Measurement of cell fusion

The cells were fixed in ethanol and stained with Giemsa Solution, then, counted for determing the fusion index by direct microscpic examination at a magnification of 400X. Randomly chosen 10 fields were counted for this purpose. The degree of fusion was determined by the ratio of the number of nuclei within the myotubes of three or more nuclei divided by the total number of nuclei.

Proteolytic activity assay

The cells were sonicated and centrifuged, and the supernatants were used for analysis. The reaction was started with the addition of 0.1M Tris-HCl buffer (pH 7.4) and 1% azocasein. After the incubation for 4 hrs at 30°C, the reaction was terminated by adding 1 ml of 10% trichloroacetic acid. The mixture was then centrifuged at 3,000 g for 15 min. The supernatant was mixed with equal volume of 0.5M NaOH and its absorbance at 440 nm was measured. One protease unit was defined as the amount of proteolytic enzymes that shows the same A_{440} by 1 μg of trypsin (Charney and Tomarelli, 1947).

Preparation of antiserum

Albino rabbits were immunized with purified ovoinhibitor (Sigma). About 0.5 ml of the ovoinhibitor solution (1 mg/ml in phosphate buffered saline) was emulsified with the equal volume of Freund's complete adjuvant, and injected intramuscularly. After 10 days, 250 μg of the ovoinhibitor in Freund's incomplete adjuvant was injected sub-cutaneously. Third injection was done samely as the second injection abter 3 weeks. Blood was collected by heart-puncture. Isolation of serum from the blood was carried out as described by Johnstone and Thorpe (1982).

Gel electrophoresis and Western blot analysis

The SDS-polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli (1970) using discontinuous gel system which consists of 3% stacking and 10% separating gel.

Western blot analysis was carried out by the method of Towbin *et al.*(1979) with slight modification. The proteins separated by electrophoresis were electrophoretically transferred to nitrocellulose sheets. The sheets were soaked in 5% bovine serum albumine (0.9% NaCl/10 mM Tris-HCl, pH 7.4) for 2 hrs at room temperature to saturate additional

protein binding sites. They were then incubated in the first antibody solution for 2 hrs at room temperature. After the blots were washed in saline solution containing 0.1% Triton X-100 for 1 hr they were incubated for 2 hrs at room temperature in the second antibedy solution (Horseradish peroxidase-conjugated goat Ig G). For the color reaction, the blots were soaked in 8 ml of 0.05 M Tris-HCl (pH 7.2) containing 2 mg of 3-3′-diaminobenzidine tetrahydrochloride and 2 μl of H_2O_2. In case of using ^{125}I-Protein A (2×10^5 cpm; 100 Ci/mol), the blots were washed in saline and dried throughly with a hair drier. The blots were exposed to Kodak X-Omat R film for 6 days. The radioactivity retained on the band was counted in liquid scintillation spectrometer (Packard Instrument Co., Downers Gove, III.).

RESULTS

Effect of ovoinhibitor on cell fusion

To observe the effect of ovoinhibitor on myoblast differentiation, the ovoinhibitor at final concentration of 0.1 and 1 μg/ml were added to the culture dishes at 34 hr and 72 hr after cell seeding. As shown in Figs. 1 and 2, the myoblast fusion was moderately inhibited by ovoinhibitor. The effect of inhibition was slightly more effective at high concentiation of ovoinhibitor (1 μg/ml). However, the ovoinhibitor showed no influence on cell proliferation.

Changes in intracellular concentiations of ovoinhibitor

The induction of the antibodies against the ovoinhibitor was certified by a double-diffusion analysis (Fig. 3). Fig. 4 showed the intracellular concentration of ovoinhibitor determined by electrophoresis and Western blot during myoblast differentiation. The intracellular concentration of ovoinhibitor was highest at 48 hr after cell seeding, thereafter, it was gradually decreased. It is note worthy that the concentration of ovoinhibitor was considerably decreased in periods from 48 hr to 72 hr after cell seeding, during which the fusion was

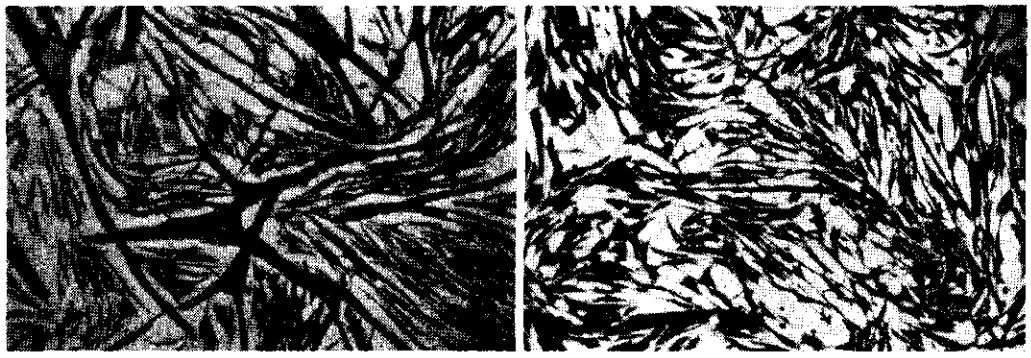

Fig. 1. The patterns of the differentiation of myoblast at 72 hr after cell seeding. A, control; B, ovoinhibitor treated (1μg/ml).

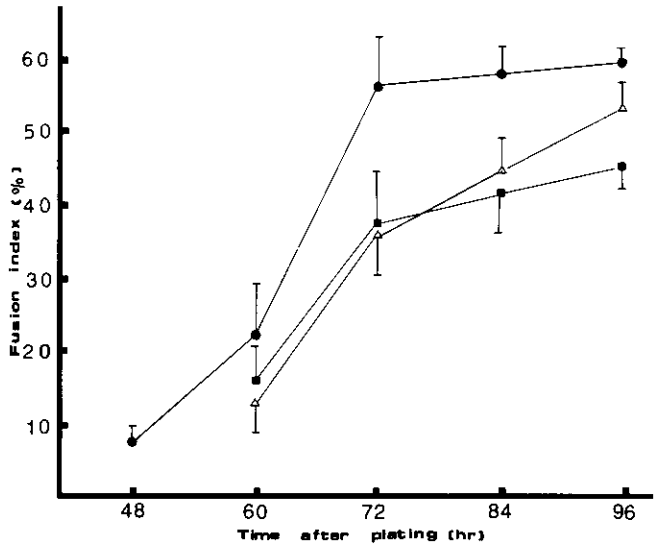

Fig. 2. Effect of trypsin inhibitor on myoblast fusion. The ovoinhibitor was added at 30 hrs and 72 hrs after cell seeding. •—•, control; △—△, 0.1 μg/ml; ■—■, 1μg/ml.

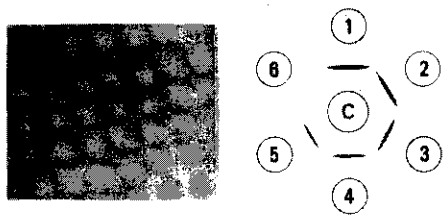

Fig. 3. Double-diffusion analysis on 1% agarose Quchterlony plate. C, 30μl of antiserum; 1, 2, 3, 4, 5, 6, 30μl of ovoinhibitor with 0, 1/2, 1/4, 1/8, 1/16, and 1/32 dilution respectively.

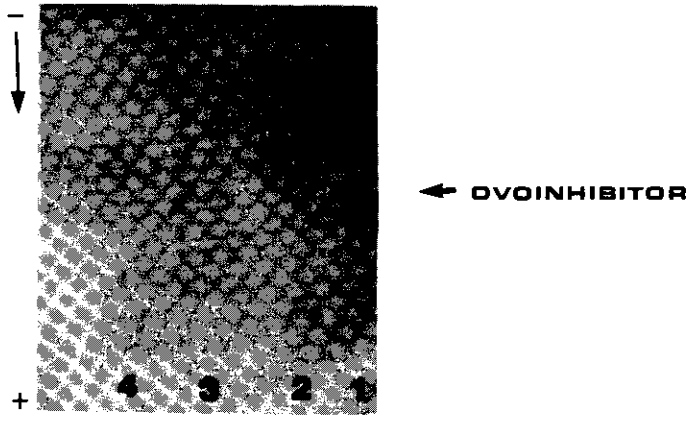

Fig. 4. Detection of ovoinhibitor by horseradish peroxidase-conjugated goat Ig G as second antibody during myoblast differentiation. Lane 1, 96 hr; 2, 72 hr; 3, 48 hr; 4, 24 hr myoblast after plating.

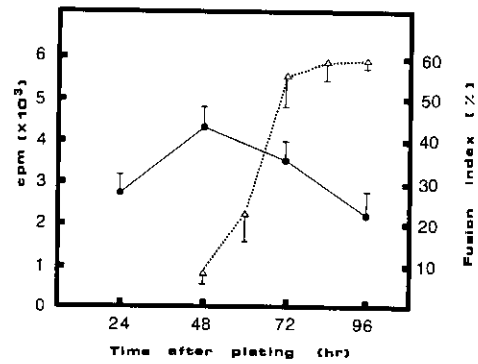

Fig. 5. The profile of ovoinhibitor activities during myoblast differentiation. The radioactivity of ^{125}I-protein A as second antibody was counted with liquid scintillation spectrometer. △···△, fusion index of control; ●—●, concentration of ovoinhibitor.

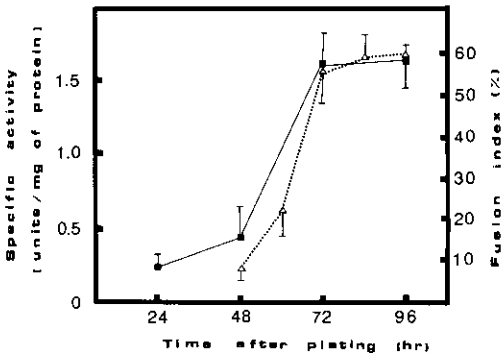

Fig. 6. The pattern of proteolytic activities during myoblast differentiation. ■—■, proteolytic activity; △···△ fusion index of control.

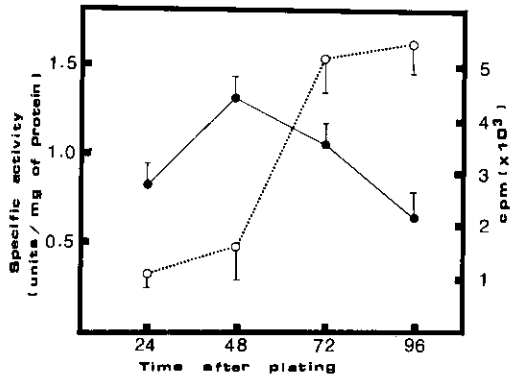

Fig. 7. Comparison of the pattern of the proteolytic activity with that of the concentration of ovoinhibitor. ○···○, proteolytic activity; ●—●, concentration of ovoinhibitor.

progressed most actively. It appeared that intracellular concentration of the ovoinhibitor was rather inversely proportional to the degree of myoblast fusion(Fig. 5).

Changes in the activities of protease and protease-inhibitor

To determeine the overall proteolysis in differentiating muscle cells, casein-hydrolysis was measured using the extracts obtained from the cells. As shown in Fig. 6, the proteolytic activity increased rapidly in the cells proceeding the fusion event and leveled off as the cells finish their fusion process. Of particular interest was that the kinetic mode of the increase in proteolysis nearly overlaped with that of the increase in fusion index. By contrast, the level of ovoinhibitor sharply declined as the fusion progresses (Fig. 7). Therefore, it wer likely that the activity of protease is inversely regulated with that of the protease-inhibitor during the fusion of myoblasts.

DISCUSSION

The fusion of mononucleate myoblasts is a multistep process including the following separable components: (1) cell migration, recognition, alignment, and (2) membrane fusion loading to cytoplasmic continuity (Nameroff and Munar, 1976; Knudsen and Horwitz, 1977). Both components, called recognition-alignment and fusion, have been studied extensively. It has been reported that differentiation of myoblasts was not only regulated by the intrinsic program in myoblasts, but can also easily be controlled by simple environmental changes. Although a number of intrinsic as well as extrinsic agents that exert influence on differentiation of myoblasts have been reported (Den *et al.*, 1975; Nameroff and Munar, 1976; Gilfix and Sanwal, 1980; David *et al.*, 1981; Wakelam and Pette, 1982; Couch and Strittmatter, 1983), none of their function for the myogenic process has been elucidated so far. Therefore, the present study was aimed to establish the role of protease and protease-inhibitor in the myoblast differentiation.

For this purpose, ovoinhibitor was chosen to see its effect on the fusion of myoblasts since it is one of the major yolk protein, with which the chick embryo develops. The present study clearly demonstrates that ovoinhibitor is capable of preventing the fusion of myoblasts (Figs. 1 & 2). Therefore, it seems possible that the protease-inhibitor blocks the activity of a certain secreted protease which may be essential for the myogenic process. However, it is yet to be clear the role of ovoinhibitor added to the culture medium.

To determine more precisely the involvement of ovoinhibitor and protease in fusion, two approaches have been used in this study: (1) the measurement of the level of ovoinhibitor using immunoblot, and (2) the direct assay of proteolytic activity in the cultured cells. These procedures enables to demonstrate that the proteolytic activity concomitently increases with the fusion of myoblasts. Furthermore, the increase in the proteolysis appeared to be inversely proportional to the level of the protease-inhibitor. These results clearly suggest that the fusion requires the proteolysis, which might be masked by the presece of protease-inhibitor during the proliferation period.

It has been reported that there occurs an extensive redistribution of membrane proteins during the fusion of myoblasts (Herman and Fernandez, 1978). The redistribution of membrane proteins could be catalyzed by a protease that is inhibitable by ovoinhibitor. In other words, when myoblasts are committed to fuse, the level of ovoinhibitor drops and therefore the activity of protease incrases. As the activated protease hydrolyzes the membrane proteins to expose possibly their hydrophobic domains, the cells can initiate fusion through the hydrophobic interation between the adjacent cells. Therefore, we suggest that a balance of the level of protease to that of protease-inhibitor may role as a signal for the augment of myogenic cell fusion, although much is required for understanding the detailed mechanism of their action.

ABSTRACT

The effects of protease and protease-inhibitor on the fusion of myoblasts were investigated *in vitro* by the measurement of fusion index, the level of ovoinhibitor using immunoblot, and the activity of protease in the extract of cells in culture.

Exogenously added ovoinhibitor was capable of blocking the fusion of myoblasts, perhaps by inhibiting the activity of a certain secreted protease that may be essential for the fusion.

The intracellular level of ovoinhibitor was maximum at 24 hr after cell seeding, but gradually declines as the fusion proceeds, showing that the level of protease-inhibitor is inversely proportional to the fusion index. On the other hand, the proteolytic activity was sharply increased during the fusion period, and then leveled off as the cells form myotubes.

These results suggest that the protease-and-protease-inhibitor system participates in myoblast fusion as a regulatory system.

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