Korean J. Zool. 31:95-103, 1988

Alterations in the Level of Fibronectin and its Receptors during Chick Myoblast Differentiation

Chang Yong Chung and Man-Sik Kang

Dept. of Zoology, Seoul National University, Seoul 151-742, Korea

Alterations in the amount of fibronectin during chick myogenesis were investigated. The amount of fibronectin as measured by immunoblotting was found to decrease during the process.

As a first step in answering the precise mode of change in the level of fibronectin during myogensis, the interaction of 28,000 dalton(28 kDa) amino terminal fragment of fibronectin as well as 85,000 dalton (85 kDa) fragment with myoblasts was examined. The specific binding of ^{125}I-28 kDa fragment to myoblasts was time-dependent and reached a maximum within 60 min. Unlabelled 28 kDa fragment inhibited the binding of ^{125}I-28 kDa fragment, whereas 85 kDa fragment containing adhesion promoting activity did not inhibit it. This finding suggests that the 28 kDa fragment interacts with the matrix assembly receptors but not with the cell adhesion receptors. Accordingly, the decrease in the level of fibronectin is likely to correlate with the fall of fibronectin receptors on the myoblasts.

KEY WORDS: Myogenesis, Fibronectin receptor, Amino terminal fragment

The differentiation of skeletal muscle *in vivo* and *in vitro* is accompanied by the fusion of mononucleated myoblasts into multinucleated myotubes. The fusion of myoblasts is certainly a multistep process, including a minimum of the following separable components: 1) cell migration, recognition, and alignment, and 2) membrane fusion leading to cytoplasmic continuity (Nameroff and Munar, 1976: Knudsen and horwitz, 1977). These lead to a series of investigations of the surface elements that may be involved in myoblast migration, recognition, and alignments.

Several authors have reported investigations of the cell surface alterations which occur during myogenesis (Hynes *et al.*, 1976; Moss *et al.*, 1978; Walsh and Phillips, 1981). As a result of these observations, it is claimed that certain proteins are involved in the fusion process. The best characterized of these is fibronectin. Fibronectin is a major extracellular connective tissue component of muscle (Stenman and Vaheri, 1978; Walsh *et al.*, 1981). The function of fibronectin in muscle tissue is not known, but in general the possible functions of fibronectin include promotion of cell adhesion, regulation of cell shape, and guidance of cell migration (Hynes, 1981 for review). Several studies of muscle cell surface in tissue culture have yielded data on the levels of fibronectin during myogenesis. Hynes *et al.* (1976) found an increase in fibronectin levels in postfusion myotube cultures. Moss *et al.* (1978) found a similar increase in fibronectin levels.

In contrast, immunocytochemical observations on the rat L_6 muscle cell line and on cloned human muscle cells indicate that as myoblasts fuse they lose their cell surface fibronectin (Chen, 1977; Furcht *et al.*, 1978; Walsh *et al.*, 1981). A similar result was obained by Kang *et al.* (1983, 1985). Furthermore, addition of exogeneous fibronectin to rat myoblast cultures inhibited fusion (Podleski, 1979) and fibronectin was also found to prevent the fusion block in the tunicamycin-treated myob-

This work was supported by the Basic Science Research Institute Program, Ministry of Education, 1987.

last cultures (Chung and Kang, 1987). The balance of these evidence suggests that the loss of fibronectin from the surface of myoblasts is important in the regulation of myoblast fusion and that its continued presence is inhibitory to myogenesis.

There are, however, a number of unanswered questions concerning the precise mode of change in fibronectin levels during myogenesis. The disappearance of fibronectin from the surface of fusing myoblasts is probably not the result of a change in the rate of synthesis of fibronectin (Gardner and Fambrough, 1983). It is likely that the fusing myoblasts and young postfusion myotubes are producing fibronectin, which is not retained by the cells. Thus, we have been particularly concerned with the cell surface receptors for fibronectin. In the present report, we have attempted to identify the receptor for fibronectin in myoblasts and to examine the interactions between fibronectin fragments and its receptors.

Materials and Methods

Materials

RPMI 1640 medium, horse serum, and antibiotics (penicillin-streptomycin solution and fungizone) were obtained from Gibco Laboratories. Tissue culture dishes and cluster plated were from Nunc. ^{35}S-methionine and $Na^{125}I$ were from Amersham, ^{125}I-protein A from New England nuclear, nitrocellulose papers (pore size, 0.45 μm) from Shleicher and Shuell. Trypsin (DPCC-treated), thermolysin, heparin-agarose, gelatin-agarose, and other reagents were obtained from Sigma.

Cell Culture

Myoblast cultures were prepared according to the methods of O'Neill and Stockdale (1972) with minor modifications. Birefly, breast muscle from 12-day old embryos were dissected out, minced, and digested with 0.1% trypsin (Gibco) for 30 min and dispersed by repeated pipetting. Cells were collected by centrifugation and suspended in RPMI 1640 medium supplemented with 10% horse serum, 10% chick embryo extract, and 1% antibiotics (811 medium). The cells were preplated on collagen-coated dishes for 15 min to remove fibroblasts. The cell suspension was then filtered through a four-fold lens paper to remove undissociated cells. Approximately 5×10^5 cells per ml were inoculated on collagen-coated dishes. Medium was changes after 24 hr with RPMI 1640 medium containing 10% horse serum, 2% embryo extract, and 1% antibiotics (8102 medium). Plated cells were placed in a humidified incubator in the atmosphere of 95% air and 5% CO_2 at 37°C.

Measurement of Cell Fusion

At appropriate times, cells were washed three times with phosphate-buffered saline (PBS) and were fixed in a mixture of ethanol, formaldehyde, and acetic acid (20 : 1 : 1, V/V) for 5 min. After rinse with distilled water, the cells were stained with hematoxylin for 5 min and washed in a tap water thoroughly. The degree of fusion was determined by the ratio of the number of nuclei within the myotubes with three or more nuclei to that of nuclei as seen under microscope at 400X. Ten fields were randomly chosen for each dish.

Electrophoresis

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was essentially the same as described by Laemmli (1970) except that the separating gel consisted of 8-14% polyacrylamide gradient. Protein quantitation was performed by the procedure of Lowry *et al.* (1951) or Bradford (1976) with bovine serum albumin as a standard.

Immunochemical Detection of Fibronectin

For producing anti-fibronectin antibodies, rabbit antiserum against electrophoretically pure horse serum fibronectin was prepared as described by Chung and Kang (1987). Myoblast homogenates (80 μg of protein) were electrophoresed in 8-14% polyacrylamide slab gels. After electrophoresis, the proteins were transferred onto nitrocellulose paper at 45V for 90 min in a trans-blot apparatus assembled as described by Stott *et al.* (1985). The papers were incubated in 5% (W/V) bovine serum albumin at room temperature for 2 hr and then in the antibody solution at 4°C overnight. They were washed with 50 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl and 0.1% Triton X-100, and were incubated in ^{125}I-protein A solution (2×10^5 cpm/ml). After incubation, the nibrocellulose papers were washed three times with the same buffer,

dried, and exposed to X-ray film for autoradiography. After autoradiography, the nitrocellulose strip was counted in a liquid scintillation spectrometer.

Purification of Fibronectin and its Fragments

Fibronectin was purified from horse serum by sequential affinity chromatography on gelatin-agarose and heparin-agarose with elution by 4 M urea and 0.5 M NaCl as descrbed by Engvall and Ruoslahti (1977).

The 85,000 dalton (85 kDa) cell binding fragment was generated by tryptic digestion of serum fibronectin and purified by the method of Johansson (1985) with minor modifications. Briefly, fibronectin (1 mg/ml) in 10 mM Tris-HCl (pH 7.4) containing 0.14 M NaCl, 1 mM $CaCl_2$, and 0.02% NaN_3 was digested with DPCC-treated trypsin (2 μg/ml) for 120 min at 37°C. The digestion was terminated by addition of phenylmethanesulfonylfluoride (PMSF) to a final concentration of 1 mM. The digest was dialyzed against 10 mM sodium phosphate (pH 7.4) containing 0.14 M NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, and 0.2 mM PMSF, and sequentially applied to columns of heparin-agarose and gelatin-agarose equilibrated with the same buffer. The unbound materials were dialyzed against 10 mM sodium phosphate (pH 7.0) containing 50 mM NaCl, and loaded to a DEAE-sepharose column. The DEAE-sepharose column was eluted with a linear gradient of NaCl from 50 to 400 mM in this buffer. The fraction which contained cell binding fragment were pooled and dialyzed against 10 mM sodium phospahte (pH 7.4) containing 2 mM dithiothreitol (DTT). After dialysis, the peptides were fractionated on a Sephacryl S-200 column. The peak containing cell binding fragment was pooled and stored at −20°C for further investigations.

Thermolysin digstion of purified fibronectin was performed according to Sekiguchi and Hakomori (1983). Fibronectin (1 mg/ml) in 25 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA, 50 mM NaCl, and 2.5 mM $CaCl_2$ was digested with 5 μg/ml of thermolysin for 4 hr at 22°C. The digestion was terminated by adding EDTA (final concentration of 5 mM). The digest in 10 mM sodium phosphate (pH 7.4) containing 0.14 M NaCl and 4.5 mM KCl was applied on gelatin-agarose column. The unbound fractions were pooled and loaded onto heparin-agarose. The bound materials were eluted with 0.5 M NaCl in the same buffer and dialyzed against 10 mM sodium phosphate (pH 7.4). After dialysis, peptides were chromatographed on a Sephacryl S-200 column. The fractions containing 28,000 dalton (28 kDa) amino terminal fragment were pooled and stored for analysis.

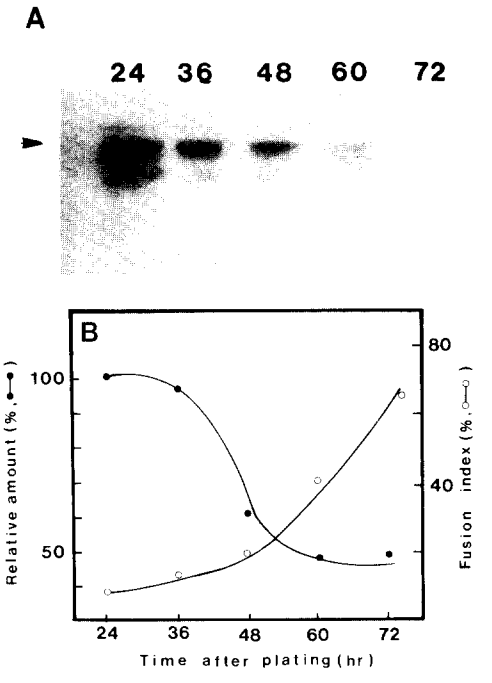

Fig. 1. Immunochemical detection of fibronectin at varying stages during myogenesis. (A) Immunoautoradiography of fibronectin. Numerals at top represent the time after myoblast seeding. (B) Change in the amount of fibronectin. The radioactivity of fibronectin bands on the nitrocellulose strips and the fusion index were presented. The radioactivity of 24-hr cultures is referred to as 100%.

Attachment Assay

Attachment assay was performed by the procedure of Ehrismann *et al.* (1982). Each 10-cm dish was inoculated with 4×10^6 cells in 8 ml of 811 medium supplemented with ^{35}S-methionine (2 μ Ci/ml). After 24 hr, myoblasts were dissociated by treating EGTA to a final concentration of 2 mM. Cells decanted from dishes were pooled, diluted with 10 mM N-2-hydroxyethylpiperazine-N′-2-

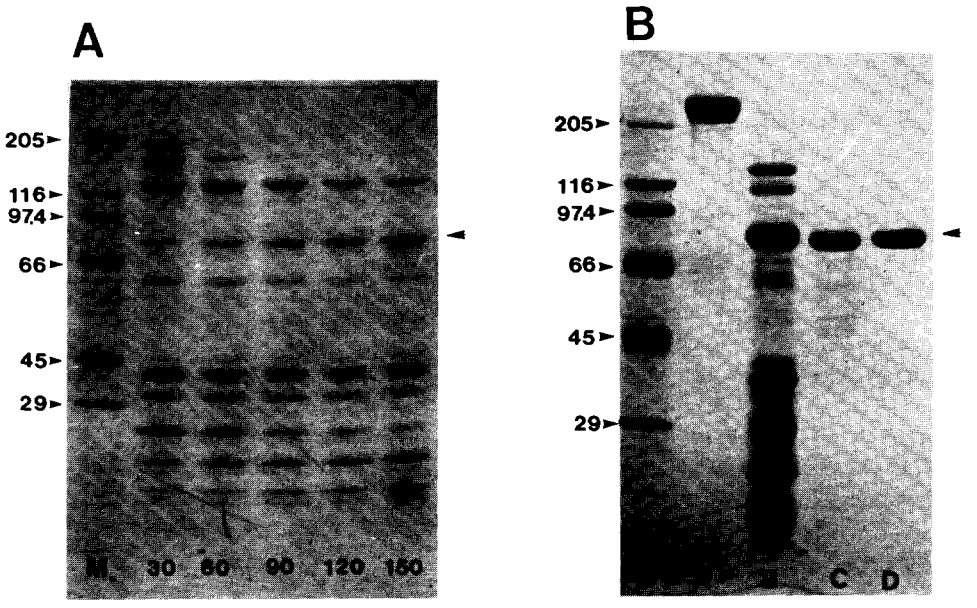

Fig. 2. Purification of 85 kDa cell binding fragment. (A) Time course of trypsin digestion of fibronectin. M, molecular weight markers; arrow on the right, 85 kDa fragment. (B) SDS-PAGE of the trypsin digest for 120 min. M, molecular weight markers; A, purified horse serum fibronectin; B, fragments not bound either gelatin-agarose or heparin-agarose; C, fragments from DEAE-sepharose; D, 85 kDa fragment fractionated on Sephacryl S-200.

ethanesulfonic acid (HEPES), pH 7.4, 0.137 M NaCl, 4.7 mM KCl, 0.65 mM $MgSO_4$, and 1.2 mM $CaCl_2$ (attachment buffer). Then the cells were used in determining the extent of cell attachment to the precoated 24-well plates. Each well was precoated by incubating for 1 hr at 37°C with 100 μl/well of protein solution to be tested, and then incubated with 0.1% bovine serum albumin for 1 hr at 37°C to block nonspecific binding sites. This solution was removed before seeding of the cells. Assay was started by pipetting 0.2 ml aliquot of cell suspension (1×10^5 cells) into each well. The extent of attachment was determined after 3 hr at 37°C as follows: The medium was decanted and the wells were gently washed three times with attachment buffer. Attached cells were then lysed with 0.2 ml of 4% SDS, and the radioactivity of lysate was determined by a liquid scintillation spectrometer.

Iodination of Fragments

200 μg of 85 kDa fragment and 100 μg of 28 kDa fragment were iodinated with 1 mCi of $Na^{125}I$ by chloramine T method (Greenwood *et al.*, 1963). The 28 kDa amino terminal fragment was labelled to a specific activity of 2×10^7cpm/μg and 85 kDa cell binding fragment to a specific activity of 1.3×10^7 cpm/μg. The integrity of the labelled peptide was ascertained by SDS-PAGE followed by autoradiography.

Binding of ^{125}I-fragment to Myoblasts

All binding assays were done in RPMI 1640/HEPES supplemented with 0.2% bovine serum albumin (binding medium). Myoblast cultures were rinsed three times with prewarmed RPMI 1640 and incubated with binding medium containing ^{125}I-fragment at 37°C. Extraction of cell layers was performed by the procedure of McKeown-Longo and Mosher (1983). After incubation, cultures were rinsed four times in ice-cold RPMI 1640/PEPES, and cell layers were either sequentially extracted in 1% deoxycholate followed by 4% SDS or directly in 4% SDS. Deoxycholate extraction was done in 0.02 M Tris-HCl (pH 8.3) containing 2 mM PMSF and 2 mM EDTA. The radioactivity of extracts was

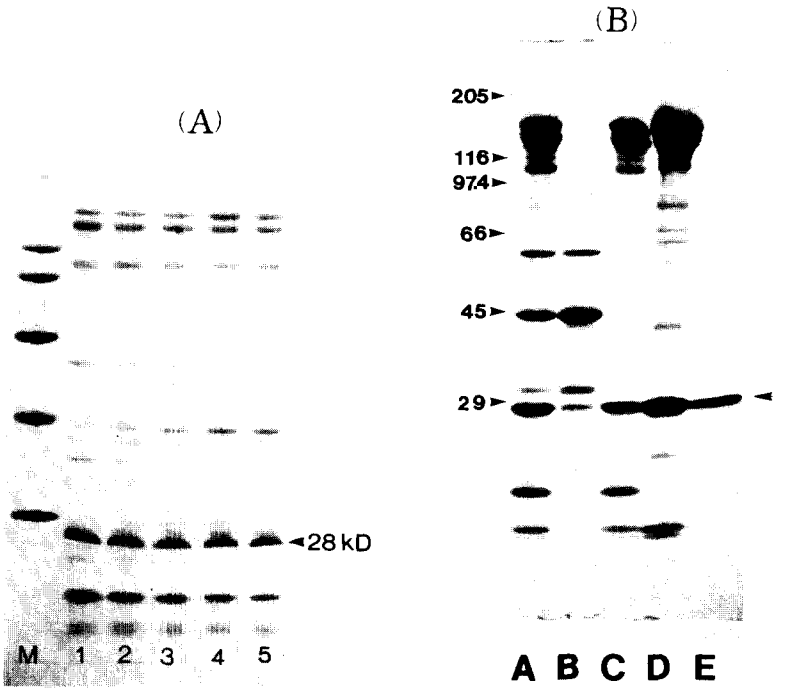

Fig. 3. Purification of 28 kDa amino terminal fragment. (A) Time course of thermolysin digestion of fibronectin. M, molecular weight markers; arrow on the right, 28 kDa fragment. (B) SDS-PAGE of the thermolysin digest for 4 hr. A, thermolysin digests; B, fragments not bound to gelatin; C, fragment bound to gelatin; D, fragments bound to heparin; E, 28 kDa fragment fractionated on Sephacryl S-200.

determined by a gamma counter.

Results

Changes in the Fibronectin Levels during Myogenesis

In order to investigate the level of fibronectin in the progress of myogenesis, the immunoblotting method was employed. As shown in Fig. 1, the level of fibronectin decreased with the progress of myoblast fusion. This result is in good agreement with earlier observations (Chen, 1977; Walsh *et al.*, 1981).

Properties of Fibronectin Fragments

Adhesion promoting activity was associated with the 85 kDa fragment which was accumulating during the incubation of fibronectin with trypsin (Fig. 2). Since 85 kDa fragment does not bind to gelatin or heparin, it must be originated from the central region of the fibronectin arms (Ehrismann *et al.*, 1982; Hayashi and Yamada, 1983). The 28 kDa fragment was one of the major fragments accumulating during thermolysin digestion (Fig. 3). The 28 kDa fragment bound to heparin and seemed to be identical to the amino terminal fragment which had been described by others (Sekiguchi and Hakomori, 1983; Zardi *et al.*, 1985).

Attachment Assay

We have examined the adhesion promoting activity of fibronectin and its fragments. Intact fibronectin and 85 kDa cell binding fragment promoted cell adhesion but little activity was associated with the 28 kDa amino terminal fragment (Fig. 4).

Time Course of ^{125}I-28 kDa Fragment Binding

The ^{125}I-28 kDa fragment bound to myoblasts in a time dependent manner at 37°C (Fig. 5). Bound ^{125}I-28 kDa fragment bacame associated with the cell layer in two distinct pools. The label-

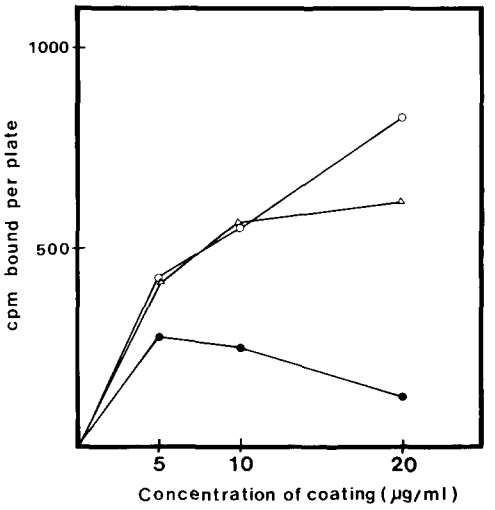

Fig. 4. Attachment of myoblasts to fibronectin, cell binding fragment or amino terminal fragment. Myoblasts prelabelled with ^{35}S-methionine were allowed to attach to plastic tissue culture cluster wells coated with fibronectin, 85 kDa fragment or 28 kDa fragment for 3 hr at 37°C. The attached cells were lysed with 4% SDS and the radioactivity was determined. ○—○, fibronectin; △—△, 85 kDa; ●—●, 28 kDa.

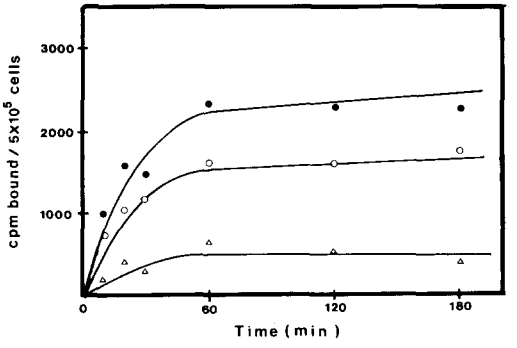

Fig. 5. Time course of specific binding of ^{125}I-28 kDa fragment to myoblasts. Cells were incubated with binding medium containing ^{125}I-28 kDa fragment (1×10^6 cpm/ml, 50 ng). At indicated times, cell layers were extracted to determine ^{125}I-28 kDa fragment in pool I or in pool II. Nonspecific binding was determined in the presence of 10 μg of fragment. Assay was done at 36 hr after cell seeding. ●—●, total specific binding; ○—○, specific binding to cell surface; △—△, specific binding to extracellular matrix.

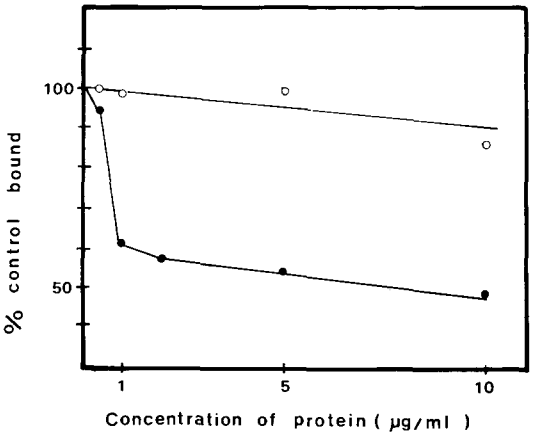

Fig. 6. Inhibition of binding of ^{125}I-28 kDa fragment to myoblasts by unlabelled 28 kDa fragment or 85 kDa fragment. Cells were incubated with ^{125}I-28 kDa fragment (1×10^6 cpm/ml) in binding medium and with increasing concentrations of unlabelled 28 kDa or 85 kDa fragment. Medium of control plates contained only albumin. Cultures were incubated for 120 min at 37°C, rinsed and scraped into 1 ml of 4% SDS. Assay was done 36 hr after cell seeding. ○—○, unlabelled 85 kDa fragment; ●—●, unlabelled 28 kDa fragment.

led fragment bound to the deoxycholate-soluble, cell surface pool (pool I) or to the deoxycholate-insoluble, extracellular matrix (pool II). Binding of the amino terminal fragment to pool I reached a steady state by 1 hr, whereas the binding to pool II was far less. The total observed binding after 120 min was specific to a level of 60 percent, as judged by the amount of binding in the presence of 200-fold molar excess of unlabelled amino terminal fragments.

Discussion

Fibronectin, a major extracellular connective tissue component of muscle cells, exerts a profound effect on the myoblast differentiation. This fibronectin mediates the myoblast-substratum attachment (Chiquet *et al.*, 1979; Ehrismann *et al.*, 1982). It was also suggested that the formation of eleongated myotubes in a certain spatial arrangement may be regulated by fibronectin (Chiquet *et*

al., 1981). In addition, it was reported that the amount of cell surface fibronectin decreases after the fusion of myoblasts to form myotubes (Hynes *et al.*, 1976; Chen, 1977; Walsh *et al.*, 1981) and that the fibronectin added exogeneously partially prevented the fusion block in tunicamycin-treated myoblast cultures (Chung and Kang, 1987). These results suggest that fibronectin plays a role in preparing the cells to fuse but its level gets reduced as the fusion proceeds.

As shown in Fig. 1, the level of fibronectin is being decreased as myoblasts differentiate. This result is in good agreement with the previous observations (Walsh *et al.*, 1981; Kang *et al.*, 1985). Yet we are unable to clearly document the mechanism for the decrease and/or disapperance of fibronectin. It was also claimed that fibronectin synthesis, as a fraction of total protein synthesis, remains constant before and after myoblast fusion (Gardner and Fambrough, 1983). Thus, the lack of surface fibronectin is thought not due to the change of fibronectin synthesis. With these implications in mind, we have examined the possibility that the change of fibronectin receptors leads to the loss of fibronectin.

It was suggested that there is a "matrix assembly site" on fibronectin for a "matrix assembly receptor" on substrate-attached cells and a "cell adhesion site" on fibronectin for a "cell adhesion receptor" on suspended cells (McKeown-Longo and Mosher, 1985). Antibody and fragment inhibition studies suggested that a site in the amino terminal 70,000 dalton sequence of fibronectin is important in matrix assembly (McDonald *et al.*, 1982; McKeonwn-Longo and Mosher, 1985). Inhibition studies using tryptic fragments derived from the 70,000 dalton fragments, however, suggested that the principal binding site is within the 27,000 dalton amino terminal region (McKeown-Longo and Mosher, 1985). Since myoblast cultures were grown on collagen substratum in our culture system, we had to rule out the binding of fibronectin to collagen. Thus, we have used the 28 kDa amino terminal fragment and 85 kDa cell binding fragment to identify the fibronectin receptor on myoblasts.

Although many investigations on the cell adhesion receptor have been reported (Johansson, 1985; Akiyama, *et al.*, 1985), the cell adhesion receptor interacting with 85 kDa cell binding fragment could not be observed in myoblast cultures (data not shown). Fibronectin and purified 85 kDa fragment mediated cell adhesion but 28 kDa amino terminal fragment did not exhibit cell adhesion activity (Fig. 4). This result suggests that the receptor interacting with 28 kDa fragment is different from cell adhesion receptor interacting with 85 kDa cell binding fragment.

It was reported that two pools of bound fibronectin in cell layers were defined on the basis of their differential solubility in 1% deoxycholate (McKeown-Longo and Mosher, 1983). The deoxycholate-soluble pool (pool I) was proposed to represent fibronectin binding to receptors on the cell surface, and the deoxycholate-insoluble pool (pool II) was proposed to represent fibronectin incorporated into the detergent-insoluble extracellular matrix. Furthermore, pool I binding seemed to be required for the transfer of fibronectin to pool II because fibronectin did not accumulate into the matrix in the absence of cells. The 28 kDa fragment was found to bind more to pool I than to pool II. Furthermore, the 28 kDa fragment binding to myoblasts was specific because it was not inhibited by 85 kDa cell binding fragment and was not due to modification of the fragment by radioiodination.

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(Accepted March 30, 1988)

계배 근원세포 분화에 따른 Fibronectin의 수준과 그 수용체의 변화

정창용 · 강만식 (서울대학교 자연과학대학 동물학과)

배양 계배 근세포의 분화과정에서 fibronectin 양의 변화를 immunoblotting 법을 써서 정량해 본 결과 근세포의 분화에 따라 감소함을 알 수 있었다. 이처럼 근분화과정에서 fibronectin의 수준이 변하는 이유를 알아보기 위해서 fibronectin의 28,000 dalton (28 kDa)의 amino terminal fragment와 85,000 dalton (85 kDa)의 cell binding fragment의 근원세포와의 상호작용에 관해 조사하여 보았다.

^{125}I-28 kDa fragment가 근원세포와 특이하게 결합하는 양상은 시간 경과에 따라 변하여 60분 이내에 최대 수준에 도달하였다. 아울러, 비표지 28 kDa fragment는 ^{125}I-28 kDa fragment의 결합을 억제하였으나, 85 kDa fragment의 결합은 억제하지 않았다. 이러한 사실은 28 kDa fragment는 fibronectin 수용체와 결합하고 cell adhesion 수용체와는 결합하지 않음을 암시하는 것이다. 따라서 fibronectin 수준의 감소는 근원세포에 존재하는 fibronectin 수용체의 감소와 연관되는 현상으로 추정할 수 있었다.