MODIFICATION OF ADHESION BETWEEN CELL AND EXTRACELLULAR MATRIX CORRESPONDING TO CONFORMATIONAL CHANGES OF CELL-SPREADING PROTEINS IN SERUM

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CONTENTS

ABSTRACT

I have examined the modification of adhesion between cell and extracellular matrix mediated by fibronectin and vitronectin. The conformational change of the cell-binding domain in fibronectin corresponding to the modification of the cell-binding activity by collagen or heparin is quantitatively represented in terms of fluorescence depolarization. The rotatory relaxation time of the whole, the domain, and the group with the fluorescent probe can be detected with three kinds of the probes with different lifetime. In contrast, modification of adhesion mediated by vitronectin corresponding to artificial conformational change of vitronectin by sodium dodecyl sulfate (SDS) was quantitatively observed. The cell-binding activity and adsorptive activity of vitronectin onto polystyrene, involved in the adhesion, are quantitatively represented by the percentage of cell spreading and the absorbance from enzyme-linked immunosorbent assay, respectively. From the above results, a method which quantitatively corresponds the modification of adhesion to the change of intramolecular rotation is established. The knowledge acquired in this study, concerning not only the affinity of the adhesion site, but also the change of interrelation between the site and substrate brings about better understanding of the modification of adhesion. This established method gives a clue to elucidate the mechanism of morphogenesis without any change in the composition of the system, and it should be helpful in the application of cell technology.

I. GENERAL INTRODUCTION

It is well known that cell adhesion precedes morphogenesis in multicellular organisms. There are two types of cell adhesion (Alberts et al., 1983). One is the type where the cell directly adheres to the other cell. In the other type, there are extracellular matrices between the cells, i. e. "adhesion between cell and extracellular matrix", which I will simply refer to as "adhesion". The adhesion involves the extracellular matrix bound onto the cell and adsorbed onto connective extracellular matrix.

Animal adhesive cells are unable to proliferate when the cells can not adhere onto the tissue culture plate. Those cells will finally perish (Stoker et al., 1968). Cell proliferation requires adhesion. The adhesion proceeds at multipoints between the cell and the connective extracellular matrix. The cell flattens and projects lateral cytoplasm (Fisher & Solursh, 1979; Folkman & Moscona, 1978). This phenomenon is called spreading. The spreading shows a sign of active phase of the cell. During chick embryonic development, neural crest cells spread and migrate through a network of extracellular matrices, and show cell differentiation from neural crest cell to pigment cell or sympathetic nerve cell at its final point (Weston, 1970). The migration of neural crest cells in chick embryo are inhibited by injection of antibodies which recognize the site for binding of extracellular matrix to cell surface (Bronner-Fraser, 1986). These results indicate that cell spreading is indispensable to morphogenesis.

Cell spreading is promoted by some glycoproteins such as laminin (Timple et al., 1979), fibronectin (Mosesson & Umfleet, 1970), vitronectin (Hayman et al., 1983) etc. which can be isolated. Both fibronectin and vitronectin are found broadly in vivo (Stenman & Vaheri, 1978; Hayman et al., 1983). Especially, serum contains both of the above proteins at high concentrations (approximately 0.2 mg/ml) (Mosesson & Umfleet, 1970; Shaffer et al., 1984).

Removal of carbohydrates from fibronectin does not influence its cell-spreading activity (Olden et al., 1979). The proteinous part of fibronectin is composed of structural units (domains) (Hayashi & Yamada, 1983; Sekiguchi & Hakomori, 1983) which adsorb onto the respective connective extracellular matrices, for example, collagen (Engvall & Ruoslahti, 1977) and heparin (Yamada et al., 1980), and bind to cells. The amino acid sequence of one of the cell-binding sites in the cell-binding domain of fibronectin is known to be Arg-Gly-Asp (Pierschbacher & Ruoslahti, 1984).

Sequence of the cell-binding site in vitronectin is similar to that of the site in fibronectin (Suzuki et al., 1985; Jenne & Stanley, 1985). Vitronectin is adsorbed onto collagen (Izumi et al., 1988) and onto heparin (Hayashi et al., 1985) as fibronectin is. However, the domain structure of vitronectin is not yet fully understood.

The function of the domains is not always independent. Adsorption of fibronectin onto heparin or collagen enhances the activity of binding fibronectin to hepatocyte

(Johansson & Höök, 1984). In addition, adsorption of fibronectin onto heparin enhances the activity of adsorption of fibronectin onto collagen (Johansson & Höök, 1980). These facts indicate that adhesion between the cell and the extracellular matrix is modified by a different extracellular matrix. Modification of adhesion influences cell spreading and in its turn influences morphogenesis.

It is interesting to measure the conformational changes of fibronectin corresponding to the modification of adhesion. Some conformational changes of local regions corresponding to the modification have been reported (Williams et al., 1982; Ankel et al., 1986). However, these results fail to compare the whole fibronectin with the one part in respect of the conformational changes. In the second chapter, corresponding to the modification of the cell-binding activity, the rotatory relaxation time of the whole, the domain, the group with a fluorescent probe is measured by the method of fluorescence depolarization. Comparing these rotatory relaxation time, the intramolecular rotation of the cell-binding domain is observed corresponding to the modification.

In the third chapter, in contrast, modification of cell-binding activity and adsorption activity caused by artificial conformational changes is measured quantitatively. Vitronectin was treated by sodium dodecyl sulfate (SDS), which is known to expose the inner hydrophobic groups to the surface of protein. Adsorptive activity onto hydrophobic polystyrene and cell-binding

activity were quantitatively observed from enzyme-linked immunosorbent assay and cell-spreading assay, respectively. The effect of SDS treatment on the percentage of cell spreading in fibronectin and vitronectin are compared.

In the fourth chapter, a method which quantitatively corresponds the modification of adhesion to the change of intramolecular rotation is established. From this fruit, a prospect to analyze the mechanism of morphogenesis in the future and the applied faces of modification of adhesion are discussed.

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II. INTERACTION BETWEEN CELL-BINDING DOMAIN AND EXTRACELLULAR MATRIX-BINDING DOMAIN OF FIBRONECTIN DETERMINED BY FLUORESCENCE DEPOLARIZATION

1. Introduction

Fibronectin is one of the main glycoprotein which involved in adhesion between cell and connective extracellular matrix. The plasma fibronectin molecule is composed of two long polypeptide chains, α and β, covalently linked via two disulfide bonds near their carboxyl termini (Hynes & Yamada, 1982). Each strand consists of well-defined structural domains, connected by short, flexible, proteinase-sensitive polypeptide segments (Alexander et al., 1979; McDonald & Kelly, 1980; Ruoslahti et al., 1979). Each domain specifically binds to the cell, or collagen or gelatin, heparin or heparan sulfate, or fibrin (Hayashi & Yamada, 1983; Sekiguchi & Hakomori, 1983).

On the other hand, it has been reported that when heparin or collagen is bound to its specific domain, the inhibitory effect of soluble fibronectin on hepatocyte attachment to fibronectin coated substrate is greatly enhanced (Johansson & Höök, 1984). The report suggested that each domain in fibronectin is not quite independent, but interacts with each other.

Some conformational changes induced by ligand binding to fibronectin have been observed. The average change of a whole molecule has been detected by circular dichroic measurement (Österlund et al., 1985). On the other hand, the changes of a partial region have also been detected (Williams et al., 1982; Ankel et al., 1986), and are described as follows. Fluorescence polarization of dye (lifetime τ=12 ns), labeled at the NH_2-terminus, decreases

by combining collagen to the collagen-binding domain (Williams et al., 1982). The report suggested that the binding of collagen changes the molecular conformation of fibronectin to a more relaxed or flexible state. Also when heparin is bound to the heparin-binding domain, the same suggestion was proposed from the results using the electron spin resonance spin-label method (Ankel et al., 1986).

I took notice of interaction among the domains. The interaction of the heparin- or gelatin-binding domain with the cell-binding domain is probably causing the conformational change of the cell-binding domain. I tried to detect the conformational change of the cell-binding domain. Conformational change is caused by the intramolecular rotational change along the strand. The rotational change was detected by photometry of fluorescence polarization of three kinds of dye. Each dye, with different lifetimes (τ=5,20 and 100 ns), was labeled at a free sulfhydryl group in the cell-binding domain. Interaction of the heparin- or gelatin-binding domain with the cell-binding domain is discussed.

2. Materials and Methods

1) Preparation of the dye-fibronectin complex.

Plasma fibronectin was purified from porcine plasma according to the method of Hayashi (Hayashi & Yamada, 1982).

The three kinds of fluorescent dye with various orders of fluorescent lifetime were labeled at a free sulfhydryl

group in the cell-binding domain of fibronectin (Hayashi & Yamada, 1983). The labeling, modified from the method of Lay (Lay & Tooney, 1984) was performed in the reaction mixture (1 mg/ml fibronectin, 10^{-5} M dye) at room temperature for 10 min after denaturation of fibronectin in 3 M guanidine hydrochloride. Unreacted dye and guanidine hydrochloride were removed by dialyzing the reaction mixture against 10 mM Tris-HCl buffer/0.15 M NaCl(pH 7.4) for 1 h for first change, for 2 h for second and third changes and overnight for the last change.

[N-(1-anilinonaphtyl-4)] maleimide (ANM, fluorescent lifetime τ=5 ns (Kanaoka et al., 1973), Teika-Seiyaku Co.), [N-(3-fluoranthyl)] maleimide (FAM, τ=20 ns (Kanaoka et al., 1976), Teika-Seiyaku Co.), and [N-(3-pyrene)] maleimide (PRM, τ=100 ns (Weltman et al., 1973), Molecular Probe Co.) were used for label.

2) Characterization of dye-labeling on fibronectin.

Content of dye per monomer of fibronectin was determined by absorption photometry of FAM-fibronectin complex (FAM-FN). The photometry was performed with the absorption coefficient of 1.28 mg $ml^{-1} \cdot cm^{-1}$ at 280 nm for plasma fibronectin (Mosesson & Umfleet, 1970) and 12000 $M^{-1} \cdot cm^{-1}$ at 362 nm for FAM (Kanaoka et al., 1976).

The content of labeled free sulfhydryl group per monomer of fibronectin was determined. To do so, unlabeled free sulfhydryl group was measured by Ellman's method (Ellman, 1959). The procedure was as follows; FAM,

fluorescent dye was added to fibronectin solution under denaturation of fibronectin in 3 M guanidine hydrochloride. At various intervals, 5, 5'-ditiobis (2-nitrobenzoic acid) solution was added to determine the unlabeled free sulfhydryl group contents. The amount of dinitrophenol anion produced corresponding to the amount of unlabeled free sulfhydryl groups was calculated from absorption coefficient of 1.36×10^4 M^{-1} cm^{-1} at 412 nm (Ellman, 1959). It was checked that the content of labeled free sulfhydryl groups was in agreement with that of FAM containing monomer of fibronectin.

To determine the dye-labeling site, FAM-FN was digested by L-1-tosylamido-2-phenylethyl chlorometyl ketone-treated trypsin (Sigma). A solution of 0.5 mg/ml FAM-FN in 30 mM NaCl/1 mM $CaCl_2$/50 mM Tris-HCl (pH 7.0) was incubated in indicated concentration of trypsin at 30 °C. At indicated intervals, digestion was terminated by addition of soybean trypsin inhibitor (type I-S, Sigma) with concentrational ratio of 1.2 : 1 (trypsin inhibitor/trypsin) and immersion in ice bath. The tryptic digestions were electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gel and reduced by 0.1 M ditiothreitol using a discontinuous Laemmli buffer system (Laemmli, 1970; Studier, 1973). The polyacrylamide gel was composed of 7.5 %/12 % separation gel and 4 % stacking gel. After electrophoresis, FAM-labeling site was determined by comparing the gel under fluorescent illumination at 360 nm excitation and after protein staining of Coomassie brilliant blue R (Sigma).

Cell-spreading assay of FAM-FN was performed according to Grinnell's method (Grinnell et al., 1977). 50 μl of various concentration of fibronectin or FAM-FN was preincubated with 150 mM NaCl/10 mM Tris-HCl (pH 7.4) in plastic tissue culture microwell plate (96 wells, Nunc) for 60 min at 37 °C. After removal of the sample solution, 100 μl of BHK(baby hamster kidney) 2 x 10^4 cells in adhesion medium (150 mM NaCl/1 mM $CaCl_2$/3 mM KCl/0.5 mM $MgCl_2$/6 mM Na_2HPO_4/1 mM KH_2PO_4) was incubated for 45 min at 37 °C. Cells were fixed with 2 % glutaraldehyde, 2 % formalin, 5 % sucrose in Dulbecco's PBS. Spreading cells were counted by inverted phase-contrast microscopy (Olympus model IMT). Heparin- or gelatin-binding activity was judged using the heparin or gelatin affinity column.

3) Photometry of fluorescence polarization.

Fluorescence polarization was detected by photometry (Baba & Ishizaka, 1970). Light from a Xenon lamp (500 W) was passed through a band-pass filter for exciting each dye. The maximum wavelength of excitation light were 350 nm (ANM), 370 nm (FAM), and 340 nm (PRM), and all full width at half maximum was 50 nm.

The photometry of fluorescence polarization of 0.2 mg/ml dye-fibronectin complex in 10 mM Tris-HCl buffer/0.15 M NaCl (pH 7.4) was performed at several temperature of points. The inverse of fluorescence polarization (1/P): fluorescence depolarization, was plotted against the

ratio(T/η) of the absolute temperature(T) to the viscosity(n) of water. The viscosity of water in literature value (Kaye & Laby, 1973) was used at several points of temperature. Temperature of the sample was measured by a thermo-couple. The relation between 1/P and T/η was obtained from data by the least-squares method. The rotatory relaxation time of the dye-fibronectin complex at 20 °C was calculated from the slope of the linear relation at the point of 20 °C and the fluorescent lifetime.

The solution containing heparin (5 mg/ml, 166.9 units/mg, Wako Pure Chem.) or gelatin(10 mg/ml, Wako Pure Chem.) each of which binds to its specific domain of the dye-fibronectin complex was added to the sample solution(3 ml of dye-fibronectin complex in 10 mM Tris-HCl/ 0.15 M NaCl (pH 7.4). The fluorescence polarization of the dye-fibronectin-gelatin or -heparin complex was measured and compared with that of the dye-fibronectin complex at 20 °C.

The photometry of fluorescence polarization was designed in a way to prevent the scattered light. The intensity of the scattered light was eliminated from the intensity of fluorescent light by using a reference cuvette containing unlabeled fibronectin, heparin, or gelatin at the same concentration as the sample cuvette.

3. Results

1) Characterization of dye-labeling on fibronectin.

The content and the site of labeled dye on fibronectin was determined.

Porcine plasma fibronectin contained about 1.6 free sulfhydryl groups per monomer of fibronectin (data not shown). This result was in agreement with observation of human plasma fibronectin (Smith et al., 1982). When a solution of FAM, fluorescent dye was added in denatured porcine plasma fibronectin, the contents of unlabeled free sulfhydryl group established equilibrium at 0.6 group per monomer of fibronectin (data not shown). Because average of free sulfhydryl groups were 1.6, the content of labeled free sulfhydryl group was 1.0 group. On the other hand, the content of FAM in monomer of fibronectin was in agreement with content of labeled free sulfhydryl groups. Therefore, one free sulfhydryl group per monomer of fibronectin was specifically labeled with FAM.

The labeling site was determined from tryptic digestion of FAM-FN. After electrophoresis of digestions, the gel was observed under fluorescent illumination (Fig. 1A) and then stained (Fig. 1B). A fluorescent band showing the cell-binding domain clearly appeared in the 75 kDa fragment (Fig. 1A and B) (Hayashi & Yamada, 1983). This result indicated that fluorescent dye was labeled at a free sulfhydryl group in the cell-binding domain. Since no fluorescent band appeared in 34 kDa fragment which should be the fibrin-binding domain (Hayashi & Yamada, 1983), containing another

free sulfhydryl group (Fig. 1A and B), FAM was not labeled at the fibrin binding domain. In addition, fluorescent bands less than 75 kDa were probably digestions of the 75kDa fragment.

Biological activity of FAM-FN was examined. The cell-spreading activity (Fig. 2) and the gelatin- or heparin-binding activity (data not shown) of FAM-fibronectin complex was equivalent to that of intact fibronectin. Labeling at a free sulfhydryl group in the cell-binding domain did not affect the biological activity.

2) Electronic state around the dye labeled at the free sulfhydryl group in the cell-binding domain.

The effect of heparin or gelatin on the electronic state around the dye labeled at the free sulfhydryl group in the cell-binding domain was tested by spectro-photometry. The maximum fluorescent wavelength of ANM-fibronectin complex (ANM-FN) with excitation at 360 nm was 435 nm. The maximum wavelength did not shift by adding heparin or gelatin (data not shown), although the maximum fluorescent wavelength of ANM sensitively shifts when the electronic state is changed (Kanaoka et al., 1973). Therefore, this result suggests that the electronic state was not affected by heparin or gelatin, binding to its domain. The fluorescence polarization of the dye-fibronectin complex is independent from the changes in the intrinsic polarization of the binding dye, and depends on the rotation of the dye.

3) The rotatory relaxation time of the dye-fibronectin complex.

The rotatory relaxation time of PRM-fibronectin complex (PRM-FN) with the longest fluorescent lifetime of 1×10^2 ns, was 7×10^2 ns at 20 °C (Fig. 3a). The rotatory relaxation time of FAM-FN with the medium length of fluorescent lifetime of 20 ns, was 1×10^2 ns at 20 °C (Fig. 3b). The linear relation between P and T/η changed at 40 °C. This result suggests a conformational change of the cell-binding domain or part of the fibronectin molecule that contains the domain at 40 °C. The rotatory relaxation time of ANM-FN with the shortest fluorescent lifetime of 5 ns, was 10^2 ns (Fig. 3c), similar to FAM-FN.

Neither the fluorescence of ANM with short fluorescent lifetime, FAM with long lifetime, nor PRM with longer lifetime on fibronectin depolarized as much as the free rotating fluorescence. In other words, the dye did not rotate freely through its fluorescent lifetime. It was found that each dye was firmly fixed in the cell-binding domain. In addition, the rotatory relaxation time of PRM-FN indicates the rotation of the whole or part of the fibronectin molecule containing the cell-binding domain. On the contrary, the rotatory relaxation time of ANM-FN and FAM-FN indicates rotation of the cell-binding domain or part of the fibronectin molecule containing the domain.

4) The effect of heparin on fluorescence polarization.

When heparin was added in the solution of PRM-FN

solution (Fig. 4a), fluorescence polarization tended to increase, as was naturally expected due to an increase in effective volume by binding heparin and/or increase of solvent viscosity. It was found that the rotation of whole or part of the fibronectin molecule containing the cell-binding domain through fluorescent lifetime of 100 ns was suppressed by combining heparin to the heparin-binding domain.

On the other hand, when heparin was added in the solution of ANM-FN (Fig. 4c) or FAM-FN (Fig. 4b), on the contrary, the fluorescence polarization tended to decrease, that is, slightly depolarize through the fluorescent lifetimes of 5 or 20 ns, respectively. It was found that the rotation of the cell-binding domain or of that part of the fibronectin molecule that contains the domain, was slightly promoted by combining heparin to the heparin-binding domain.

5) The effect of gelatin on fluorescence polarization.

When 5.0×10^{-3} mg/ml gelatin was added to the solution of PRM-FN, the fluorescence polarization was increased. However, the fluorescence polarization tended to decrease at higher concentration, but did not decrease under the fluorescence polarization of PRM-FN (Fig. 5a). It was found that the rotation of the whole or of part of the fibronectin containing the cell-binding domain through fluorescent lifetime of 100 ns was not promoted by combining gelatin to

the gelatin-binding domain.

On the other hand, when gelatin was added in the solution of ANM-FN (Fig. 5c) or FAM-FN (Fig. 5b), on the contrary, the fluorescence polarization tended to decrease, that is, slightly depolarize through each fluorescent lifetime of 5 or 20 ns, respectively. It was found that the rotation of the cell-binding domain or of that part of the fibronectin molecule containing the domain, was slightly promoted by combining gelatin to the gelatin-binding domain.

4. Discussion and Conclusion

I tried to observe interaction among the domains in the fibronectin molecule. An interaction of the heparin- or gelatin-binding domain with the cell-binding domain must induce the conformational change of the cell-binding domain. The conformational change was detected by photometry of fluorescence polarization.

Fibronectin is composed of two long polypeptide chains (Hynes & Yamada, 1982). Each chain consists of well-defined structural domains, connected by short, flexible, proteinase-sensitive polypeptide segments (Alexander et al., 1979; Ruoslahti et al., 1979; McDonald & Kelley, 1980). The order of domains, proceeding from the NH_2-terminus, is a strong heparin- or heparan sulfate-binding domain, a gelatin- or collagen-binding domain, a cell-binding domain, a weak heparin- or heparan sulfate-binding domain, and a fibrin-binding domain (Hayashi & Yamada, 1983; Sekiguchi & Hakomori, 1983). Fibronectin is a long, thin and flexible

strand (Erickson et al., 1981; Williams et al., 1982).

The cell-binding domain contains a free sulfhydryl group (Hayashi & Yamada, 1983). This free sulfhydryl group is located in the center of fibronectin molecule, and is situated at a distance of about 300 amino acid residues from the cell-binding site, which is in the tetrapeptide sequence (Arg-Gly-Asp-Ser) toward the NH_2-terminus (Kornblihtt et al., 1985).

I took notice of the free sulfhydryl group in the cell-binding domain, and the sulfhydryl group was labeled with three kinds of fluorescent dye, the lifetime of which is different. Since the free sulfhydryl group is buried in the fibronectin molecule (Smith et al., 1982), it was labeled with the dye under denaturation (Lay & Tooney, 1984).

In this experiment, when heparin or gelatin was bound to its specific domain, the maximum fluorescent wavelength of ANM, labeled at the free sulfhydryl group, did not shift. It was confirmed that the intrinsic polarization of the dye at the free sulfhydryl group was stable in the state in which heparin or gelatin bound to the domain. The fluorescence polarization depends on the rotation of the dye.

From the results of fluorescence polarization of these dye-fibronectin complex, it was found that each dye was firmly fixed in the cell-binding domain. The fact agrees with the results of electron spin resonance spectrum of spin-label fibronectin (Lay & Tooney, 1984). The

fluorescence polarization depends on the rotation of the whole or that part of the fibronectin molecule that contains the cell-binding domain, or the cell-binding domain alone.

By a photometry of fluorescence polarization of FAM- or ANM-FN, it was found that the rotation of the cell-binding domain or of that part of the fibronectin molecule that contains the domain, was slightly promoted by combining gelatin, which is denatured collagen, to the gelatin-binding domain. It has been reported that when collagen was bound to the collagen-binding domain, fluorescence polarization of dye labeled at NH_2-terminus was decreased (Williams et al., 1982). In this experiment, it is significant that the rotatory relaxation time of not only NH_2-terminus, which is apt to rotate, but also the cell-binding domain in the center of the long and thin fibronectin molecule reduced.

By a photometry of fluorescence polarization of PRM-FN, it was found that the rotation of the whole or part of the fibronectin molecule containing the cell-binding domain through fluorescent lifetime of 100 ns was suppressed by combining heparin to the heparin-binding domain. Using a photometry of fluorescence polarization of FAM- or ANM-FN, on the other hand, it was found that the rotation of the cell-binding domain or of that part of the fibronectin molecule containing the domain, was slightly promoted by combining heparin to the heparin-binding domain. Thus, the rotatory relaxation time of the cell-binding domain or of that part of the fibronectin molecule containing the domain was reduced. The fact agrees with the results from electron

spin resonance spin-label method (Ankel et al., 1986).

I attempt a further analysis concerning the reduction of the rotatory relaxation time of the cell-binding domain or of that part of the fibronectin molecule containing the domain. The rotatory relaxation time, ρ, is proportional to the viscosity, η, of the environment surrounding the rotating molecule and inversely proportional to the temperature, T, giving $\rho=V\eta/kT$ (Perrin, 1926) where k is the Boltzmann constant and V is the effective volume of a rotatory unit. Since the temperature was kept constant, the viscosity of the sample solution did not change. Therefore, the reduction of the rotatory relaxation time must be a result of shrinkage of the effective volume, which indicates a conformational change of the cell-binding domain or in that part of the fibronectin molecule containing the domain.

It is concluded that each domain of fibronectin is not quite independent, but interacts with each other. The interaction of the heparin- or gelatin-binding domain with the cell-binding domain induced the conformational change of the cell-binding domain or that part of the fibronectin molecule containing the domain.

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6. Figures and Legends

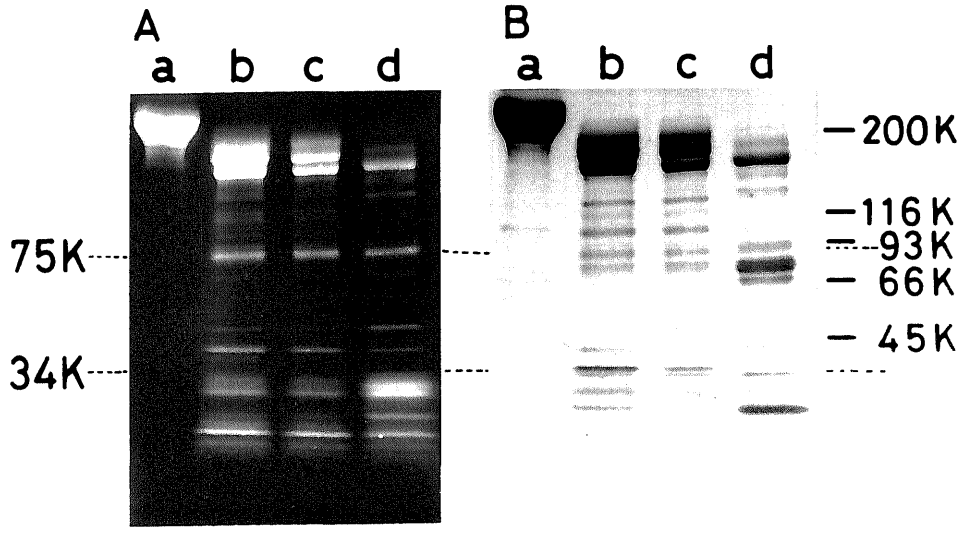
A
a b c d
B
a b c d
75K
34K
200K
116K
93K
66K
45K

Figure 1. Fragmentation of FAM-fibronectin complex by trypsin. Fragments were analyzed in a 4 %/7.5 %/12 % SDS-polyacrylamide gel. FAM-labeling site was determined by comparing the gel under fluorescent illumination at 360 nm excitation (A) and after protein staining with Coomassie brilliant blue R (B). FAM-fibronectin complex (0.5 mg/ml) in 1 mM $CaCl_2$, 30 mM NaCl, and 50 mM Tris-HCl (pH 7.0) was digested at 30 °C by trypsin at enzyme/substrate ratios (w/w) of 0.2 % for 30 min (b), 0.2 % for 45 min. (c), 2 % for 30 min (d). Lane a is fibronectin before digestion by trypsin. 75 kDa fragment is the cell-binding domain, and 34 kDa fragment is the fibrin-binding domain. Standards of molecular weight of protein are; 200 kDa for myosin, 116 kDa for galactosidase, 93 kDa for phosphorylase, 66 kDa for bovine serum albumin and 45 kDa for ovalbumine.

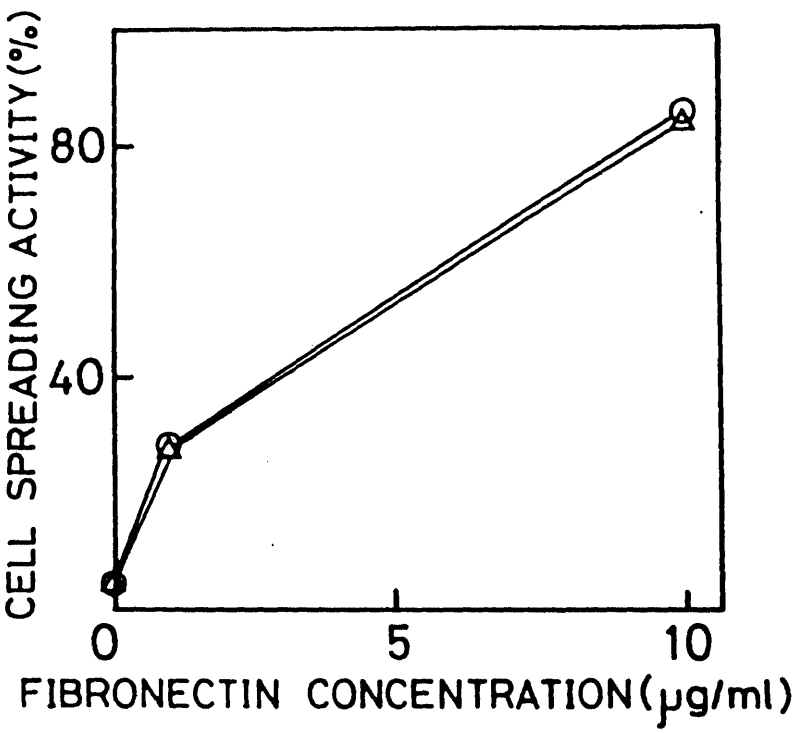
CELL SPREADING ACTIVITY (%)
80
40
0
5
10
FIBRONECTIN CONCENTRATION (μg/ml)

Figure 2. Cell-spreading activity of FAM-fibronectin complex. The cell-spreading activity was examined for baby hamster kidney cells with FAM-fibronectin complex (△) and intact fibronectin (○).

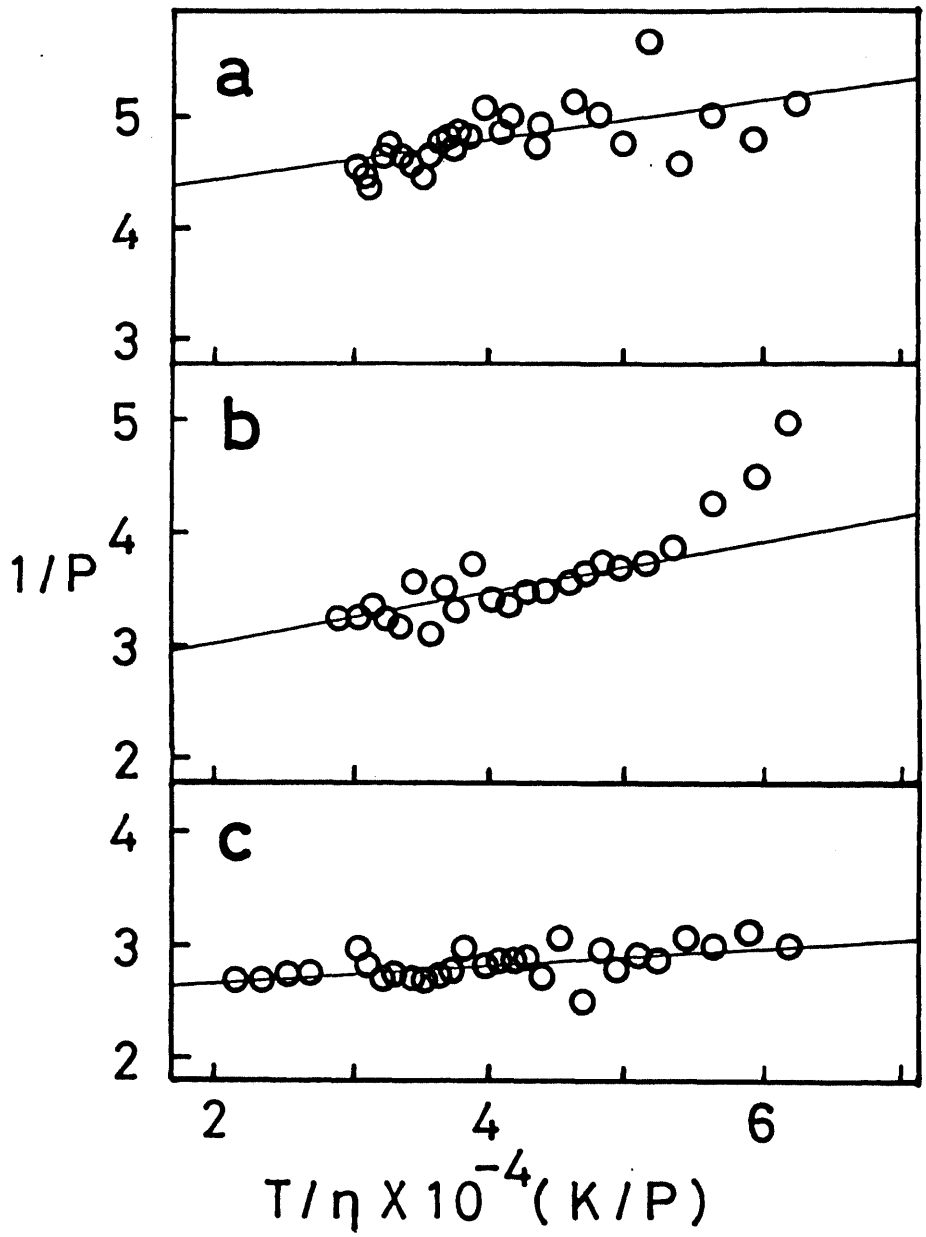
a
b
c
5
4
3
5
4
3
2
4
3
2
1/P
2
4
6
T/η X 10^-4 (K/P)

Figure 3. Perrin plots for the dye-fibronectin complex. T/η, the ratio of absolute temperature (K) to viscosity (poise) was varied by changing the sample temperature. $1/P$ is the inverse of fluorescence polarization: fluorescence depolarization. a:PRM-fibronectin complex (0.3 mg/ml), b:FAM-fibronectin complex (0.2 mg/ml), c:ANM-fibronectin complex (0.2 mg/ml).

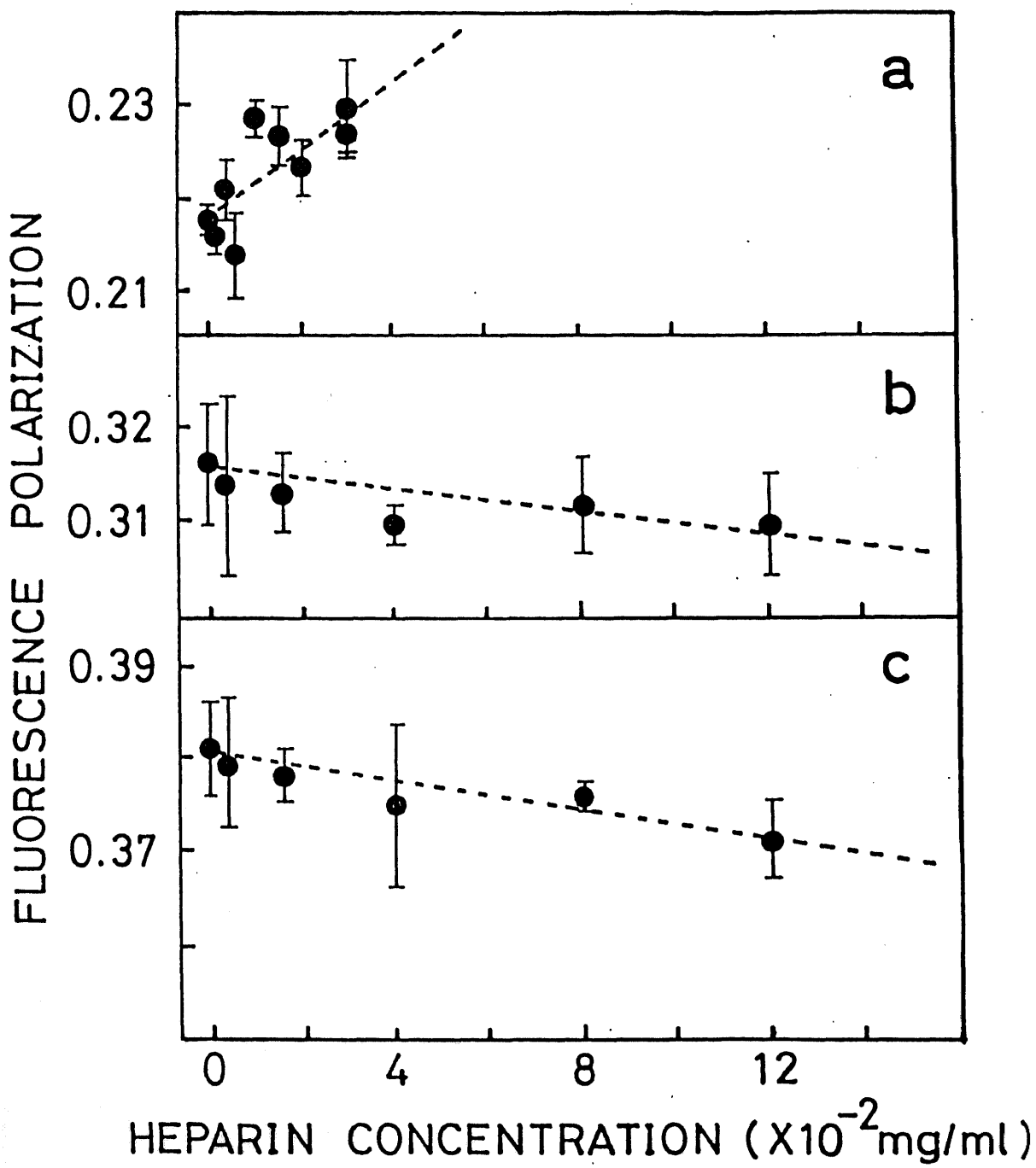
a
b
c
0.23
0.21
0.32
0.31
0.39
0.37
FLUORESCENCE POLARIZATION
0
4
8
12
HEPARIN CONCENTRATION (X10^{-2}mg/ml)

Figure 4. The effect of heparin on the degree of fluorescence depolarization at 20 °C. a:PRM-fibronectin complex (0.3 mg/ml), b:FAM-fibronectin complex (0.2 mg/ml), c:ANM-fibronectin complex (0.2 mg/ml)

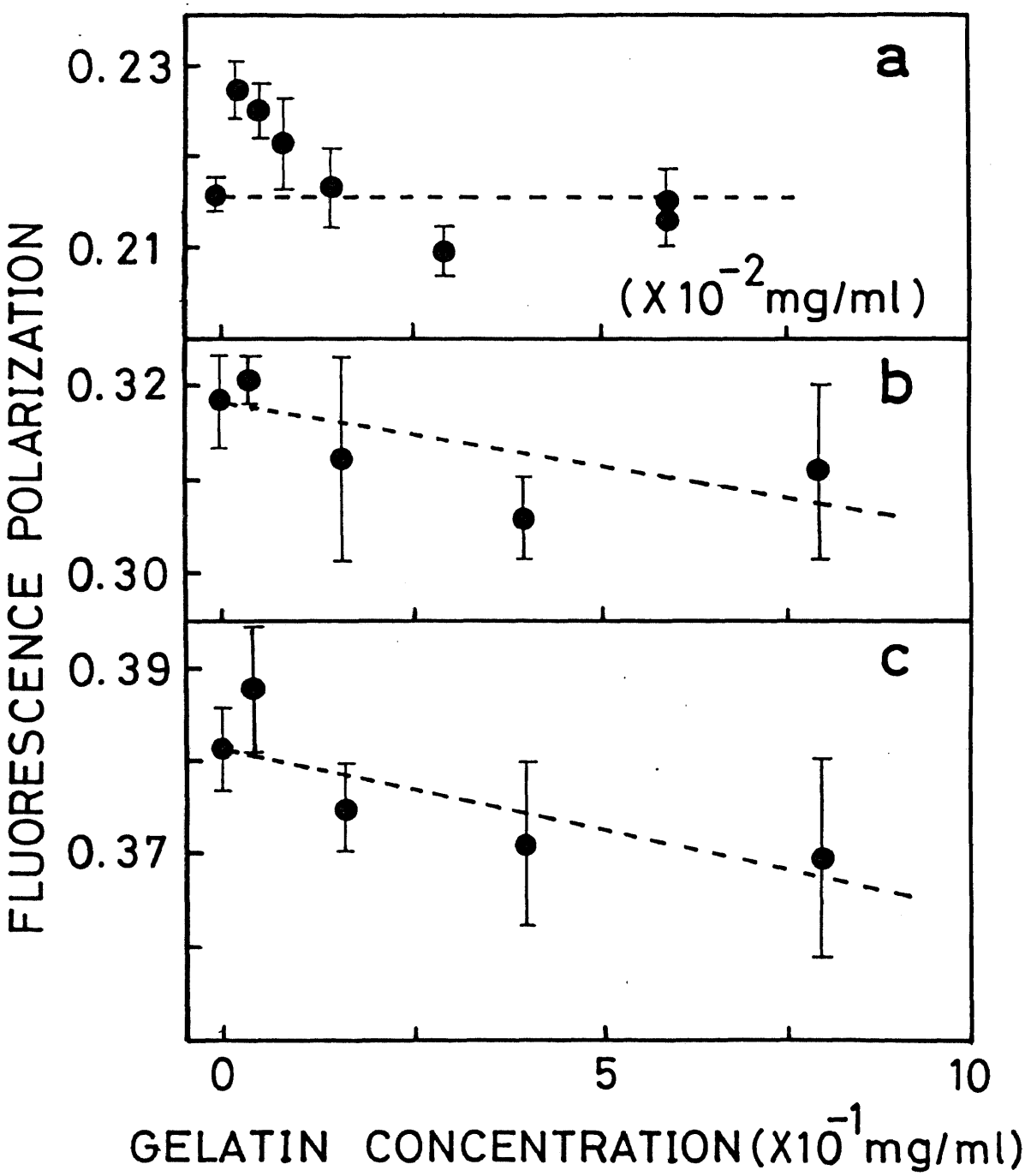
0.23
0.21
a
(X10^{-2}mg/ml)
0.32
0.30
b
0.39
0.37
c
FLUORESCENCE POLARIZATION
0
5
10
GELATIN CONCENTRATION (X10^{-1}mg/ml)

Figure 5. The effect of gelatin on the degree of fluorescence depolarization at 20 °C. a:PRM-fibronectin complex (0.3 mg/ml), b:FAM-fibronectin complex (0.2 mg/ml), c:ANM-fibronectin complex (0.3 mg/ml)

III. Adsorption of Vitronectin in Human Serum onto Polystyrene is Augmented by Sodium Dodecyl Sulfate

1. Introduction

It is well known that a variety of animal cells can be grown *in vitro* with synthetic media supplemented with animal sera. Most cultured cells need to attach and spread on a substrate to proliferate *in vitro* (Folkman & Moscona, 1978; Fisher & Solursh, 1979). Adhesive factors are provided by serum in the medium and/or secreted by cultured cells themselves (Grinnell et al., 1977; Barnes et al., 1980; Knox & Griffiths, 1980; Hayman et al., 1982).

The adhesive factors in serum are glycoproteins mostly identified as fibronectin (Hynes & Yamada, 1982; Akiyama & Yamada, 1987) and as vitronectin (Barnes & Silnutzer, 1983; Hayman et al., 1983). Vitronectin is also termed serum spreading factor (Barnes & Silnutzer, 1983), S-protein (Podack & Müller-Eberhard, 1979), and epibolin (Stenn, 1981). Both adhesive glycoproteins are different in their molecular sizes, amino acid sequences, and cDNA sequences (Barnes et al., 1983; Suzuki et al., 1984; Suzuki et al., 1985; Jenne & Stanley, 1985). Their antibodies do not cross-react with each other (Barnes et al., 1983; Hayman et al., 1983). Fibronectin spreads cells in more expanded peripheral cytoplasm of cells than vitronectin does (Yatohgo et al., 1988a).

In fetal bovine serum mostly used for cell culture, vitronectin rather than fibronectin appears to be a main adhesive glycoprotein, as is reported below. Human plasma vitronectin is known to adsorb avidly to polymers (Collins et al., 1987). Cell-attachment activity attributable to

vitronectin is 8 - 16-fold greater than that of fibronectin in the cell-blotting assay of fetal bovine serum (Hayman et al., 1985). Fibronectin concentration in fetal bovine serum is only about 0.03 mg/ml (Hayman & Ruoslahti, 1979), probably about 1/7 of the vitronectin concentration (Hayman et al., 1985). Vitronectin is approximately 10-fold greater in the specific activity for cell spreading than fibronectin (Barnes & Silnutzer, 1983; Yatohgo et al., 1988a). Fibronectin in human serum adsorbs onto tissue culture plates and induces cell spreading in lower serum concentrations than 1 % (Grinnell & Feld, 1982). But, it adsorbs very little at 10 % serum (Grinnell & Feld, 1982), which is the serum concentration mostly used for cell culture. At concentrations of 3 % serum and above, cell spreading is mediated by vitronectin (Knox, 1984).

However, little is known about characteristics of vitronectin adsorption onto tissue culture polystyrene plates from serum. A modified sandwich ELISA termed ELISA-SDS (Akama et al., 1986) has been previously developed to quantitate vitronectin in human serum reliably. During treatment of human serum with sodium dodecyl sulfate (SDS) for the quantitation, I have noticed that vitronectin in human serum became highly adhesive to the polystyrene plates after treatment with SDS. In this chapter I describe that SDS activated the adsorption of vitronectin in human serum to the polystyrene plates and the adsorbed vitronectin could spread cultured cells.

2. Materials and Methods

1) Whole Serum, Fibronectin-depleted Serum, and Vitronectin-depleted Serum.

Human plasma was clotted with the addition of a final concentration of 20 mM $CaCl_2$ at room temperature for 1 h then at 4 °C overnight and centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting serum was stored frozen until use. Protein concentration of the whole serum was 64 mg/ml. Fibronectin-depleted serum was obtained as a flow-through fraction of gelatin-Sepharose affinity chromatography of the whole serum (Engvall & Ruoslahti, 1977). Fibronectin concentration in the serum extensively decreased from 6.3 μg/mg of total protein to 0.024 μg/mg, while vitronectin concentration in the serum was kept at 3.8 μg/mg of total protein almost similar to the value in the whole serum of 4.1 μg/mg. Vitronectin-depleted serum was obtained as a flow-through fraction after two-cycles of anti-vitronectin-Sepharose 4B column chromatography. Vitronectin concentration in the serum decreased from 4.1 μg/mg of total protein to 0.29 μg/mg. However, fibronectin concentration in the serum also decreased from 6.3 μg/mg to 1.3 μg/mg. The shortage of fibronectin was supplied with pure fibronectin up to 6.3 μg/mg for the vitronectin-depleted serum. The concentrations of vitronectin and fibronectin in the above sera were determined with ELISA-SDS (Akama et al., 1986) and sandwich ELISA (Sato & Hayashi, 1986), respectively.

2) Preparation of Fibronectin, Vitronectin, and Their Antibodies.

Purified vitronectin from outdated human plasma was obtained from Prof. Hayashi, Ochanomizu University (Yatohgo et al., 1988a). Fibronectin was purified from outdated human plasma using a gelatin affinity column (Engvall & Ruoslahti, 1977; Hayashi & Yamada, 1982). The concentrations of pure vitronectin and pure fibronectin were determined from absorbance measurements at 280 nm with a 1 cm-path length cell using $E_{1mg/ml}$ value of 1.38 (Dahlbäck & Podack, 1985) and 1.28 (Mosesson & Umfleet, 1970), respectively. Rabbit anti-human vitronectin antibody and rabbit anti-human fibronectin antibody, and their conjugation with horseradish peroxidase were obtained from Prof. Hayashi, Ochanomizu University (Akama et al., 1986; Sato et al., 1986).

3) Adsorption and Quantitation of Vitronectin.

In the standard procedure, human serum was diluted to 0 - 4 % in phosphate-buffered saline (PBS) containing 0.1 % SDS and boiled for 5 min. Polystyrene 96-well plate for ELISA (No. MS-3496F, Sumitomo Bakelite, Tokyo) was incubated with 50 µl of SDS-treated serum and non-treated serum at room temperature for 1 h. After rinsing with PBS 3 times, the plate was incubated with 1 % (w/v) bovine serum albumin in PBS at room temperature for 1 h. After rinsing with PBS 3 times again, the plate was incubated with horseradish peroxidase-conjugated anti-vitronectin antibody diluted at

1/2000 in PBS containing 1 % bovine serum albumin at room temperature for 1 h. After rinsing with PBS 5 times, an orange color was generated with the incubation of 100 µl of 0.4 mg/ml o-phenylenediamine, 2.5 mM H_2O_2, 0.1 M citric acid, and 0.2 M Na_2HPO_4 for 10 min in the well. The adsorbed vitronectin on the plate was quantitated by the absorbance at 492 nm for the orange color.

4) Cell-Spreading Activity.

BHK cells were grown in Dulbecco's modified Eagle medium supplemented with 10 % calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin sulfate at 37 °C in a 5 % CO_2-humidified air mixture. Polystyrene 96-well plate for tissue culture (No. 167008, Nunclon Delta SI, Nunc, Denmark) was incubated at room temperature for 1 h with 50 µl of human serum, fibronectin-depleted serum, vitronectin-depleted serum, pure vitronectin, and pure fibronectin at indicated concentrations before and after boiling in 0.1 % SDS for 5 min. After rinsing with PBS 3 times, the plate was incubated with 100 µl of BHK cell suspension (2×10^5 cells/ml) in a Grinnell's adhesion medium (Grinnell et al., 1977) of 150 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 0.5 mM $MgCl_2$, 6 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.3, at 37 °C for 1.5 h. After rinsing gently with PBS to remove unattached cells, attached cells were fixed with 100 µl of 2 % glutaraldehyde, 5 % formaldehyde, and 5 % sucrose in PBS at room temperature for 30 min. Percent of spread cells (number of spread cells

per 100 attached cells) in 4 areas of 0.7 x 0.7 mm^2 were counted microscopically.

To test effects of antibody on cell spreading, 50 µl of 0 - 1.5 mg/ml rabbit anti-human vitronectin antibody, rabbit anti-human fibronectin antibody, or rabbit normal IgG in PBS containing 1 % bovine serum albumin was incubated with the serum-coated wells at 37 °C for 3 h before receiving BHK cells.

3. Results

1) Increased Adsorption of Vitronectin.

A modified sandwich ELISA, termed ELISA-SDS, has been developed previously (Akama et al., 1986). In the ELISA-SDS, vitronectin was boiled in 2 % SDS for 5 min, diluted at an 1 to 20 to lower the concentration of SDS to 0.1 %, and added to the polystyrene plates pre-coated with anti-vitronectin antibody. After the establishment of ELISA-SDS, I noticed that boiling in 0.1 % SDS remarkably activated the adsorption of vitronectin in human serum to the polystyrene plates. The amount of vitronectin adsorption increased with increasing concentrations of SDS-treated serum (Fig. 1). A limited range of SDS concentrations (0.05 - 0.25 %) was effective (Fig. 2a). Although boiling was not necessary for the activation of vitronectin adsorption, I always boiled human serum in 0.1 % SDS in the following experiments to ensure the SDS treatment.

2) Cell-Spreading activity on the Adsorbed Plates.

The polystyrene plates incubated with human serum were examined for cell-spreading activity using BHK cells. Interestingly, BHK cells spread on the plates coated with SDS-treated serum. The effective range of SDS concentrations for cell spreading (Fig. 2b) was in good agreement with that for vitronectin adsorption (Fig. 2a). Human serum treated with more than 0.5 % SDS induced neither vitronectin adsorption nor cell spreading. When 4 % serum treated with 0.5 % SDS was serially diluted, both cell spreading and vitronectin adsorption occurred at dilutions of an 1 to 32 - 64, corresponding to 0.016 - 0.008 % SDS (data not shown). Therefore, the lack of cell-spreading activity in more than 0.5 % SDS was mainly due to the lack of vitronectin adsorption, but not to denaturation of its binding activity to cells. Percentage of cell spreading increased with increasing concentrations of SDS-treated serum and saturated with around 3 % serum (Fig. 3). These results suggest that cell-spreading activity adsorbed onto the plates from SDS-treated serum was attributed to vitronectin.

Without treatment with SDS, serum also made the polystyrene plates active for cell spreading. The maximal activity of cell spreading was observed at lower concentrations of native serum ranging from 0.1 to 0.5 % (Fig. 3), which were in good agreement with the profile of fibronectin adsorption reported (Grinnell & Feld, 1982). Therefore, this activity could be probably attributed to at

least fibronectin in the serum.

Morphology of BHK cells spread on the coated plates was shown in Figure 4. Cell spreading was promoted by native 0.5 % serum (Fig. 4a) but not by native 4 % serum (Fig. 4b), and inversely by SDS-treated 4 % serum (Fig. 4d) but not by SDS-treated 0.5 % serum (Fig. 4c). Cell attachment was sparse only in a well coated with native 4 % serum (Fig. 4b). Fibronectin (Fig. 4e) promoted wider lateral expansion of the peripheral cytoplasm of spread cells than vitronectin did (Fig. 4f), as is found very recently (Yatohgo et al., 1988a). Native 0.5 % serum (Fig. 4a) promoted slightly wider lateral expansion of the peripheral cytoplasm of spread cells than SDS-treated 4 % serum did (Fig. 4d). The latter shapes resembled ones obtained by pure vitronectin (Fig. 4f), and native 0.5 % serum (Fig. 4a) took shapes between that of the pure fibronectin (Fig. 4e) and that of the pure vitronectin (Fig. 4f). The morphology of spread cells suggests that vitronectin dominated the cell spreading in SDS-treated 4 % serum. The cell-spreading activity induced by native 0.5 % serum can not be attributed to only vitronectin.

3) Fibronectin and Vitronectin in the Adsorbed Cell-Spreading Activity.

Cell-spreading activity on the polystyrene plates coated with human serum was examined more specifically with respect to fibronectin and vitronectin which are major cell-spreading glycoproteins in human serum. Both anti-

fibronectin antibody and anti-vitronectin antibody interfered with cell spreading induced by native 0.5 % serum (Fig. 5a). Cell spreading induced by SDS-treated 4 % serum was also interfered by anti-vitronectin antibody but not by anti-fibronectin antibody (Fig. 5b).

Action of fibronectin and vitronectin in the native and SDS-treated sera were also examined using whole serum, fibronectin-depleted serum, and vitronectin-depleted serum. When treated with SDS, all types of 0.5 % sera and vitronectin-depleted 4 % serum did not promote cell spreading, while fibronectin-depleted 4 % serum promoted cell spreading in a similar degree to that with whole 4 % serum (Table 1). These results indicate that the cell-spreading activity in SDS-treated sera attributed to only vitronectin. This result was consistent with the result that cell spreading induced by SDS-treated 4 % serum was interfered by anti-vitronectin antibody but not by anti-fibronectin antibody (Fig. 5b).

Without treatment with SDS, on the other hand, all types of 4 % sera did not promote cell spreading substantially (Table 1). This result suggests that neither fibronectin nor vitronectin could sufficiently adsorb to the plates probably due to the abundant presence of serum albumin. Lower concentrations of sera promoted cell spreading. The percentage of cell spreading induced by fibronectin-depleted 0.5 % serum and vitronectin-depleted 0.5 % serum was approximately 1/3 and 1/5 of that induced by

whole 0.5 % serum (Table 1). This result suggests that fibronectin and vitronectin were additively active each other for cell spreading in native serum, as was consistent with the result of antibody interference (Fig. 5a).

4) Adsorption and Cell-spreading Activity of SDS-treated Pure Vitronectin.

Pure vitronectin after boiling with 0.1 % SDS was adsorbed onto polystyrene plates as native pure vitronectin was (Fig. 6). Adsorption of pure vitronectin onto the polystyrene plates was interfered by the presence of bovine serum albumin. The interference was much greater for native pure vitronectin than for SDS-treated pure vitronectin (Fig. 6).

I examined whether SDS-treated pure vitronectin adsorbed onto plates retained cell-spreading activity. Unexpectedly, the cell-spreading activity of SDS-treated pure vitronectin remarkably decreased (Table 2). I supposed that serum protein influenced the cell-spreading activity of vitronectin. Then, bovine serum albumin was added to pure vitronectin before boiling with SDS. The percentage of cell spreading increased up to 47.0 %, though the relative amount of adsorbed vitronectin decreased from 2.11 to 1.50 (Table 2). In addition, a similar phenomenon was observed, when bovine serum albumin was added to SDS-treated vitronectin before plate incubation (Table 2). These results suggest that the effect of bovine serum albumin was activation of the cell-spreading activity of pure vitronectin rather than

protection of that from SDS treatment.

4. Discussion

The adsorption of vitronectin in human serum to the polystyrene plates intended for tissue culture and ELISA. From these experiments, I have found that 0.1 % SDS remarkably activated the adsorption of vitronectin in human serum to the plates. My novel developed method provides an efficient coating of vitronectin to the polystyrene plates without purification of vitronectin.

The adsorbed vitronectin in human serum was active for cell spreading and a trace amount of SDS possibly adsorbed was not toxic. However, SDS-treated pure vitronectin did not retain the cell-spreading activity, and recovered the activity when bovine serum albumin was added to pure vitronectin before or after boiling with 0.1 % SDS (Table 2). These results suggest that resistance of cell-spreading activity of vitronectin for boiling in 0.1 % SDS was not due to an intrinsic property of vitronectin, but depended on a support by serum protein.

In morphology of spread cells, fibronectin induces larger lateral expansion of peripheral cytoplasm of spread cells than vitronectin (Yatohgo et al., 1988a). Some, but not all, reported that the RGD peptides-coated substrate induces poorer cell spreading in several aspects including the area of spread cells, number and size of focal contacts, and development of actin cables (Izzard et al., 1986; Woods

et al., 1986; Singer et al., 1987; Streeter & Rees, 1987; Obara et al., 1988). They suggest that a second site, heparin-binding domain of fibronectin, is required for full spreading of cells. Although vitronectin exposed its heparin-binding site, area of cells spread by vitronectin is similar to that by the RGD peptides rather than by fibronectin (Yatohgo et al., 1988a). Thus, heparin-binding activity does not simply support full spreading of cells.

There is yet an unsolved problem. Although vitronectin was adsorbed more in native 4 % serum than in 0.5 % serum (Fig. 1), the 4 % serum did not promote cell spreading whereas the 0.5 % serum did (Fig. 3). There are two possibilities. First, I used tissue culture plates which have high efficiency of cell attachment. With native 4 % serum, the cell-attachment activity was strongly inhibited by the serum protein (Fig. 4b). The cell-spreading activity, as well as cell-attachment activity, might have been inhibited by the abundant presence of serum protein. Second, in native 0.5 % serum, both fibronectin and vitronectin worked in the cell-spreading activity (Fig. 5a and Table 1). Grinnell and Feld have reported that the amount of fibronectin adsorption onto plates are maximized at 0.1 % serum, and that it is then decreased with increasing serum concentration (Grinnell & Feld, 1982). The decrease of fibronectin adsorption might be contributed to the decrease of cell-spreading activity in native 4 % serum. The problem will be elucidated further in the near future.

The anti-vitronectin antibody used in this study reacts

with vitronectin dependent on its conformation (Akama et al., 1986). Therefore, adsorbed vitronectin assayed in this study should be interpreted as relative values. To clarify more quantitatively, I plan to use purified and radiolabelled preparations of vitronectin and fibronectin. In the purified preparations, conformational change of vitronectin will be also measured after a coupling of fluorescent dyes to vitronectin as applied to fibronectin in the second chapter.

Barnes et al. (Barnes et al., 1980) have reported that vitronectin supports cell growth in a variety of cell lines in serum-free medium. In an application for the serum-free cell culture, treatment of serum with SDS will provide an instant and specific coating of tissue culture polystyrene plates with vitronectin. Although bovine and porcine sera instead of human serum have not examined yet, they appear to contain similar concentrations of vitronectin (Hayman et al., 1985; Yatohgo et al., 1988b). Therefore, coating the plates with bovine or porcine sera in the presence of 0.1 % SDS is economically suitable for the serum-free cell culture. I also have used neither 10 % serum nor fetal bovine serum yet. The same conditions as for routine cell culture should be studied in the near future with respect to examination on the adsorption of fibronectin and vitronectin as well as on the morphology of spread cells.

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6. Tables

Table 1. Cell-spreading Activity of Fibronectin- and Vitronectin-depleted Sera

Concentrations of serum	Treatment with SDS	Cell spreading (%)		
		Whole serum	Fibronectin -depleted serum	Vitronectin -depleted serum
0.5 %	untreated	58.3	20.9	11.5
	treated	0.0	0.0	0.0
4 %	untreated	1.6	0.0	5.4
	treated	44.7	36.9	0.0

Fibronectin concentrations in whole, fibronectin-depleted, and vitronectin-depleted sera were 6.3 μg/mg, 0.024 μg/mg, and 6.3 μg/mg of total protein, respectively. Vitronectin concentrations in whole, fibronectin-depleted, and vitronectin-depleted sera were 4.1 μg/mg, 3.8 μg/mg, and 0.29 μg/mg of total protein, respectively. Concentration of sera was normalized by the protein concentration of 64 mg/ml as 100 %. Treatment of sera with SDS was performed with boiling in 0.1 % SDS for 5 min. Cell-spreading activity was examined as described in the legend of Figure 2.

Table 2. Effect of Bovine Serum Albumin on Cell-spreading Activity of Vitronectin

Coating condition	Cell spreading (%)	Adsorbed vitronectin
bovine serum albumin (BSA)	1.5	-
vitronectin	49.4	1.99
vitronectin→boiling with SDS	3.3	2.11
vitronectin+BSA→boiling with SDS	47.0	1.50
vitronectin→boiling with SDS→+BSA	47.2	1.44

To examine the effect of bovine serum albumin on the cell-spreading activity of pure vitronectin, the albumin was added to SDS-treated pure vitronectin (8 μg/ml) before or after boiling with 0.1 % SDS. The concentration of the albumin was 0.25 %. Polystyrene plates were incubated with above samples. Amount of adsorbed vitronectin and its cell-spreading activity were examined as described in the legend of Figure 1 and Figure 2, respectively.

7. Figures and Legends

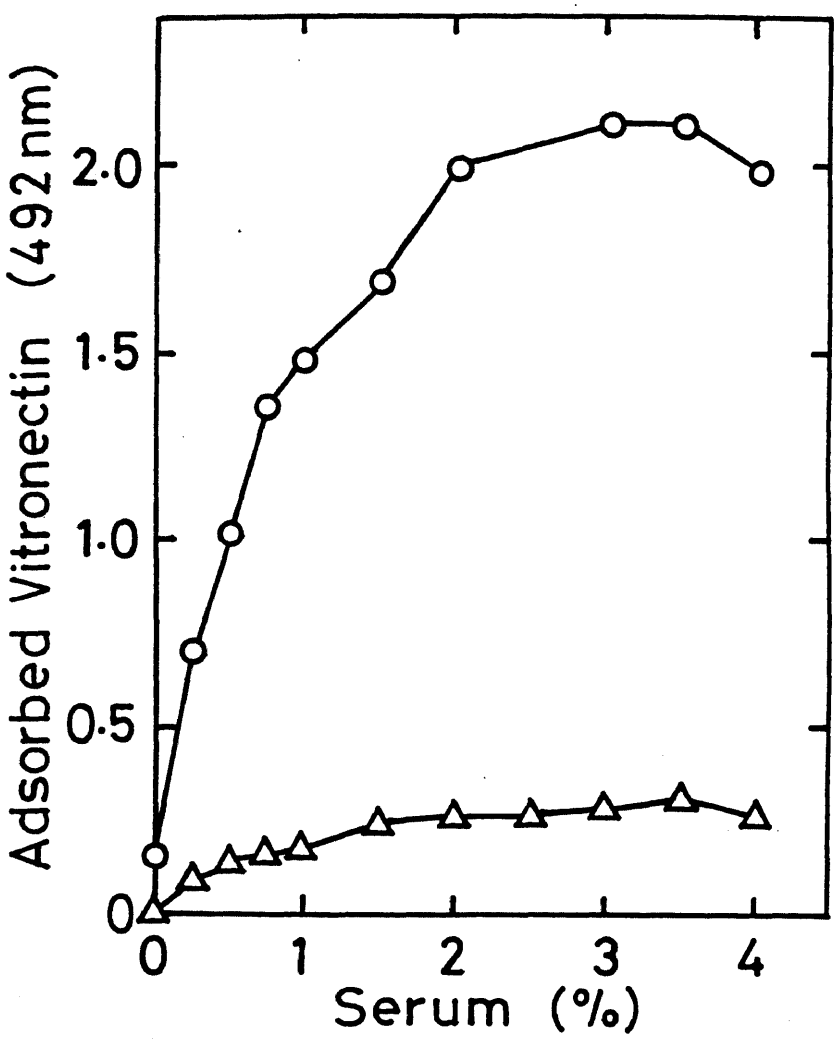
Adsorbed Vitronectin (492nm)
2·0
1·5
1·0
0·5
0
0
1
2
3
4
Serum (%)

Figure 1. Adsorption of SDS-treated vitronectin in human serum. Human serum was diluted to 0 - 4 % in PBS containing 0.1 % SDS and boiled for 5 min. An ELISA 96-well plate was incubated with 50 μl of SDS-treated serum and native serum at room temperature for 1 h. The plate was rinsed with PBS and blocked with 1 % bovine serum albumin in PBS at room temperature for 1 h. After rinsing with PBS again, the plate was incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-vitronectin antibody at an 1 to 2000 dilution in 1 % bovine serum albumin. After rinsing with PBS, an orange color was generated with the incubation of 100 μl of 0.4 mg/ml o-phenylenediamine, 2.5 mM H_2O_2, 0.1 M citric acid, and 0.2 M Na_2HPO_4 for 10 min at room temperature. The adsorbed vitronectin on the plate was expressed as the absorbance at 492 nm for the orange color. SDS-treated serum; ○, and native serum; △.

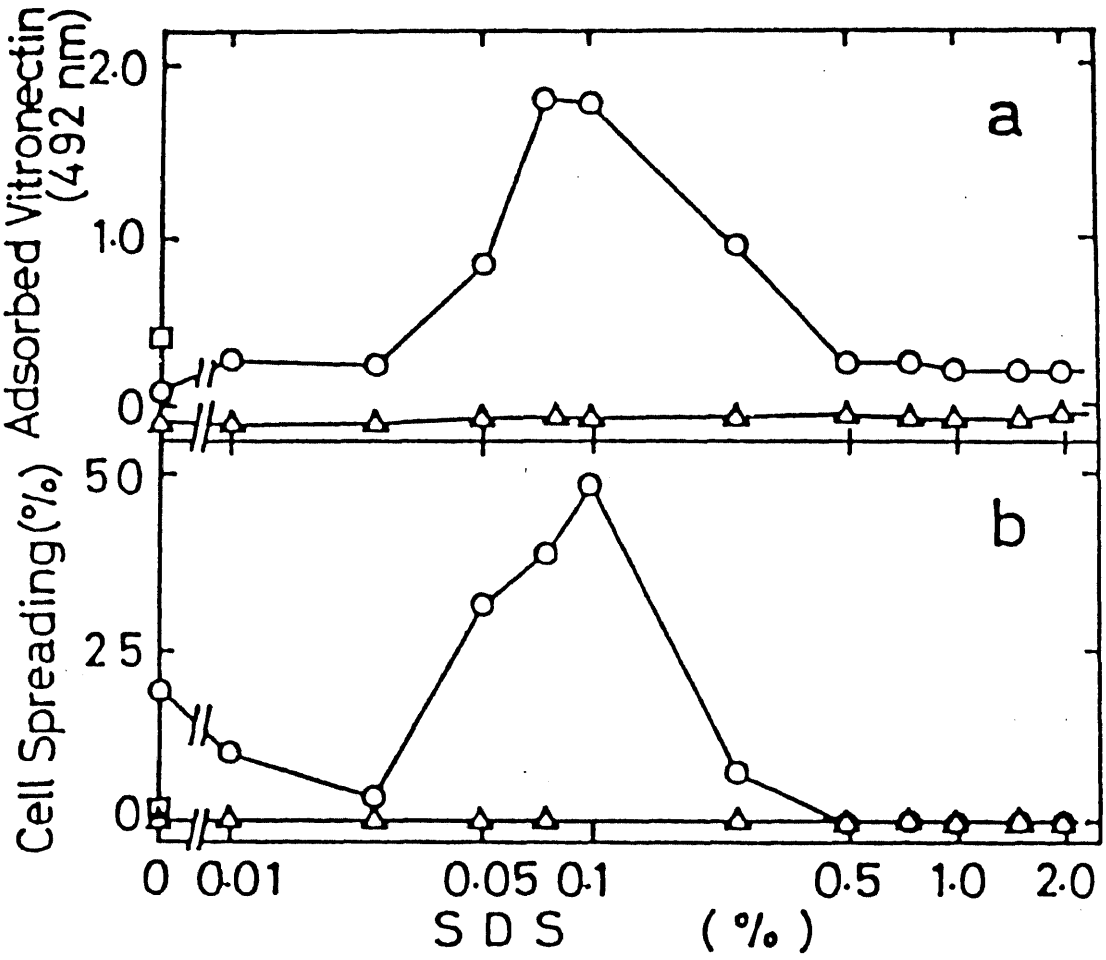
Adsorbed Vitronectin (492 nm)
2·0
1·0
0
a
50
25
0
b
Cell Spreading (%)
0
0·01
0·05
0·1
0·5
1·0
2·0
S D S (%)

Figure 2. Effective concentrations of SDS for adsorption of vitronectin and cell-spreading activity in human serum. Four % human serum was boiled in concentrations from 0 to 2 % SDS for 5 min. (a) Adsorption of vitronectin was examined as described in the legend of Figure 1. (b) Cell-spreading activity of the same sample was examined. A 96-well tissue culture plate was incubated with 50 μl of the above SDS-treated 4 % serum at room temperature for 1 h. The plate was rinsed with PBS and incubated with 100 μl solution containing 2 x 10^4 BHK cells at 37 °C for 1.5 h. The percentage of cell spreading was quantitated microscopically. SDS-treated 4 % serum; ○, SDS-treated 0.25 % bovine serum albumin as controls; △, and non-treated 4 % serum; □.

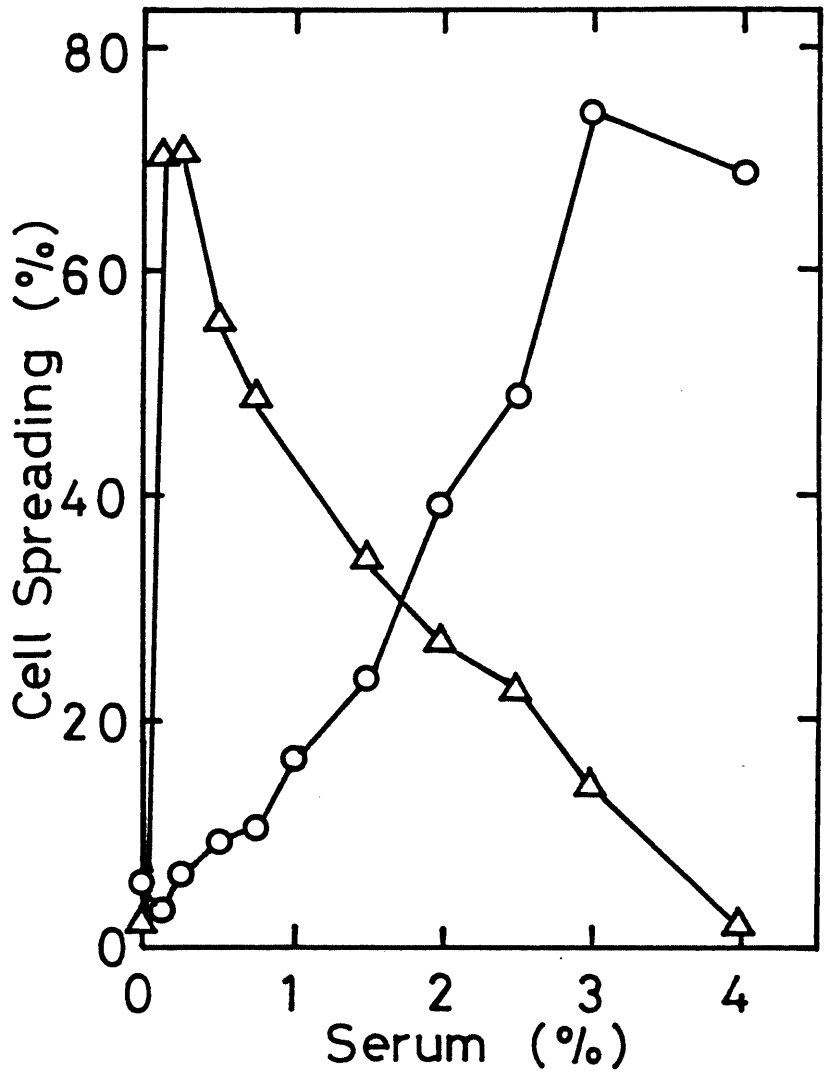
80
60
40
20
0
Cell Spreading (%)
0
1
2
3
4
Serum (%)

Figure 3. Relationship between serum concentrations and cell-spreading activities. Cell-spreading activity in human serum with or without 0.1 % SDS treatment was examined as described in the legend of Figure 2. SDS-treated serum; ○, and native serum; △.

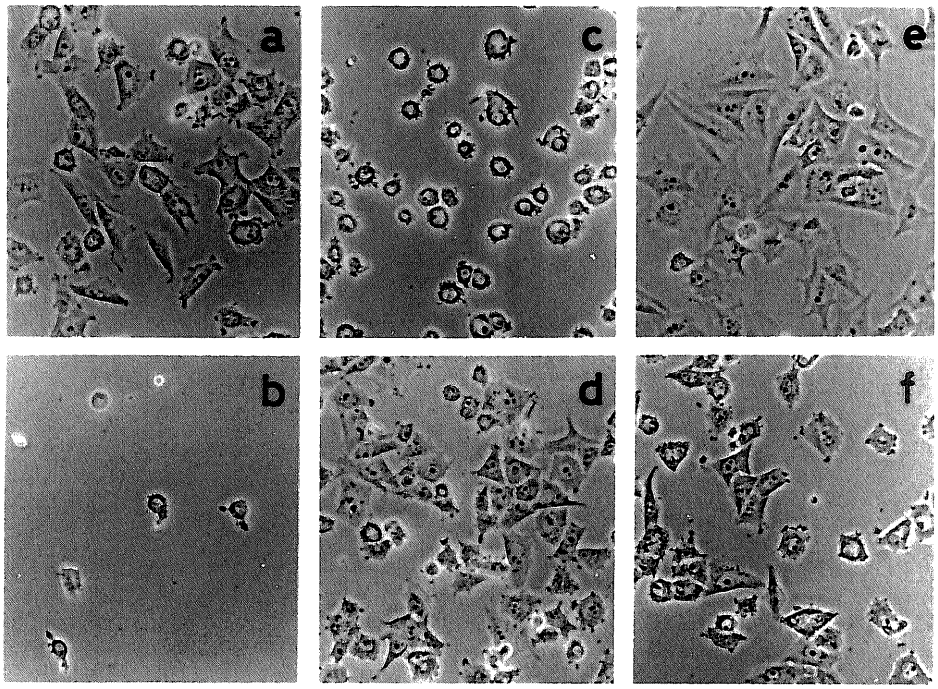
a
c
e
b
d
f

Figure 4. Morphology of spread BHK cells induced by SDS-treated serum, non-treated serum, fibronectin, or vitronectin. Photographs (a - d) correspond to the result in Figure 3. a; native 0.5 % serum, b; native 4 % serum, c; SDS-treated 0.5 % serum, d; SDS-treated 4 % serum, e; 8 μg/ml fibronectin, and f; 8 μg/ml vitronectin.

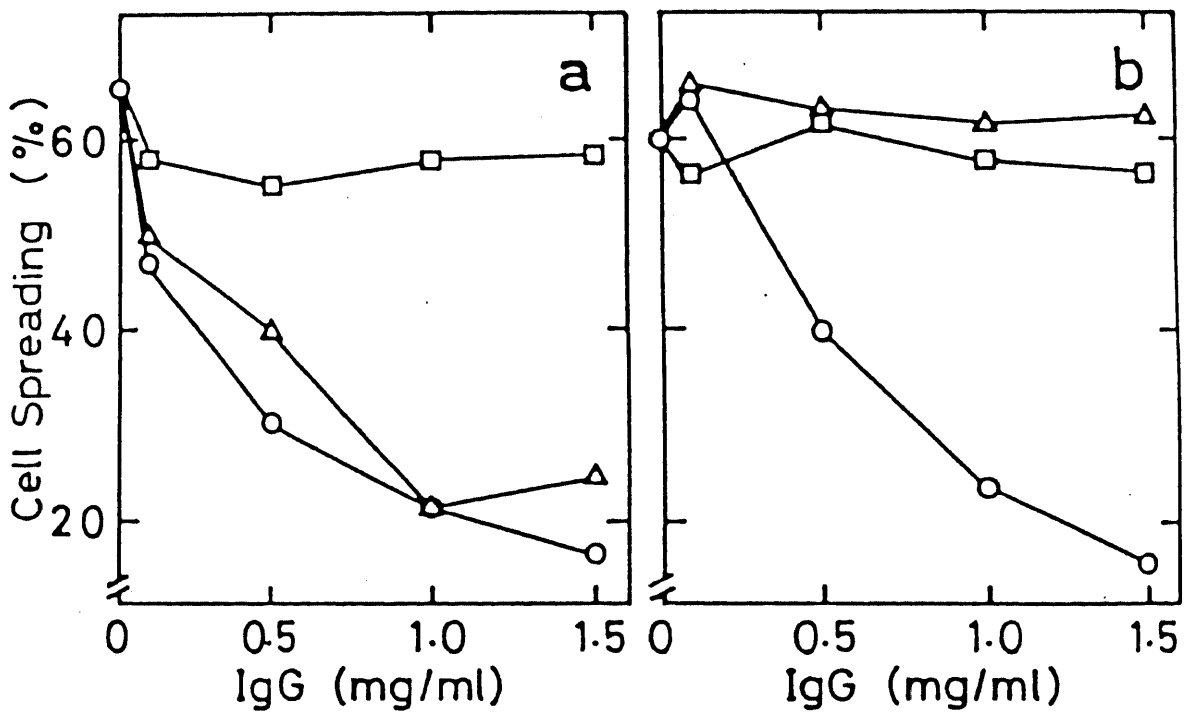
a
b
Cell Spreading (%)
60
40
20
0
0.5
1.0
1.5
IgG (mg/ml)
0
0.5
1.0
1.5
IgG (mg/ml)

Figure 5. Inhibition of cell-spreading by antibodies. Cell-spreading activity was examined as described in the legend of Figure 2, except for the treatment with antibodies as follows. A serum-coated 96-well tissue culture plate was incubated with 50 μl of 0 - 1.5 mg/ml anti-vitronectin antibody, anti-fibronectin antibody, or normal IgG in 0.1 % bovine serum albumin at 37 °C for 3 h before receiving BHK cells. (a) The plate was coated with native 0.5 % serum. (b) The plate was coated with SDS-treated 4 % serum. Anti-vitronectin antibody; ○, anti-fibronectin antibody; △, and normal IgG; □.

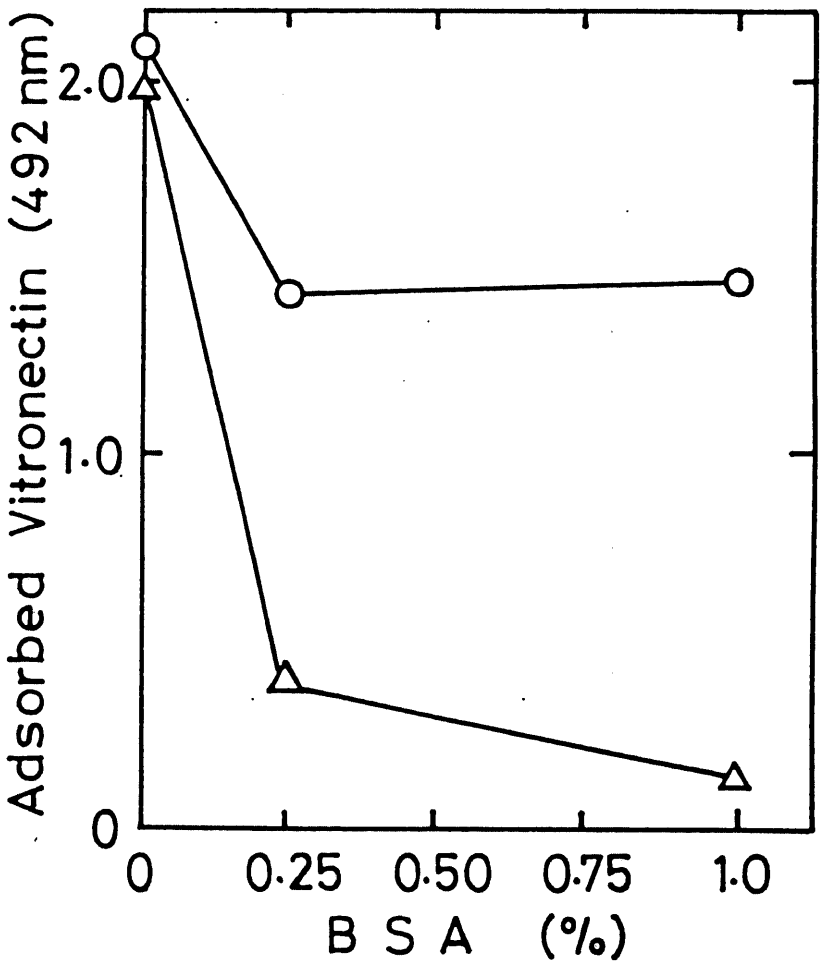
Adsorbed Vitronectin (492 nm)
2·0
1·0
0
0
0·25
0·50
0·75
1·0
B S A (%)

Figure 6. Adsorption of pure vitronectin in the presence of bovine serum albumin. Adsorption of 8 μg/ml native pure vitronectin and 8 μg/ml SDS-treated pure vitronectin in the presence of indicated concentration of bovine serum albumin was examined as described in the legend of Figure 1. SDS-treated pure vitronectin; ○ , native pure vitronectin; △.

IV. GENERAL DISCUSSION AND CONCLUSION

Conformational changes of fibronectin corresponding to modification of adhesion was quantitatively measured. Binding activity of the cell-binding domain is enhanced by adsorption of fibronectin onto collagen (gelatin) or heparin (Johansson & Hook, 1984). Conformational change of the cell-binding domain corresponding to the enhancement was analyzed by the method of fluorescence polarization. The rotatory relaxation time of the whole, the domain, and the group can be detected with three kinds of fluorescent probe with different lifetime. The result indicated that the promotion of the intramolecular rotation of the cell-binding domain corresponds to the enhancement of the binding activity.

When the conformation of vitronectin was changed by treatment of sodium dodecyl sulfate (SDS), which is known to expose the inner hydrophobic groups, adsorption of vitronectin onto hydrophobic polystyrene augmented. Since anti-vitronectin antibody did not lose the reactivity in SDS-treated vitronectin, augmentation of adsorption of SDS-treated vitronectin was quantitatively measured by absorbance from enzyme-linked immunosorbent assay (ELISA). Cell-spreading activity on polystyrene plate mediated by vitronectin was quantitatively measured by the percentage of cell spreading. The percentage of cell spreading decreased by treatment of vitronectin with SDS. Therefore, activity of adsorption augmented, and on the other hand, activity of cell binding decreased. These results show that modification of adhesion can be represented by the

quantitative changes of both cell-binding activity and adsorption activity. From the above, a method which quantitatively corresponds modification of adhesion to the change of intramolecular rotation of the adhesive part was established.

Mechanism of modification in adhesion involves the characteristics of the site in itself as well as the interrelation between the site and the substance which will bind to it. In this study, treatment of SDS led vitronectin to remarkable adsorption onto hydrophobic polystyrene. The cell-binding activity by treatment with SDS is inclined to decrease due to prevention of cell binding by exposure of hydrophobic groups around the cell-binding site (Arg-Gly-Asp). An interrelation arises from the translational movement. However, in room temperature, the velocity of the whole molecule becomes low, because the molecular weight of the adhesive complex is remarkably increased by adsorption of fibronectin onto collagen or heparin. For this reason, the frequency of collision between fibronectin-collagen or fibronectin-heparin complex and cell remarkably decreases. Experimentally, cell-binding activity is in fact enhanced (Johansson & Höök, 1984). This fact indicates that changes of interrelation without translation of adhesive complex is necessary for the enhancement of binding activity. The increase of local rotation or deformation is in agreement with the results of degree of fluorescence depolarization.

The establishment of the method which quantitatively

corresponds modification of adhesion to the change of intramolecular rotation in the adhesive part, leads to the following significance in morphogenesis. Without any change in the composition of the system, morphogenesis may be effected by the spreading activity influenced by modification in adhesion which is caused as a result of a "switching on" of fibronectin or vitronectin. The method established in this thesis gives an effective clue to elucidate the mechanism of morphogenesis.

Modification of adhesion caused by artificial conformational changes can be applied to practical use. In this study, treatment of serum with SDS provided an easy and efficient coating of vitronectin to tissue culture plate (polystyrene). It has been reported that a variety of cell lines can be cultured in serum-free medium by coating of vitronectin to plate (Barnes et al., 1980). Therefore, the fruit in this thesis greatly contributes to the development of a serum-free cell culture. Thus, artificial modification of adhesion possesses high value of application.

From the fruit in this thesis, the method which quantitatively corresponds the modification of adhesion to change of intramolecular rotation in the adhesive part was established. The modification of adhesion was represented by the percentage of cell spreading and the absorbance from ELISA. The change of intramolecular rotation in the adhesive part compared with rotation of the whole was represented by the terms of fluorescence depolarization. The knowledge acquired in this study, concerning not only

the affinity of the adhesion site, but also change of interrelation between the site and substrate brings about better understanding on the modification of adhesion. This established method gives a clue to elucidate the mechanism of morphogenesis without any change in the composition of the system, and it should be helpful in the application of cell technology.

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