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Cytochemical and Biochemical Characteristics of Cellular Adhesion in *Amoeba proteus*

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*Amoeba proteus*의 표면흡착에 관한 세포화학 및 생화학적 특성

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적 요

단백질 분해효소, neuraminidase 및 EDTA가 아메바의 배양기표면 흡착, 세포표면의 미세구조 및 생화학적 조성에 미치는 영향을 concanavalin A(con A) cytochemistry 및 SDS PAGE에 의해 조사하였다. Con A cytochemistry에 의해 세포표면 바깥쪽의 filamentous(F)층과 안쪽의 amorphous(A)층이 쉽게 구분되었다. Neuraminidase로 처리한 아메바는 대조군에 비해 용기표면 흡착성과 퍼짐이 증가하였으며 A층과 F층에 더 많은 con A결합부위가 노출되었다. Trypsin 및 proteinase K로 처리한 아메바는 각각 12시간, 48시간동안 용기표면에 부착하지 못하였으며, proteinase K의 처리는 A층의 con A결합부위 및 모든 glycoprotein을 제거시키는 효과를 낳았으며, trypsin은 세포막의 PAS염색물질에는 아무런 변화를 초래하지 않았으나 A층과 F층의 con A결합부위를 제거하였다. 이들 효소 및 EDTA처리에 의해 세포 표면의 mucopolysaccharide 일부가 분리되었다. 아메바를 monovalent con A로 처리하였을 때도 아메바는 용기표면에 부착하지 못하고 cytolysis되었다. 이상의 결과로 아메바의 용기표면 흡착에는 세포막의 glycoprotein과 A층의 mucopolysaccharide간의 상호작용에 의해서 이루어지는 것으로 보인다.

INTRODUCTION

For the normal embryonic or adult cells from avian or mammalian organisms to survive and divide *in vitro*, they stringently require adherence to a specific type of substratum,

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particular glasses or activated polystyrene plastic (Martin and Rubin, 1974). In contrast, malignant cells either transformed by oncogenic viruses or derived from tumors frequently lose anchorage dependence, adapt to grow as single cells in suspenison, and readily underlap neighboring cells during the movement across substrata (Culp and Black, 1972; Shields and Pollock, 1974).

Involvement of particular molecular components in cell to substratum adhesion has been well characterized in some cell systems such as, adherons in the embryonic chick neural retinal cells (Cole and Glaser, 1984; Cole *et al.*, 1985; Schubert and LaCorbiere, 1985; Schubert *et al.*, 1983), cell-substratum attachment antigen in myogenic and fibroblastic cells (Damsky *et al.*, 1985), fibronectin (Hynes and Yamada, 1982) and laminin (Yamada, 1983) in chick and mouse fibroblastic and embryonic cells, a 36,000 dalton protein in Madin-Darby Canine Kidney cells (Sabanero *et al.*, 1985), and heparan sulfate proteoglycan in mammalian tissues and cultured cells (Anderson and Fambrough, 1983; Cohn *et al.*, 1976; Kjellen *et al.*, 1980; Oldberg *et al.*, 1979). In most cell systems due to the complexity of the cell surface components, the mechanism of the cellular adhesion is not yet clearly understood.

It was suggested that simple eukaryotic cells such as protozoans adhere by the mechanisms that are biochemically simpler than those found in complex avian and mammalian cells (Culp and Buniel, 1978). Among the protozoans amoebae perform all of the cellular activities only when they are attached to the substratum. But their longevity is like that of transformed cells. In *A. proteus* proteins occupy 24.5% of the plasmalemma (O'Neill, 1964). The protein or mucopolysaccharide components of the plasmalemma were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Ahn and Jeon, 1982). Three mucopolysaccharide bands in stacking gel and three glycoprotein bands in resolving gel were detected by staining with periodic acid-Schiff (PAS) reagent. Four additional Coomassie blue stainable protein bands were also resolved in separating gel. Among these only one polypeptide was exposed to the outer surface accessible to iodination by lactoperoxidase. In comparison of molecular complexity of the plasmalemma amoebae appear to be far simpler than any other cell system (Muller, *et al.*, 1980a, b; Storrie, 1981). The suface fraction of *A. proteus* was partially characterized and was found to be 11.3% neutral sugar and 3.4% hexosamine (Allen and Winzler, 1973). Mannose accounted for two-thirds of the neutral sugars as quantitated by gas-liquid chromatography. Such a simplicity of the molecular composition of the plasmalemma and the cellular behavior of the amoebae would be advantageous for the study of cellular adhesion. The results could provide an insight into the molecular mechanism for the role of the cell surface in both normal and transformed cells.

In this study the effects of proteases, neuraminidase and EDTA on adhesion of amoebae on substratum, ultrastructure and biochemical composition of the cell surface were studied by concanavalin A (con A) cytochemistry and SDS PAGE.

MATERIALS AND METHODS

1. Cell Culture

Amoeba proteus was cultured in glass dishes (18 cm in diameter, 2 cm in depth) in a modified Chalkley's medium at 23°C (Ahn and Choi, 1985). Amoebae were fed three times a week with axenically cultured and washed *Tetrahymena* as food organism.

2. Treatment of Amoebae with Surface Modifying Agents.

To study the effect of surface modifying agents on the cellular behavior, morphology, and on the biochemical composition of the plasmalemma amoebae were treated with trypsin, proteinase K, neuraminidase or EDTA. All enzymes for this study were purchased from Sigma Chemical Co. (St. Louis, Mo.). For tryptic digestion cells were incubated in Chalkley's solution containing 11.5mM $CaCl_2$ and 1500 BAEE units of trypsin per ml for 10 min, then washed more than four times. For other enzymes, cells were left in Chalkley's solution containing 50 μg/ml of proteinase K (17.0 units/mg protein) or 100 μg/ml of neuraminidase (65 units/mg protein) for 10 min and 20 min, respectively. To release the components that are associated on the surface by divalent cation, cells were incubated in Ca^{2+}-free Chalkley's solution containing 3 mM EDTA for 20 min.

3. Cytochemistry of the Cell Surface.

In order to examine the changes in morphology of the cell surface cells were subjected to con A cytochemistry. Control and enzyme-treated amoebae were fixed in phosphate-buffered (0.1 M, pH 7.4) paraformaldehyde-glutaraldehyde for an hour at 4°C and washed three times at 10 min intervals in cold phosphate buffer (Ahn, 1983). Then the cells were suspended in the same buffer and stored overnight at 4°C. Prefixed cells were processed for con A-cytochemistry (Graham and Karnovsky, 1966; Guillouzo and Feldmann, 1977).

The prefixed samples were incubated with 250 μg/ml of con A in 0.1M phosphate buffer, pH 7.4 for an hour at room temperature. Then the cells were washed three times at 20 min intervals and incubated with 100 μg/ml of horseradish peroxidase (HRP; 152 units/mg solid) in phosphate buffer for an hour. After rapid washing with the same buffer, cells were incubated for 30 min in a saturated solution of diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer, pH 7.6 containing 0.01% H_2O_2. Then the cells were post-fixed in 2% buffered osmium tetroxide for 1h at room temperature. The fixed cells were dehydrated and embedded in Epon. Thin sections were obtained using a 50B microtome (JEOL), mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate. Double stained samples were examined and photographed in a CX100 electron microscope (JEOL) at 100 KV.

4. SDS-PAGE

After treatment of cells with surface modifying agents, the components released in the medium and the ones left on the plasmalemma were collected and compared by SDS-polyacrylamide gel electrophoresis. To collect the components released in the medium, enzyme

treated supernatant was filtered through Whatman No. 2 filter paper and concentrated by lyophilization. To prepare membrane fractions, cells were washed 3 times with cold Chalkley's solution and processed as previously described (Ahn and Jeon, 1985). Proteins were quantitated according to Lowry *et al.* (1951), electrophoresed and stained as described previously (Ahn and Jeon, 1982).

RESULTS

Effects on Cellular Behavior and Ultrastructure of the Cell Surface.

The behavior of the live amoebae was observed under the dissecting microscope. The appearance of the cell shape is shown in Fig. 1. Modification of the cell surface by the enzymes or EDTA at the concentration used in this study did not cause any direct cytolysis. All of those effects were reversible. Cells treated with trypsin or proteinase K were in star-shape after removing the enzymes by repeated washing and remained unattached for 12 and 48 hrs, respectively. While unattached, amoebae did not demonstrate most of the visible cellular behavior, but once attached they resumed normal cellular activities such as, cytoplasmic streaming, formation of pseudopods endo-and exocytosis showing no sign of lasting damages. On the other hand, amoebae which were treated with neuraminidase flattened and attached to the substratum firmer than the untreated control group and retained normal cellular behavior. The effect of EDTA on cell shape and cellular behavior was not conspicuously different from control group.

By con A cytochemistry, the filamentous (F) and amorphous (A) layers of the cell surface could be better visualized than those of unprocessed control (cf. Fig. 2a and 2b). The thickness of F- and A-layers after con A cytochemistry of the normal cells was measured 120nm and 80nm on the average, respectively. A-layer could be further bifurcated into the more dense and the less dense part (Fig. 2b). Each of these layers was arranged in parallel with even thickness and density throughout the cell surface. No electron dense deposit was observed when the cells were incubated with HRP or DAB alone. When the cells were treated with neuraminidase, electron density of the cell surface increased further particularly in the less dense part of A-layer (Fig. 2c). This implies that neuraminidase exposed the more con A binding mannose residues of the cell surface. When the cells were treated with trypsin or proteinase K, the overall appearance of the outer layer was quite different (Fig. 2d and e). Proteinase K removed most of the less dense part of the A-layer, but exposed the more con A binding sites on the more dense part of A- and F-layer by seeing the thickness and electron density of the reaction product (Fig. 2d). By treatment with trypsin the con A binding sites of both F- and A-layers were damaged, consequently no reaction product was detected (Fig. 2e). When the EDTA-treated cells were processed for cytochemistry the overall structure was the same as the untreated cells. In all samples, the trilaminar appearance of the plasma membrane was readily discernible with no significant change

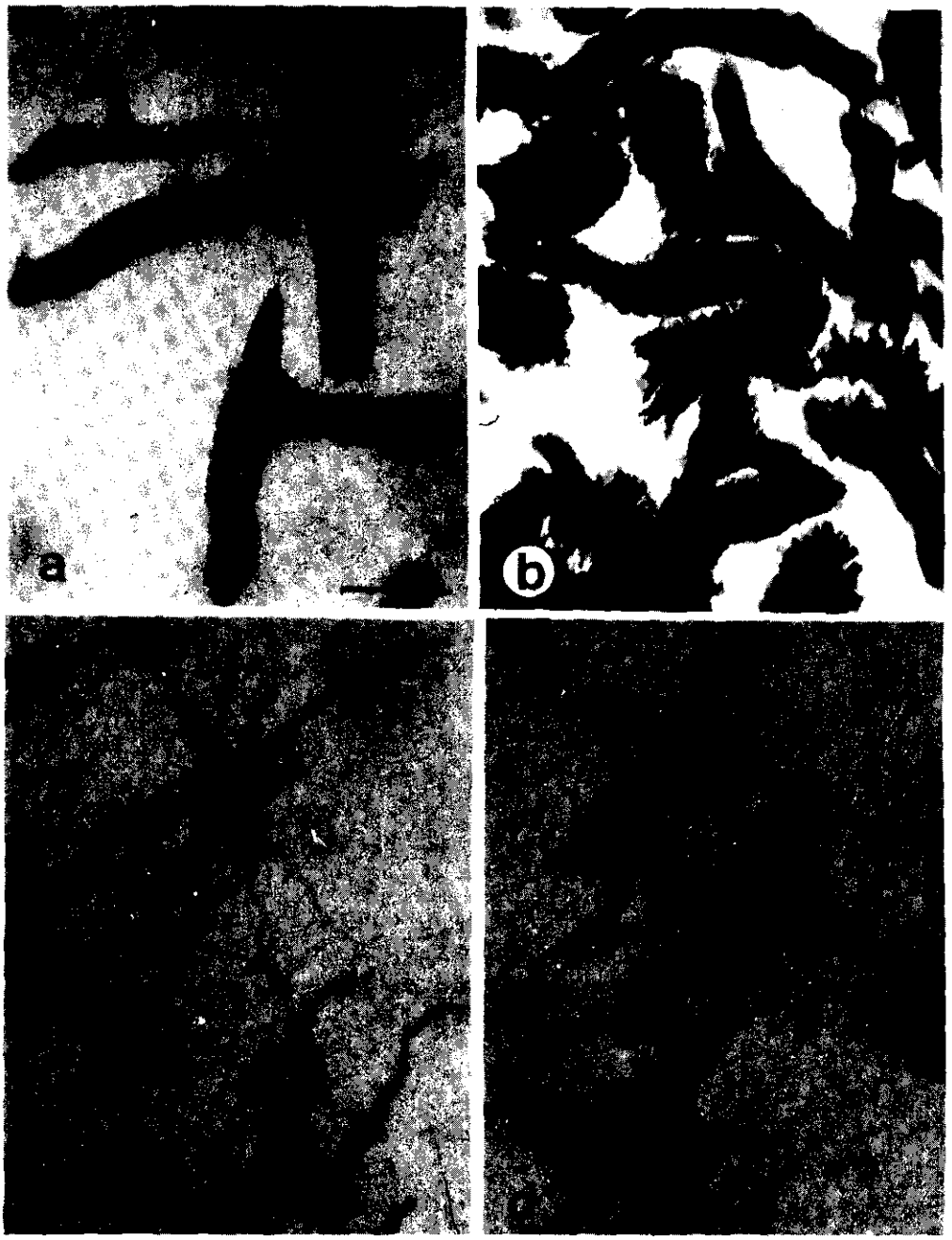

Fig. 1. Changes in morphology and adhering behavior of amoebae on the substratum after treatment with cell surface-modifying agents, untreated control (a), neuraminidase (b), proteinase K(c), and trypsin (d). In (b), cells spreaded and adhered vigorously on the substrata. Cells which were treated with proteases (c,d) did not adhere and settled at the bottom of the culture dish. All pictures are at the same magnification. The bar in (a)represents 50 μm.

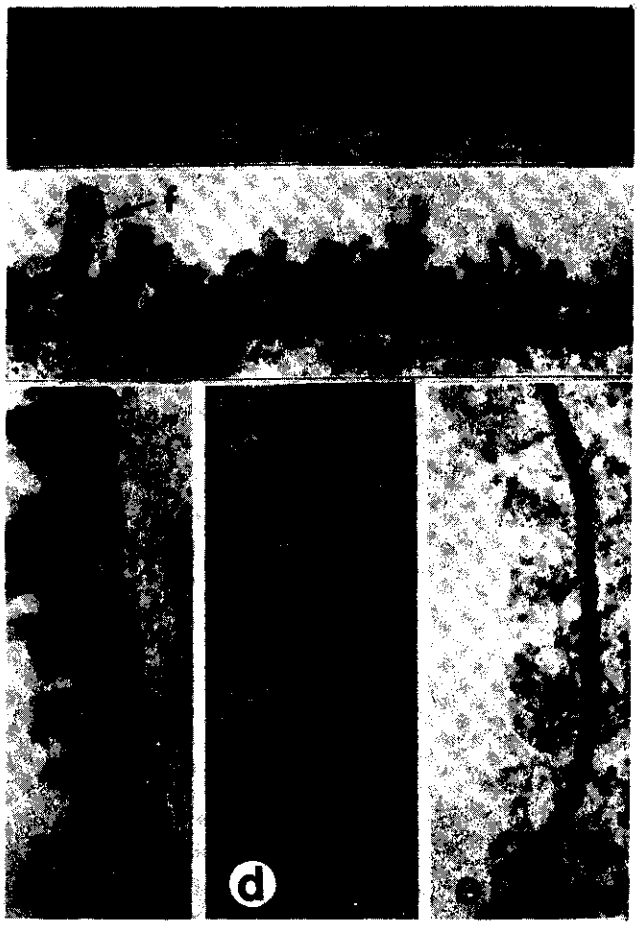

Fig. 2. Concanavalin A cytochemistry of the plasmalemma in *A. proteus*. (a) The ultrastructure of the cell surface without con A cytochemistry, (b) with con A cytochemistry, (c) neuraminidase-treated cell with con A cytochemistry, (d) proteinase K-treated cell with con A cytochemistry, and (e) trypsin-treated cell with con A cytochemistry. Plasma membrane is indicated by arrow head. "f" indicates filamentous layer, "a" and "a'" indicates bifurcated amorphous layers. All pictures are at the same magnification. The bar in (a) represents 0.1 μm.

in morphology.

Changes in Glycoproteins and Mucopolysaccharides by Surface Modification

Electrophoretic pattern of the components released in the medium and those bound to membrane was analyzed by PAS-staining (Fig. 3). Treatment of the cells with trypsin, neuraminidase or EDTA caused no qualitative difference in the profiles of glycoproteins and mucopolysaccharides by comparing with those of the untreated sample (cf, lanes n, t and e with c in Fig. 3). While proteinase K removed all of those three glycoproteins which could be resolved in separating gel (lane p of Fig. 3). But mucopolysaccharides were still resolved

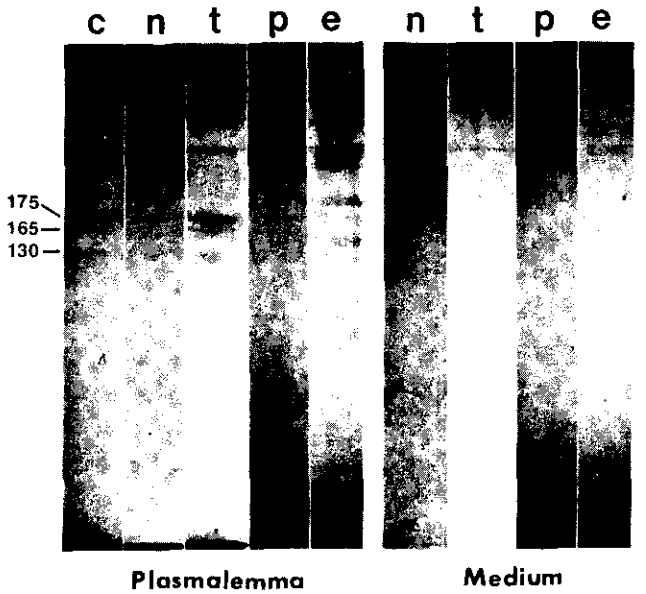

Fig. 3. PAS-stained profiles of the glycoproteins and mucopolysaccharides, which are bound to or released from the plasmalemma of amoebae by treatment with neuraminidase (n), trypsin (t), proteinase K(p), and EDTA (e). "c" represents the fraction obtained from untreated cells.

in stacking gel as three distinct bands.

Those components which were released in the medium by proteases, neuraminidase or EDTA were all resolved in the spacer gel only (Fig. 3). There was no difference on PAS-stained profiles among the samples obtained by treatment with the two proteases and neuraminidase. Since these three PAS-stained bands were not stained by Coomassie brilliant blue, they appeared to be mucopolysaccharides. On the other hand, EDTA released mucopolysaccharides that could be resolved as two bands (Fig. 3 lane e).

DISCUSSION

In the past, polysaccharides of the cell surface were visualized by various histochemical procedures in which they suffered from the limitation of specificity. Recently, lectins have been used as a probe for identification of specfic carbohydrate residues (Lis and Sharon, 1973). The reaction of horseradish peroxidase that is bound to con A results in an increased mass of DAB polymer so as to increase opacity to electron beam (Stebens, 1977). The accumulation of osmium containing deposits on A- and F- layer suggests that the technique of con A cytochemistry is a satisfactory and adequate method for the localization of con A binding mannose residues of glycocalyx (Fig. 2b). Thus the effects of surface-modifying agents were readily compared.

Amoebae which were treated with neuraminidase spread and attached better than the

untreated control. Their surface morphology was quite different from other groups. The reaction products of HRP were widely spread arounded the filaments (cf. Fig. 2c and b, d, or e). This means the more con A binding mannose residues are exposed by the enzyme. But the PAS-stained pattern of mucopolysaccharides and glycoproteins were not altered (Fig. 3) though the profile of iodinatable proteins by lactoperoxidase (LPO) was different from untreated plasmalemma (Ahn and Jeon, 1985). When the cells were treated with proteolytic enzymes, both the cellular behavior and morphology were modified drastically. But changes in PAS-stained profile of trypsin treated membranes was not significantly different from the control group. Trypsin was known to modify 175 KD glycoprotein so as to reduce LPO-iodinatibility to 1/3 of the control (Ahn and Jeon, 1985). Though the cells have lost most of the con A binding mannose residues by trypic digestion, they recovered normal behavior in 12 hr. The effect of proteinase K appeared to be more acute on cellular behavior than that of trypsin. For recovery it took about 48 hr. The plasmalemma lost all of PAS-stained glycoproteins. But the damage on con A binding sites of the cell surface was not so severe as by trypsin. For the recovery of the lost 175 KD glycoprotein it took more than 20 hr. The importance of con A binding component on cellular adhesion was further supported by observing the behavior of amoebae that were incubated with con A for 10 min at 4°C. While incubation in a conical centrifuge tube amoebae adhered each other so as to form aggregates. When the cells were incubated with chymotrypsin-treated lectin, this aggregation was not significant. Thus treated cells were unable to attach on the substrata after repeated washing of the unbound monovalent con A.

Thus the adhesion of amoebae on the substratum appears to be primarily mediated by con A binding components of the glycocalyx in A layer and secondarily by either 175KD protein alone or all three PAS-stained glycoproteins which were resolved in separating gel. These two components are not likely the same molecule by examining the DAB reaction products on the surface of proteinase K-treated cells. The larger polysaccharides which were released by EDTA are not directly related with cellular adhesion as deduced from the fact that there was not significant change in cellular behavior, morphology and protein composition of the plasmalemma. Further characterization of the mucopolysaccharides or the glycoproteins using specific antiserum or monoclonal antibodies will clarify the molecular mechanism of cellular adhesion in amoebae.

SUMMARY

The effects of proteases, neuraminidase and EDTA on adhesion of amoebae on the substratum, ultrastructure and biochemical composition of the cell surface were studied by concanavalin A (con A) cytochemistry and SDS PAGE. By con A cytochemistry the glycocalyx of the plasmalemma was easily subdivided into outer filamentous (F) layer and the inner amorphous (A) layer. On treatment with neuraminidase, amoebae attached to the

substratum and spreaded better than untreated cells exposing the more con A binding sites in A- and F-layer. When the cells were treated with trypsin or proteinase K, cells stayed unattached for 12 and 48 hr, respectively. Con A binding sites of A layer and all of those glycoproteins were removed by proteinase K. On the other hand, trypsin damaged all of the con A binding sites in both A- and F-layer without significant change in PAS-stained profile of the plasmalemma. Some of the mucopolysaccharides of the cell surface were released by these enzymes and EDTA. When the cells were incubated with monovalent con A they did not attch on the substratum and cytolysed. From these results adhesion of amoebae on the substratum appears to be mediated by the interaction of the glycoproteins and mucopolysaccharides of the A layer.

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