Korean J. Zool. 33: 108-118, 1990

Alteration of Matrix Assembly Receptor for Fibronectin During Chick Myogenesis

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Fibronectin is a glycoprotein found in the extracellular matrix as well as in the serum, and has been known to exert pronounced effect on the myoblast fusion. Our previous studies have suggested that the decrease of fibronectin levels during myogenesis is due to the decreased availability of the receptor for the 28 kDa fragment of fibronectin.

In the fusion-blocked myoblasts by EGTA, the levels of fibronetin and binding of 28 kDa fragment decreased but far less than the control level. In contrast, the levels of fibronectin and binding of 28 kDa fragment decreased to the control level in the myoblast released from the fusion block. On this account, we suggest that the decrease of fibronectin levels during myoblast fusion is closely associated with the loss or alteration of the receptor for 28 kDa fragment.

Mild trypsin treatment decreased the binding of the 28 kDa fragment to the myoblasts significantly. Similarly, the presence of gangliosides in the binding media decreased the binding of the 28 kDa fragment in a dose-dependent manner. Furthermore, gel overlay of ^{125}I-28 kDa fragment on the SDS-PAGE of the myoblast homogenates revealed that the 28 kDa fragment bound to a 43 kDa protein and to gangliosides as well. These results suggest that myoblast fusion is correlated with decrease of the receptor for the 28 kDa fragment and that the receptor might be a glycoprotein that contains glyco-conjugate found in gangliosides.

KEY WORDS: Fibronectin, 28 kDa fragment receptor, Myogenesis

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

It has been reported that alterations in the cell surface occur during myogenesis (Hynes *et al.*, 1976; Moss *et al.*, 1978; Walsh and Philips, 1981). Based on these observations, it is claimed that certain proteins are involved in myoblast fusion process. The best characterized protein involved in myoblast fusion is fibronectin. It is a major extracellular connective tissue component of muscle (Stenman and Vaheri, 1978; Walsh *et al.*, 1981). The fibronectin is a large glycoprotein present on the surface of various cell types, in connective tissue matrix, and in body fluids (including plasma). The function of fibronectin in muscle tissues is not known yet, but the possible function of fibronectin

This work was supported in part by grants from the Ministry of Education and the Korea Science and Engineering Foundation

is assumed to be the promotion of cell adhesion, regulation of cell shape, and guidance of cell migration (Hynes, 1981). 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. In contrast, immunocytochemical observations on the L6 muscle cell line as well as on cloned human muscle cells indicated that as myoblasts fuse they lose their surface fibronectin (Chen, 1977; Walsh *et al.*, 1981). Similar results were obtained using chick embryonic myoblasts (Kang *et al.*, 1983, 1985). Furthermore, addition of exogeneous fibronectin to rat myoblast culture inhibited fusion (Podleski, 1981), and fibronectin was found to prevent the fusion block in tunicamycin-treated chick myoblast 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 still a number of unanswered questions concerning the precise mode of change in the levels of fibronectin during myogenesis. The disapperance of fibronectin from the surface of fusing myoblasts is probably not the results of a change in the rate of fibronectin synthesis (Gardner and Fambrough, 1983). Therefore, it is likely that fusing myoblasts and young myotubes produce fibronectin continuously, but the fibronectin is not retained by the cells. Thus, we have been particularly concerned with the cell surface receptors for the fibronectin.

It is suggested that there is a "matrix-driven 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-Longer and Mosher, 1985). Antibody and fragment inhibition studies suggest that a site in the amino terminal 70,000 dalton sequence of fibronectin is important in matrix assembly (McDonald *et al.*, 1982; Mckeown-Longer and Mosher, 1985). Inhibition studies using tryptic fragment derived from the 70,000 dalton fragments, however, suggest that the principal binding site lies within the 27,000 dalton amino terminal region (McKeown-Longo and Mosher, 1985).

In this report, we have used 28 kDa amino terminal fragment to identify "matrix assembly receptor" in chick myoblasts and to investigate the relationship between "matrix assembly receptor" and myogenesis.

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 24-well cluster plates were from Nunc, ^{35}S-methionine and Na^{125}I from Amerhsam, ^{125}I-protein A from New England Nuclear, and nitrocellulose (NC) papers (pore size: 0.45 μm) from Schleicher and Schuell. Trypsin (DPCC-treated), thermolysin, heparin-agarose, gelatin-agarose, and other reagents were from Sigma Chemical Co.

Cell Culture

Myoblast cultures were prepared according to the method of O'Neill and Stockdale (1972) with minor modifications. Briefly, breast muscles from 12-day-old chick embryos were dissected out, minced, and digested with 0.1% trypsin for 30 min and dispersed by repeated pipetting. Cells were collected by centrifugation and suspended in RPMI 1640 medium spplemented with 10% horse serum, 10% chick embryo extracts, and 1% antibiotics (811 medium). The cells were preplated on collagen coated dishes for 20 min to remove fibroblasts. The cell suspension was filtered through a four-fold lens paper to remove undissociated cells. Approximately 5×10^5 cells per ml were inoculated on collagen coated dishes. After 24 hr the medium was replaced with RPMI 1640 medium containing 10% horse serum, 2% embryo extracts, 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.

Measurment of Cell Fusion

At appropriate times, the cells were washed three times with phosphate buffered saline (PBS)

and 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 of three or more nuclei divided by the total number of nuclei as seen under a microscope at × 400. Ten fields were randomly chosen for each dish.

EGTA Treatment and Switch to Normal Medium

At 24 hr after plating, 0.7 mM EGTA was treated to each dishes. EGTA-treated myoblasts were kept for 24 to 60 hr and then switched to control medium to see whether release from the fusion block occurs.

Extracts of Cell Cultures

Dishes were removed from the incubator at appropriate times, washed three times with PBS and frozen at −20°C for later extraction. Prior to electrophoresis, dishes were thawed and scraped by a rubber policeman.

Gel Electrophoresis

The discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was essentially the same as described by Laemmli (1970) except that separation gel consisted of 8-14% polyacrylamide gradient. Samples were dissolved in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and were boiled for 3 min. Electrophoresis was carried out with constant current of 40-50 mA for 5-8 hr. Protein was determined by the procedure of Lowry *et al.* (1951) or Bradford (1976) with bovine serum albumin as a standard.

Immunochemical Detection of Fibronectin

Myoblast homogenates (80 μg of protein) were subjected to eletrophoresis in 8-14% gradient polyacrylamide slab gels. After electrophoresis, the proteins were transferred to NC papers 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 3 hr and then in the antibody solution at 4°C overnight. They were washed with buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 0.1% Triton X-100. They were then incubated in ^{125}I-protein A solution (2×10^5 cpm/ml). After incubation, the NC papers were washed four times with the buffer, dried and exposed to X-ray film for autoradiography. Following the autoradiography, the NC papers were cut out and the radioactivity on them was counted in a liquid scintillation counter.

Purification of Fibronectin and 28 kDa Amino Terminal Fragment

Fibronectin was purified from horse serum by gelatin-agarose and heparin-agarose affinity chromatography with elution by 4M urea and 0.5M NaCl as described by Engvall and Ruoslahti (1977). The 28 kDa amino terminal fragment was generated by thermolysin digestion and pruified by the method of Sekiguchi *et al.* (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 disgested with 5 μg/ml of thermolysin for 2 hr at 22°C. The digests in 10 mM sodium phosphate buffer, pH 7.6, containing 0.13 mM NaCl and 4.5 mM KCl were applied on a gelatin-agarose column. The unbound fractions were pooled and loaded on a heparin-agarose column. The bound materials were eluted with 0.5 M NaCl in the same buffer and dialysed against 10 mM sodium phosphate buffer, pH 7.4. After dialysis, the peptides were subjected to chromatography on Sephacryl S-200 column. The fractions containing 28 kDa amino terminal fragment were pooled and stored at −70°C for further use.

Iodination of 28 kDa Fragment

20 μg of 28 kDa fragment was iodinated with 1 mCi of Na^{125}I by chloramin T method (Greenwood *et al.*, 1963) to a specific activity of 1×10^7 cpm/μg.

Binding of ^{125}I-28 kDa Fragment to Cultured Myoblasts

Binding assays were done in RPMI 1640/HEPES medium supplemented wth 0.2% (W/V) bovine serum albumin (binding medium). Myoblast cultures were rinsed three times with pre-

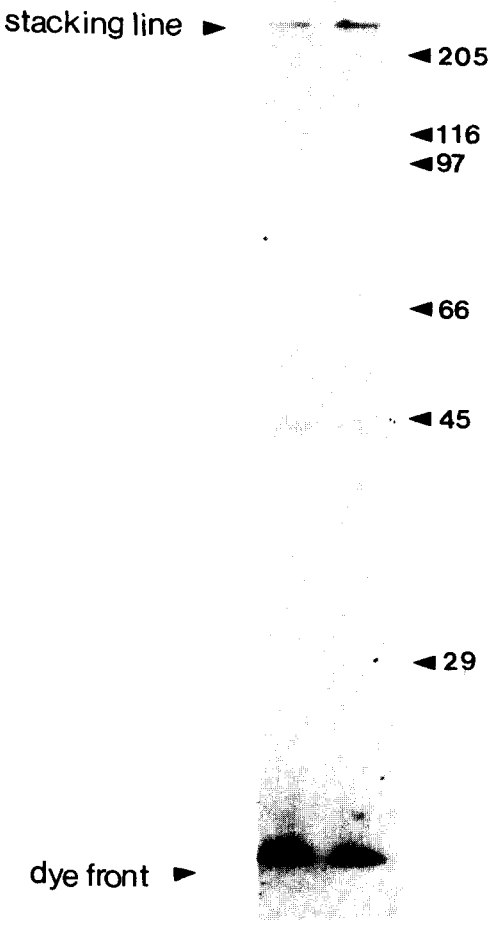

Fig. 9. Detection of candidate receptor proteins for 28 kDa fragment with an ^{125}I-28 kDa fragment gel overlay technique. A major band that appears to be the receptor is indicated by an arrow at left. Stacking line and dye front appear to contain proteoglycan and gangliosides, respectively.

fragment containing adhesion promoting activity did not inhibit it. This finding suggests that the 28 kDa fragment interacts with the receptor different from cell adhesion receptor (140 kDa complex) which interacts with 85 kDa cell binding fragment (Chung and Kang, 1988). It was also reported that the amount of cell surface fibronectin decreased 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).

In the present study, we have investigated the availability of possible receptors for 28 kDa fragment to elucidate the correlation between fibronectin receptor and myoblast fusion by employing the cell surface binding assay using ^{125}I-28 kDa fragment to myoblasts decreased gradually following fusion. It is consistent with the decrease in the amount of fibronectin (Fig. 3). Myoblasts treated with EGTA adhered each other but did not fuse. The levels of specific binding of ^{125}I-28 kDa fragment in EGTA-treated cells were not decreased compared to the control. This result is consistent with the immunoblotting data in Fig. 4. Thus, it seems that the receptor for ^{125}I-28 kDa fragment is correlated with the myoblast fusion. Furthermore, when EGTA was removed at 60 hr of cultivation, the levels of bound ^{125}I-28 kDa fragment decreased rapidly as myoblast fusion proceeded. These results reveal that myoblast fusion is closely correlated with the decrease of the availability of the possible receptor for 28 kDa fragment.

The myoblasts treated with trypsin revealed much less specific binding of ^{125}I-28 kDa fragment than untreated controls (Fig. 7). At the same time, treatment of gangliosides led to the decrease of specific binding of ^{124}I-28 kDa fragment in a dose-dependent manner (Fig. 8). These results suggest that the receptor of 28 kDa fragment is probably a glycoprotein, containing the glyco-conjugate that occurs in gangliosides as Matyas *et al.* (1986) indicated.

The cross-linking experiment with BHK cells originally indicated that a glycoprotein of 47 kDa was in particularly close proximity to fibronectin (Aplin *et al.*, 1981). Other experiments indicated that glycoproteins which bind to wheat germ agglutinin are a good candidate for the receptor (Oppenheimer-Marks and Grinnel, 1982). Furthermore, Urushihara and Yamada (1986) suggested that there are multiple protein components involved in the cell interaction with fibronectin. Trypsin-treated cells have been reported to inhibit certain fibronectin-mediated cell adhesion and cell binding events, suggesting the involvement of proteins in the binding of fibronectin to cells although

鷄胚 筋分化 過程에서 Fibronectin의 Matrix Assembly Receptor의 變化

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혈청을 비롯해서 extracellular matrix에 존재하는 당단백질인 fibronectin은 근세포의 융합과 밀접한 관계가 있는 것으로 알려져 있다. 본 연구실에서는 최근에 근세포가 분화하는 동안에 fibronectin의 수준이 감소되며, 이러한 감소는 fibronectin의 28 kDa fragment에 대한 수용체의 유용성이 감소하는 결과로 밝혀낸 바 있다.

본 연구에서는 근세포의 융합을 억제하는 물질로 알려진 EGTA를 이용하여 근세포의 융합과 28 kDa fragment receptor의 관계를 검토하여 보았다. EGTA를 처리한 경우 EGTA를 처리하지 않은 근세포에 비해서 fibronectin의 수준과 28 kDa fragment binding이 훨씬 적게 감소하였으며, 융합이 봉쇄된 근세포에서 EGTA를 제거하여 융합을 재개시키면 fibronectin의 수준과 28 kDa fragment의 binding이 정상 근세포 수준으로 환원되었다. 이상의 실험 결과로 볼 때 28 kDa fragment에 대한 수용체의 감소 또는 변화가 근세포의 분화과정에서 일어나는 fibronectin 수준의 감소와 연관성이 있음을 알 수 있다.

한편, 배양액 내에 trypsin을 처리한 경우에는 처리하지 않은 경우에 비해서 28 kDa fragment의 binding이 현저하게 감소되었고, gangliosides를 처리한 상태에서는 gangliosides의 농도에 정비례해서 28 kDa fragment의 binding이 감소되었다. 이 밖에 gel overlay technique을 이용하여 28 kDa fragment가 SDS-PAGE gel에서 분자량이 약 43 kDa인 단백질 및 gangliosides와 binding하는 사실을 알 수 있었다. 이러한 실험 결과를 종합하여 볼 때, 근세포의 융합은 28 kDa fragment에 대한 receptor의 감소와 관계가 있으며, 그 수용체는 gangliosides와 비슷한 당을 가지고 있는 당단백질일 것으로 추정된다.