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# Decrease of Surface Fibronectin Availability Required for Myoblast Adhesion by Tunicamycin

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# Tunicamycin에 의한 근원세포 접착에 필요한 표면 Fibronectin 유용성의 감소

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## 요 약

근세포 융합에 있어서 당단백질의 역할을 세포내 glycosylation의 저해제인 tunicamycin을 이용하여 검토하였다.

근세포가 융합하기 전 여러 시기에 tunicamycin을 0.04 μg/ml의 농도로 처리하면 세포내 glycosylation과 근세포 융합은 크게 감소되지만, 단백질 합성률과 creatine kinase 활성은 별로 변하지 않는 점으로 미루어 보아 근세포 표면의 당단백질은 세포간의 recognition과 adhesion에 관여하는 것으로 추정할 수 있었다.

따라서, 표지된 Con A 염색법을 써서 근세포 원형질막의 당단백질의 변화를 검토해 본 결과 tunicamycin을 처리한 경우 원형질막 당단백질의 대부분이 감소됨을 볼 수 있었으며, 아울러 근세포내 단백질의 분해속도는 증가하고 fibronectin은 감소하는 현상을 관찰할 수 있었다. 한편, fibronectin(20 μg/ml)을 tunicamycin과 같이 처리한 경우에는 융합이 억제되지 않았다.

이상의 결과들은 tunicamycin이 근세포가 adhesion하는데 필요한 세포막표면의 fibronectin의 유용성을 감소시킴으로써 근세포의 융합을 억제할 가능성을 제시하는 것이다.

## INTRODUCTION

Membrane fusion is a fundamental phenomenon observable in developmental biology.

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The developing muscle cells provide a useful *in vitro* system for the study of cell-cell fusion mechanism (Bischoff and Holtzer, 1969; Yaffe, 1969; O'Neill and Stockdale, 1972). In the process of skeletal muscle differentiation(myogenesis), the bipolar myoblasts initially undergo a phase of proliferation while aligning along their longitudinal axis and then fuse to form multinucleated myotubes. At approximately the same time as the onset of cell-cell fusion, the embryonic muscle cells cease DNA synthesis and cell division and initiate the elaboration of the specialized proteins associated with skeletal muscle contraction.

Knowing these changes which appear during myoblast differentiation, a number of studies have been performed to ascertain what are the changes produced in myoblast membrane which make them fusion competent. The concept has been formulated that changes of the myoblast membrane required for membrane union occur through several steps (Knudsen and Horwitz, 1977). Two of these steps are cell-cell recognition and adhesion. Membrane glycoproteins have been suggested to play an important role in the phenomenon of cell-cell recognition (Stanley and Sudo, 1981) and cell-substrate adhesion (Knudsen *et al.*, 1981), but direct evidence for this has not emerged. What kinds of changes occur during the development of recognition and fusion competence? The question has been approached by a number of workers in various ways.

Under appropriate conditions, myoblast fusion is inhibited by concanavalin A (Con A) which binds to surface glycoproteins (Den *et al.*, 1975). This implies that mannosylated glycoproteins may be involved in myoblast fusion. Cates *et al.* (1984a) have isolated two classes of Con A resistant, nonfusable $L_6$ mutants. In one, a selective reduction in the binding of $^{125}$I-Con A was shown to be due to the absence of a single polypeptide of Mr 46,000. Significantly, somatic hybrids produced by complementation not only regained the capacity to produce glycoprotein but also the ability to fuse. These observations suggested that some glycoproteins are important components in cell-cell interaction and in cell fusion as well.

The synthesis of several membrane proteins has been shown to increase during myogenesis and to decline after fusion. 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. Walsh and Phillips(1981), using several surface labelling methods, showed that synthesis of fibronectin increases upon cell fusion and declines during myotube differentiation. A similar result was obtained by Kang *et al.* (1983, 1985); the level of fibronectin decreased with cell fusion and the 165K and 93K proteins appeared at the onset of fusion as well as the increase in the number of low molecular weight proteins. Tunicamycin(TM), which is known to inhibit glycosylation of proteins by blocking the formation of N-acetylglucosaminyl pyrophosphoryl polyisoprenol (Hemming, 1977; Tkacz and Lampen, 1975), also inhibits the fusion of myoblasts (Gilfix and Sanwal, 1980). This inhibition was partially reversed when proteinase inhibitors were added with tunicamycin (Olden *et al.*, 1981).

In the present study, we have used tunicamycin to investigate the role of glycoprotein in the fusion of embryonic chick breast muscle cells in culture. In addition, we have examined the possibility that myoblast fusion is blocked when protein glycosylation is strongly impaired by tunicamycin treatment. Furthermore, fibronectin was found to prevent the fusion block in the tunicamycin-treated cultures.

## MATERIALS AND METHODS

### Materials

12-day-old hen's eggs were purchased from local poultry farm. PRMI 1640 medium, horse serum, and antibiotics (penicillin-streptomycin solution and fungizone) were obtained from Gibco. Tunicamycin and fibronectin were from Sigma Chemical Co. $^{35}S$-methionine and $^{125}I$-protein A from Amersham, $^{3}H$-galactose and $Na^{125}I$ from New England Nuclear, nitrocellulose papers (0.45 $\mu$m) from Shleicher and Schüell. All other reagents were obtained 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 muscle from 12-day-old 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 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 $5 \times 10^5$ cells per ml were inoculated on collagen-coated dishes. Medium was changed after 24 hr with RPMI 1640 medium containing 10% horse serum, 2% embryo extract, and 1% antibiotics (8102 medium). Cultures of various stages of differentiation were labelled at 36, 48, and 72 hr, which correspond to prefusion, midfusion, and postfusion stages in our culture systems, respectively.

### Metabolic Labelling of Cells

Levels of protein synthesis and glycosylation were measured by the incorporation of $^{35}S$-methionine (2 $\mu$Ci/ml) and $^{3}H$-galactose (2 $\mu$Ci/ml), respectively. Cells were labelled at 37°C for 2 hr with $^{35}S$-methionine in methionine-free PRMI 1640 medium or $^{3}H$-galactose in RPMI 1640 medium. The cells were washed twice with cold phosphate-buffered-saline (PBS) and harvested with 10% TCA, and then collected by centrifugation at 1,000g for 10 min. The TCA precipitate was dissolved in 0.3N NaOH by boiling for 2 min. Radioactivity of labelled proteins was measured in a Packard Minaxi $\beta$-Tri-Carb liquid scintillation counter.

### Measurement of Cell Fusion

At appropriate times, cells were washed three times with PBS and were fixed in a

mixture of ethanol, formaldehyde, and acetic acid (20:2:1, v/v) for 5 min. After rince 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 divided by the total number of nuclei as seen under microscope at 400X. Ten fields were randomly chosen for each dish.

**Creatine Kinase Assay**

Creatine kinase (CK) activity was estimated by the method of Koedam (1969). Dishes were washed with PBS for three times and stored at -70°C until required for analysis. Cells were scraped with a rubber policeman after the addition of Tris (100 mM)-magnesium (25 mM) buffer. The samples were sonicated and centrifuged, and the supernatants were used for analysis. Reaction was started with the addition of ADP solution and stopped by $Ba(OH)_2$-EDTA and $ZnSO_4$ solution. These mixtures were centrifuged and the supernatants were diluted with distilled water, then ninhydrin solution was added. After the addition of KOH solution, fluorescence was measured by Mark IV spectrofluorometer (excitation 405 nm; emission 520 nm).

**Preparation of Plasma Membrane**

Plasma membranes were prepared as described by Schimmel *et al.* (1973). Briefly, monolayer cultures were washed three times with cold PBS, and cells were removed from dish by scrapping with a rubber policeman and collected by centrifugation at 1,100g for 5 min. The cell collection and subsequent steps were done at 4°C. The cells were suspended in 0.25 M sucrose-2 mM triethanolamine(TEA)-HCl, pH 7.4 using 15 ml/g wet weight of cells and homogenized by 15 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,700g for 10 min and the supernatant was removed and centrifuged at 33,000g for 60 min. The pellet was resuspended in 1 ml of sucrose-TEA buffer and layered over a discontinous sucrose density gradient. The sucrose gradient was prepared by layering 0.5 ml of 40% (w/w) sucrose, followed by 1 ml each of 32%, 27%, and 20% successively. After centrifugation at 205,000 g for 90 min, membranes at the 8.3～20% and 20～27% interfaces were pooled and diluted to about 4 ml with 2 mM TEA-HCl, pH 7.4, and the proteins were then collected by centrifugation at 105,000 g for 60 min.

**Na, K-ATPase Assay**

Ouabain-sensitive Na, K-ATPase activity was determined as described by DePierre and Karnovsky (1973). Inorganic phosphate released was determined by the method of Chen *et al.* (1956). Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as a standard.

**Gel Electrophoresis**

The discontinous SDS-polyacrylamide gel electrophoresis was essentially the same as described by Laemmli (1970) except that the separating gel consisted of a 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 at 20~30 mA for 5~8 hr.

**Lectin Staining of Polyacrylamide Gels**

Lectin staining was carried out as described by Walsh *et al.* (1981) and by Mintz and Glaser (1978). After electrophoresis, gels were soaked in 50% methanol for 30 min, transferred to 50% methanol containing 0.05% glutaraldehyde and further incubated for 90 min. The fixing solution was replaced by 0.1 M NaCl, 0.03 M sodium phosphate buffer, pH 8.0, containing $NaBH_4$ at a final concentration of 20 $\mu$g/ml. After 1 hr, the gels were replenished with the same fixing solution and left at 20°C for 16 hr. The gels were then soaked in PBS containing 0.05% $NaN_3$ and 1 mg hemoglobin per ml. And then, $^{125}$I-labelled Con A, obtained by using chloramine T method (Greenwood *et al.* 1963), was added at a level of $2 \times 10^5$ cpm per ml of PBS. The gels were incubated at 20°C for 72 hr. The gels were finally stained with Coomassie blue, dried, and autoradiographed.

**Rate of Protein Degradation**

Rate of degradation of total cellular proteins was determined by measuring the release of TCA-soluble radioactive material from proteins prelabelled with $^{35}$S-methionine. Cells were grown in 811 medium for 24 hr. Medium was changed after 24 hr with 8102 medium with or without 0.04 $\mu$g/ml TM and then incubated for 6 hr. After the incubation, $^{35}$S-methionine was added to the culture to a final concentration of 2 $\mu$Ci per ml of medium and cultured for 24 hr. After removing the medium, the cells were washed three times with RPMI 1640 medium and then incubated in 8102 medium. Release of radioactive material was monitored for 20 hr. At various times during incubation, the medium from cultures was withdrawn. Cells were washed three times with cold PBS, and 10% TCA was added to plate. The original medium along with PBS washings were pooled. The TCA-soluble fractions from both the cells in the plate and washings were mixed together (acid-soluble fraction) and the radioactivity of the aliquots was measured. The TCA-extracted cells were dissolved in 0.3 N NaOH (acid-insoluble fraction) and its radioactivity was determined. The rate of protein degradation was calculated by the ratio of the radioactivity of soluble fraction/the radioactivity of insoluble fraction (S/I ratio).

**Immunochemical Detection of Fibronectin**

Myoblast homogenates (60 $\mu$g of protein) were electrophoresed in 8~14% gradient polyacrylamide slab gels. After electrophoresis, the proteins were transferred to nitrocellulose paper at 45 V for 90 min in a transblot 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 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-labelled protein A solution ($2 \times 10^5$ cpm/ml). After incubation, the nitrocellulose papers were washed three times with the same buffer, dried, and exposed to X-ray film for autoradiography. After autoradiography, the nitrocellulose paper was cut out and the radioactivity on the nitrocellulose strips was counted in a liquid scintillation counter.

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

Rabbit antiserum against electrophoretically pure horse serum fibronectin was prepared as described by Zardi *et al.* (1980). Briefly, 0.3 mg of purified fibronectin emulsified with complete Freund's adjuvant was used to immunize the rabbits. Two weeks later, the rabbits received four booster injections consisting of 0.25 mg of fibronectin in incomplete adjuvant administered at 20-day intervals. Blood was collected by heart puncture. The IgG fraction from serum was purified by using protein A-Sepharose (Pharmacia) column.

## RESULTS

### Effect of TM on Myoblast Fusion

The participation of surface glycoproteins in the fusion of undifferentiated myoblasts to form differentiated myotubes was examined by determining whether TM blocked muscle cell fusion. For these studies cells were grown in 811 medium for 24 hr and the medium was changed to 8102 medium containing varying concentrations of TM.

As shown in Fig. 1, TM at a concentration of 0.04 μg/ml was found to inhibit the fusion of myoblasts consistently, to a level less than 30% of untreated cells. Untreated myoblasts initiated fusion at around 50 hr and nearly completed by about 72 hr, but TM-treated myoblasts attained a level of 20~30% of the control after 4 days in culture (Fig. 2). At a concentration of 0.04 μg/ml, cell proliferation was only slightly affected. Prolonged exposure of myoblasts to TM and higher concentration of TM (more than 0.05 μg/ml) resulted in cytotoxic effect.

The fusion index of myoblasts treated with TM at varying times from 24 hr through 72

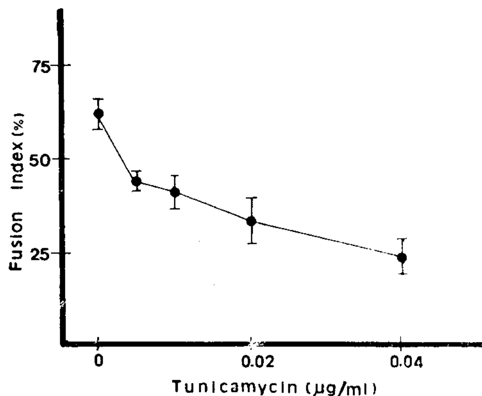


**Fig. 1.** Effect of TM at various concentrations on the fusion of myoblasts. TM was treated at 24 hr and fusion index was scored at 72 hr after myoblast seeding.

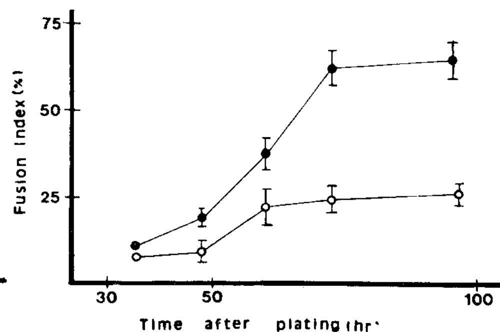


**Fig. 2.** Effect of TM on the fusion index of myoblasts. TM was treated at 24 hr after myoblast seeding at a concentration of 0.04 μg/ml. ●-●, control; ○-○, TM.

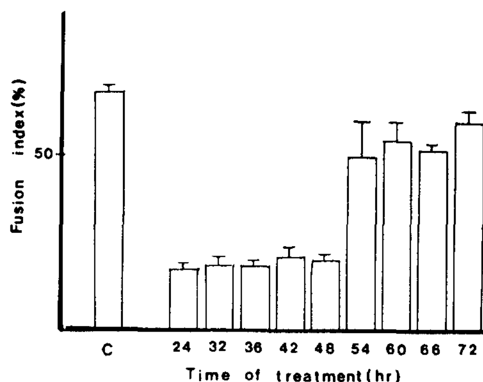


**Fig.** 3. Effect of time of TM treatment on the fusion index of myoblasts. Myoblasts were treated with TM at the designated times and scored at 96 hr after plating. C, control.

hr and scored at 96 hr is shown in Fig. 3. As is evident from Fig. 3, the inhibitory effect of TM is prominent when TM is added up to the time of onset of the fusion burst. The differential inhibition might be interpreted as the asparagine-linked, surface glycoproteins are required for the myoblast fusion and membrane glycoproteins are specifically involved in cellular recognition and adhesion. Thus, the inhibitory effect of TM is likely due to the inhibition of cellular recognition and adhesion.

**Effect of TM on the Protein Synthesis and Glycosylation**

To examine the effects of TM on the protein synthesis and glycosylation, myoblast cultures were incubated for 12, 24, and 48 hr in 8102 medium with and without TM (0.04 $\mu$g/ml), followed by the addition and incubation of labelled precursors for 2 hr. The results of the experiments are shown in Table 1. TM inhibited the incorporation of $^{3}$H-galactose into cellular glycoproteins by about 70%. Relative protein synthesis was inhibited by TM as low as 15% as shown by $^{35}$S-methionine incorporation experiments. A similar result was obtained by Olden *et al.* (1981) in quail myoblasts.

To study the pattern of protein synthesis in TM-treated myogenic cells, 24 hr-old myo-

**Table 1.** Effect of TM on the protein synthesis and glycosylation*

| Time of incubation | Treatment | $^{35}$S-methionine incorporation | | $^{3}$H-galactose incorporation | |
|---|---|---|---|---|---|
| | | cpm/$\mu$g protein | % of control | cpm/$\mu$g protein | % of control |
| 12 | Control | 366±76 | 100 | 5.66±0.29 | 100 |
| | TM | 428±102 | 116 | 4.15±0.06 | 73 |
| 24 | Control | 374±11 | 100 | 12.25±1.75 | 100 |
| | TM | 368±20 | 98 | 7.28±1.62 | 59 |
| 48 | Control | 432±33 | 100 | 5.20±0.50 | 100 |
| | TM | 404±24 | 93 | 1.34±0.13 | 25 |

* TM was added 24 hr after plating. Incorporation of radioactive materials into total TCA-insoluble material was determined as described in Materials and Methods.

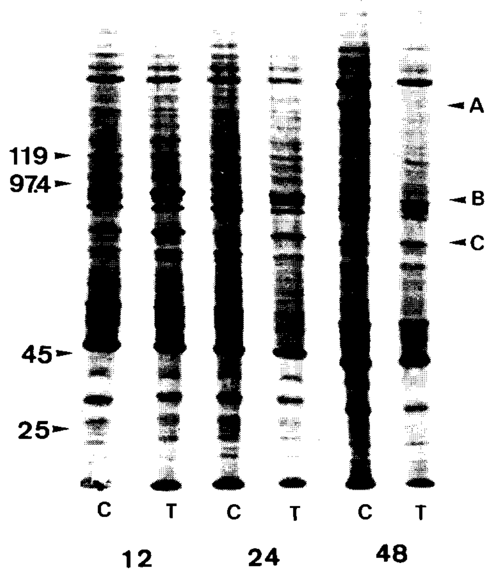


**Fig. 4.** Fluorogram of SDS-polyacrylamide slab gels of proteins synthesized in control (C) and TM treated cells (T). Numerials at the bottom represent the time after drug treatment. B and C, glucose-regulated proteins, 95K and 75K, respectively. Molecular weight markers are shown at left.

blasts were treated with TM and the cells were labelled with $^{35}$S-methionine for 2 hr at various times (12, 24, and 48 hr) after TM treatment. After labelling, the cells were washed with cold PBS and harvested with sample buffer. Electrophoresis was then performed. The results are presented in Fig. 4. The most apparent alteration observed in TM-treated cells was the marked increase of two major polypeptide bands having apparent molecular weight of 95,000 and 75,000. The electrophoretic mobilities of these bands are identical to those of the "glucose/glycosylation regulated proteins"(GRP) induced in chick cells subjected to glucose starvation or to inhibition of glycosylation (Olden *et al.* 1979a). The role of these proteins in myogenesis has not been elucidated but they might be induced via mechanism other than glucose starvation. In addition, there was a marked decrease of one polypeptide band having apparent molecular weight of 130,000 in TM-treated cultures.

**Effect of TM on Creatine Kinase Activity**

Creatine kinase (CK) activity was chosen as an index of biochemical differentiation of myoblasts, since this parameter has been known to be muscle specific and coincides quantitatively with the progress of muscle differentiation. The CK activity of both control and TM-treated myoblasts are shown in Fig. 5. In the control cultures, the level of CK activity began to increase at around 55 hr after cell plating, which corresponds to the onset of myoblast fusion. A similar increase of CK activity was found in TM-treated cultures. The relationship between the fusion of myoblast and the synthesis of muscle specific protein is

not yet fully elucidated. However, it is accepted that the expression of muscle specific proteins can be uncoupled from the fusion event. An abrupt drop in the level of CK activity at 96 hr in TM-treated cultures might be due to cytotoxic effect of TM.

### Rate of Protein Degradation in TM-treated Cultures

To examine the possible role of carbohydrate in the glycoproteins, experiments were performed to determine the rate of degradation of total cellular proteins (Fig. 6). In TM-treated cultures, protein was degraded to TCA-soluble peptides and amino acids about twice more rapidly than the control cultures. These results suggest that nonglycosylated proteins are degraded more rapidly than the glycosylated proteins.

### Staining of Glycoprotein with $^{125}$I-Con A

If glycoproteins function in the fusion or in the processes leading to fusion such as cell-cell recognition, they would reasonably be expected to locate in the plasma membrane. Therefore, we examined the effects of TM treatment on surface glycoproteins of myoblast. Plasma membrane fractions were isolated from skeletal muscle cell cultures. The purity of membrane fraction was estimated by Na, K-ATPase activity in the membrane fraction.

The specific activities of Na, K-ATPase assayed in membrane fraction obtained from the discontinuous sucrose density gradient were reproducible and were in good agreement with those originally reported by Schimmel *et al.* (1973). Membrane fractions between 8.3% and 20% (w/w) sucrose (fraction I) or between 20% and 27% sucrose (fraction II) had higher ouabain-sensitive Na, K-ATPase activity compared to other fractions (data not shown).

Total cellular proteins and plasma membrane fractions isolated from myoblasts treated

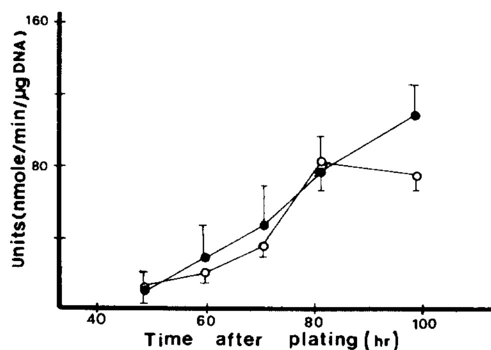


**Fig. 5.** Effect of TM on the creatine kinase activity. TM was treated at 24 hr after myoblast seeding at a concentration of 0.04 µg/ml. The medium was replaced at 72 hr with new medium containing TM. The fall of creatine kinase level at 96 hr seems to be due to the cytotoxic effect of TM. •-•, control; ○-○, TM.

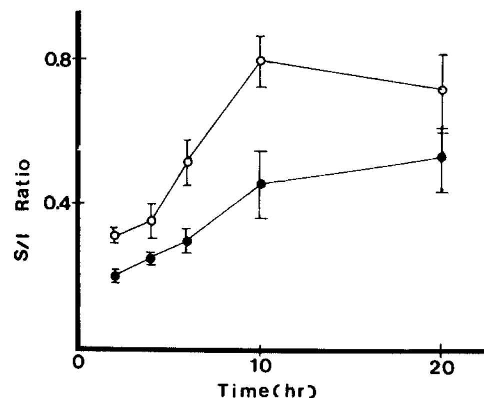


**Fig. 6.** Effect of TM on the protein degradation in the myoblasts. Rate of protein degradation was expressed in terms of S/I ratio. S/I ratio= radioactivity of TCA-soluble fraction/that of TCA-insoluble fraction. •-•, control; ○-○, TM.

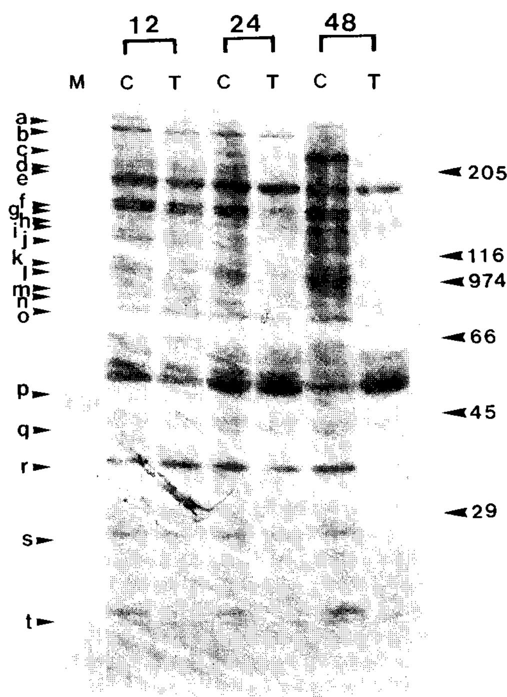


Fig. 7. Autoradiogram of cellular glycoproteins in TM-treated cultures (T) and untreated control (C). 80 μg of proteins were electrophoresed, and the gel was 'stained' with $^{125}$I-Con A. Glycoproteins affected by TM are indicated with arrows. Numerials at the top represent the time after TM treatment. The positions of molecular weight standards are indicated, and Mr's are shown $\times 10^{-3}$. 205, myosin; 116, β-galactosidase; 97.4, phosphorylase b; 66, bovine serum albumin; 45, ovalbumin; 29, carbonic anhydrase. Standards for molecular weight estimation, except ovalbumin containing mannose, did not bind $^{125}$I-Con A (lane M).

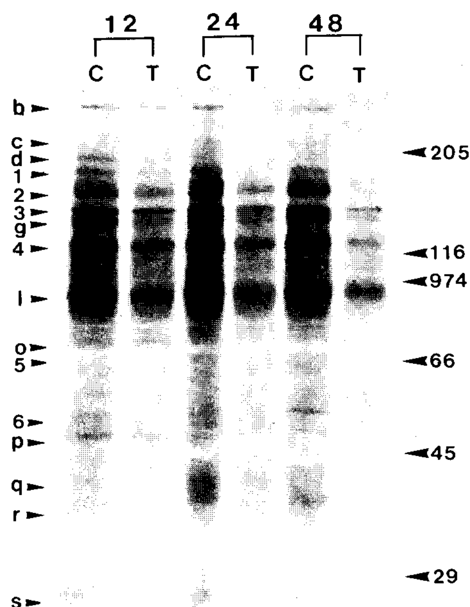


Fig. 8. Autoradiogram of plasma membrane glycoproteins in TM-treated cultures (T) and untreated control (C). 45μg of proteins were electrophoresed, and gel was 'stained' with $^{125}$I-Con A. Glycoproteins affected by TM are indicated with arrows at left.

with TM were compared with untreated controls by means of SDS-polyacrylamide gel electrophoresis, followed by $^{125}$I-labelled Con A binding (Figs. 7 and 8). Nonglycoprotein standards used for molecular weight determination were found not to bind with $^{125}$I-labelled Con A. As shown in Fig. 8, it is evident that TM treatment results in the decrease of glycosylation of most plasma membrane glycoproteins containing Con A binding sites. All the glycoproteins affected by TM are indicated by arrows in Figs. 7 and 8 and listed in

**Table 2.** Glycoproteins affected by TM.

| Apparent M.W. (×$10^{-3}$) | Cellular glycoprotein | Membrane glycoprotein | Apparent M.W. (×$10^{-3}$) | Cellular glycoprotein | Membrane glycoprotein |
|---|---|---|---|---|---|
| 250 | a | | 115 | j | |
| 230 | b (FN)* | b (FN) | 110 | k | |
| 210 | c | c | 97 | l | l |
| 200 | d | d | 90 | m | |
| 190 | e | | 87 | n | |
| 180 | | 1 | 80 | o | o |
| 165 | | 2 | 70 | | 5 |
| 155 | | 3 | 52 | | 6 |
| 150 | f | | 48 | p | p |
| 140 | g | g | 40 | q | q |
| 135 | h | | 35 | r | r |
| 130 | i | | 24 | s | s |
| 120 | | 4 | 14 | t | |

* Fibronectin

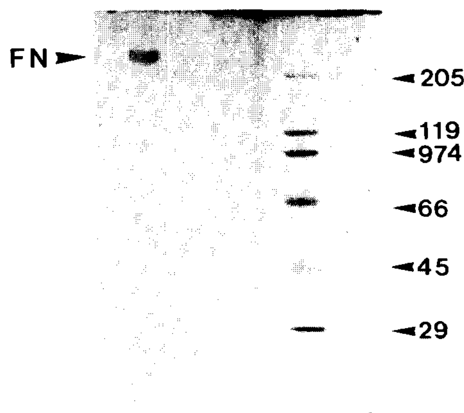


**Fig. 9.** SDS-PAGE of purified horse serum fibronectin (FN). Samples containing 8 $\mu g$ of purified FN were run on 8~14% polyacrylamide gradient gel as described in Materials and Methods. The gel was stained with Coomassie brilliant blue. Positions of molecular weight markers are shown for comparison.

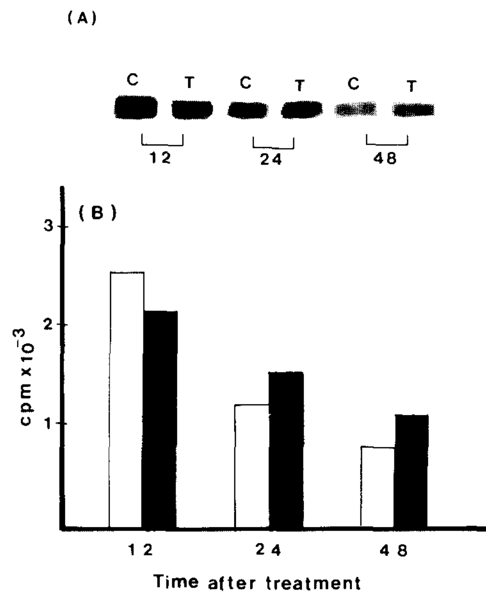


**Fig. 10.** Immunochemical detection of FN at varying stages after TM treatment. (A) Immunoautoradiography of FN. Autoradiographs represent the FN transferred to nitrocellulose paper and stained by FN antibody and $^{125}$I-protein A. C, control; T, TM-treated culture. (B) Radioactivity of FN bands on the nitrocellulose strips. □, control; ■, TM.

Table 2.

**Immunochemical Detection of Fibronectin**

In order to investigate the level of fibronectin in the TM-treated cultures, the immunoblotting method was employed. Fibronectin was purified by sequential gelatin-agarose and heparin-agarose affinity chromatography as described in Materials and Methods. Fibronectin obtained with this procedure was electrophoretically homogeneous(Fig. 9). Rabbit antiserum against horse serum fibronectin was prepared as described in Materials and Methods.

The results of immunoblotting are shown in Fig. 10. In control cultures, the level of fibronectin decreased as the cell fusion proceeded. Similar results were obtained by many workers(Chen, 1977; Walsh and Philips, 1981). Despite that the fusion was blocked in TM-treated myoblasts, the level of fibronectin was found to decrease also. This result suggests that the nonglycosylated fibronectin seems to be more sensitive to proteolysis than fibronectin containing carbohydrate.

**Effect of Fibronectin on the TM-induced Fusion Inhibition**

The previous results suggested that the inhibitory effect of TM might be due to the prevention of cellular recognition and adhesion. Because fibronectin plays an important role in adhesion of myoblasts, the effects of fibronectin on TM-treated myoblasts were examined. The cultures incubated with fibronectin plus TM did not exert a profound inhibitory effect on myoblast fusion as compared to those incubated with TM alone (Figs. 11 and 12). The fibronectin added exogeneously prevented the inhibitory effect of TM in a dose dependent manner. In the control cultures, however, fibronectin inhibited myoblast fusion only slighlty (approximately 10%). An abrupt drop in the fusion index at 96 hr in the cultures treated with fibronectin plus TM might be attributed to the cytotoxic effect of TM.

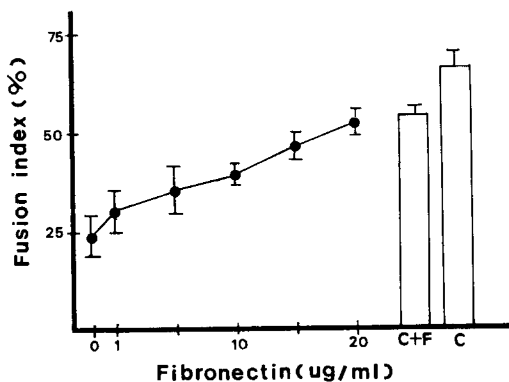


**Fig. 11.** Effect of FN at various concentrations on the fusion of myoblasts treated with TM. FN restores TM-induced inhibition of myoblast fusion. TM and FN were added at 24 hr after myoblast seeding, and fusion index was scored at 72 hr. C, control; F, FN.

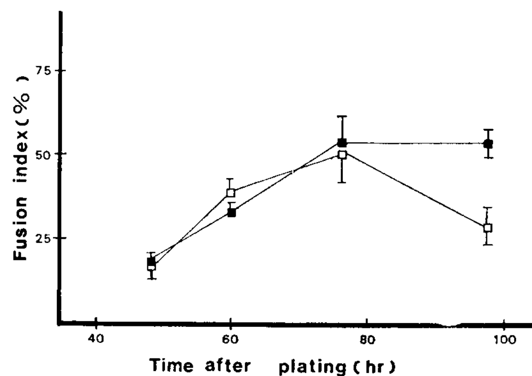


**Fig. 12.** Effect of simultaneous treatment of FN and TM on the fusion of myoblasts. The drugs were added at 24 hr after myoblast seeding. The medium was replaced at 72 hr. FN was treated at a concentration of 20 $\mu$g/ml. ■-■, FN; □-□, TM plus FN.

## DISCUSSION

In order to examine whether surface glycoproteins with asparagine-linked oligosaccharides are required for alignment and fusion of embryonic muscle cells in culture, effects of TM on the process of differentiation were followed. TM severly inhibited myoblast fusion at noncytotoxic concentrations. This inhibition was apparently not due to the toxicity of the drug because TM inhibits neither $^{35}$S-methionine incorporation nor growth rate. TM inhibited not only $^{3}$H-galactose incorporation but also fusion itself, suggesting that the fusion-specific glycoprotein was not synthesized in the presence of TM. This results is in good agreement with those obtained by Olden *et al.* (1981) and Cates *et al.* (1984b). The inhibitory effect of TM is prominent when TM is added prior to the onset of fusion burst. These results suggest that TM exerts its effect on the myoblast adhesion step, thereby blocking myotube formation. Thus, the asparagine-linked, surface glycoprotein is involved specifically in cellular recognition and adhesion. This finding is consistent with the previous observation of Knudsen (1985) that TM blocks the specific, $Ca^{2+}$-dependent aggregation of fusion competent myoblasts.

Meanwhile, TM is not likely to affect the biochemical differentiation of myoblasts. The creatine kinase activity was found to be similar, regardless of the presence or absence of TM. Many studies have revealed that in cells cultured with a low calcium medium by treating either Chelex or EGTA, the cell fusion was blocked but muscle specific protein synthesis was continued to increase (Turner *et al.*, 1976; Moss and Strohman, 1976).

Fibronectin, a major extracellular connective tissue component of muscle cells, mediates myoblast-substratum attachment; one region of the molecules binds directly to the cell surface, while the others bind to collagen, glucosaminoglycan and to other fibronectin molecules (Ehrismann *et al.*, 1982). It is suggested that the formation of elongated myotubes in certain spatial arrangement may be regulated by fibronectin (Chiquet *et al.*, 1981). It was shown that the amount of cell surface fibronectin decreases after the fusion of myoblasts to form myotubes (Hynes *et al.* 1976; Chen, 1977). In the previous works from our laboratory (Kang *et al.* 1983, 1985), it was demonstrated that the level of fibronectin, in fact, decreases after the fusion. Furthermore, Podleski *et al.* (1979) showed that when fibronectin was maintained at high levels, the fusion of $L_6$ myoblasts was inhibited. Also shown by the same authors was that when surface fibronectin was removed by trypsin one day before cells began to fuse, inhibition of fusion was observed, whereas trypsinization on the day fusion began enhanced fusion. Presumably fibronectin plays a role in preparing the cells to fuse but then reduces in amount as the fusion proceeds.

We have examined the membrane glycoproteins affected by TM. As shown in Fig. 8, TM decreased the glycosylation of most plasma membrane glycoproteins including fibronectin. Olden *et al.* (1979b) found that no apparent difference was detected between glycosylated

and nonglycosylated fibronectins, when tested for activity in mediating the attachment of cells to collagen, and for effectiveness in hemagglutination of sheep erythrocytes. However, nonglycosylated fibronectin was shown to be more sensitive to protease than glycosylated one. It was also shown in the present study that enhanced protein degradation by protease was observed (Fig.6), and the decrease in the amount of fibronectin in TM-treated cultures was apparent (Fig. 10). These results suggest that nonglycosylated fibronectin degrades more rapidly than glycosylated one. Therefore, TM-treated cells are expected to exhibit reduced amounts of surface-associated fibronectin. A similar result was obtained by Butters *et al.* (1980) in baby hamster kidney (BHK) fibroblasts. It is likely that the reduced amounts of surface fibronectin is not sufficient to mediate cellular adhesion, and thus the fusion of myoblasts is inhibited by poor adherence of cells. To check this possibility we investigated the effect of exogeneous fibronectin on TM-treated cultures. As shown in Fig. 10, exogeneous fibronectin (20 $\mu$g/ml) partially prevented the effect of TM. These results indicated that disappearence of surface fibronectin resulted in the inhibition of myoblast fusion. However, our findings do not rule out the possibility that oligosaccharide units of fibronectin have some role in myoblast fusion, and that other glycoprotein affected by TM plays an important role in the recognition and adhesion of myoblast.

The major conclusion to be derived from this work is that TM apparently inhibits fusion by decreasing the availability of surface fibronectin required for myoblast adhesion.

## ABSTRACT

We have investigated the role of glycoprotein in the fusion of embryonic chick breast muscle cells in culture by using TM to inhibit the glycosylation of proteins.

TM (0. 04 $\mu$g/ml) blocked protein glycosylation and strongly inhibited fusion when added to cultures of differentiating muscle cells before the onset of fusion, but had no apparent effect on protein synthesis and creatine kinase activity. These results revealed that the asparagine-linked, surface glycoprotein is involved especially in cellular recognition and adhesion.

Staining of glycoproteins in the plasma membrane of myoblasts with $^{125}$I-labelled Con A revealed that most membrane glycoproteins were decreased in their relative amounts following TM treatment. In addition, TM treatment enhanced the rate of protein degradation and decreased the level of fibronectin in fusion blocked myoblasts. Fibronectin (20 $\mu$g/ml) added exogeneously prevented partially the fusion block in TM-treated cultures.

These results suggest the possibility that TM inhibits fusion by decreasing the availability of surface fibronectin required for myoblast adhesion.

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