Effect of Preadsorbed Proteins on Cell Adhesion to Polymer Surfaces

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Adsorption of three different proteins and adhesion of cells onto various substrates in the presence of serum proteins were studied. Both the maximal protein adsorption and the maximal cell adhesion were observed on surfaces with water contact angle around 70°. Preadsorption of serum albumin prevented cell adhesion to all the substrates, whereas preadsorbed fibronectin (FN) enhanced cell adhesion to all the substrates, independent of their water wettability, except for poly(vinyl alcohol) and acrylamide-grafted films. Competitive adsorption of FN from mixed proteins, ranging from 0.03 to 0.07 µg/cm², markedly influenced cell adhesion in the presence of serum. These results suggest that the effect of the water wettability of surfaces on cell adhesion in the presence of serum should occur through protein layers adsorbed directly to the substrate surfaces.

INTRODUCTION

The biocompatibility of artificial organs and surgical implants is intimately related to cellular interactions with biomaterials used in the construction of medical devices. Therefore, a deep understanding of cellular interactions with biomaterial surfaces is crucial for the successful development of biomaterials. Indeed, a large number of studies on cell adhesion to various substrate surfaces have been reported (1–7). It has been found that cell adhesion strongly depends on the substrate chemistry; for example, on the water wettability and the surface charge (8–15).

In a previous paper (16), we found that cell adhesion onto different polymeric substrates was governed by the surface wettability of the polymeric substrates as well as by their ionic properties. Maximal cell adhesion occurred on surfaces having moderate water wettability, and adhesion decreased both on surfaces with higher and on those with lower water wettabilities. In the study, a serum-free system was used in order to avoid the effect of proteins on the cell adhesion to the substrate, because, as is well known, plasma proteins instantly adsorb on the surface when the substrate comes in contact with serum. However, such protein adsorption cannot be prevented when materials are in contact with tissue and blood. Moreover, it has been shown that adsorbed proteins, such as fibronectin, laminin, and vitronectin, play an important role in the subsequent cell adhesion (17–29).

Thus, it is important to study cellular interactions with biomaterials in association with protein adsorption.

Since protein adsorption from serum or plasma involving many different proteins at different concentrations is a complex phenomenon that is difficult to control, we adopt an approach in which substrates preadsorb one, two, or three proteins competitively. We selected three proteins, albumin, γ-globulin, and fibronectin, for this work. Albumin and γ-globulin have the highest concentrations in serum, whereas fibronectin is an important protein for cell adhesion to substrates in spite of its quite low concentration in serum (18).

In a living body, there are many different cells, such as fibroblasts, platelets, and white blood cells. For the cell adhesion test in this study, we use the L cell because it is a very simple and stable cell line whose nature is well known and it has been used for cell adhesion tests by many researchers.

As mentioned in a previous paper (16), the water wettability of the surface is one of the most important factors when a biomaterial is designed. Many investigators have discussed the relationship between water wettability and protein adsorption, or between water wettability and cell adhesion. But there are few reports that have studied protein adsorption in connection with cell adhesion. The present study describes the effect of protein preadsorption on cell adhesion to substrates of different water wettabilities.

EXPERIMENTAL

Materials

Commercial films listed in Table 1 were used as polymeric substrates for cell adhesion. A commercial cover glass for optical microscopic observation was utilized as the glass. All the substrates were purified by Soxhlet extraction with methanol for 12 h. Grafted films were prepared by graft polymerization of acrylamide (AAm) onto a high density polyethylene (PE) film using a corona-pretreatment technique.

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as described previously (16). To effect the coupling of collagen (atelocollagen) and plasma fibronectin onto the surface of the PE film, acrylic acid was first graft-polymerized to the film surface using the corona-pretreatment technique to introduce carboxyl groups onto the surface. The carboxyl groups introduced were reacted with a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and then the film was put in a solution of each protein for covalent immobilization. After immobilization, the modified films were thoroughly washed in phosphate buffered saline (PBS) to remove unimmobilized proteins. The surface density of the immobilized protein, measured by the radioisotope labeling method, is about 3.0 \( \times 10^6 \) Al for collagen, and 1.0 \( \times 10^6 \) Al for fibronectin.

**Contact Angle Measurement**

Water contact angles were measured at 25°C for dried films and glass with the sessile drop method. Redistilled water (10 \( \mu l \)) was gently placed on the sample films kept in a desiccator after purification. After 30 s, the contact angle was read directly using a goniometer. At least five readings on different parts were averaged. The results are given in Table 1.

**Protein Adsorption**

Bovine serum albumin (Alb, crystallized) purchased from Miles Laboratories Inc., bovine \( \gamma \)-globulin (IgG) purchased from Sigma, and plasma fibronectin (FN) isolated in our laboratory from frozen human plasma by affinity chromatography with gelatin–Sepharose (30) were used in the adsorption experiment. Their purities were checked by SDS-polyacrylamide gel electrophoresis (31). Labeling of proteins with \( ^{125}I \) (New England Nuclear, 100 mCi/ml) was performed using the chloramine-T method (32), and uncorporated iodide was removed by gel filtration on a G-25 Sephadex column (Pharmacia), preequilibrated with a buffer solution containing 2.5 mg/ml of respective protein, to reduce nonspecific adsorption of the radiolabeled protein to the column matrix. The eluted protein fraction was diluted with the “cold” protein to a concentration of 5 to 50 mg/ml, stored at 4°C, and used within 10 days after iodination.

Protein adsorption was carried out in 0.01 \( M \) PBS of pH 7.4, as follows. Films of each polymer \((1 \times 1 \ cm^2)\) were immersed in PBS and then the solution was degassed by vacuum pump until bubbles stopped appearing on the film’s surface. The stock protein solution was added to the above solution until a given concentration of protein was reached \((3 \ mg/ml \ for \ Alb \ and \ 0.3 \ mg/ml \ for \ FN)\). Three to four pieces of the polymer film were immersed in a test tube containing the protein solution and allowed to stand in a water bath kept at 37°C for 3 h for protein adsorption. At the end of incubation, the protein solution was exchanged with a PBS solution containing no protein, the films being always kept in solution in order to prevent the films from being in direct contact with the interface between air and the protein solution. After the absence of radioactivity in the solution was confirmed, one piece of film was taken out of the solution with forceps and rinsed with PBS solution while waving 30 times at a constant speed. Then, the radioactivity on the film was measured with a \( \gamma \)-ray scintillation counter to determine the amount of protein adsorbed. In the case of competitive adsorption, solutions containing two or three mixed proteins, one radiolabeled and the other(s) unlabeled, were used in place of the single protein solution. The competitive protein adsorption was performed in a fashion similar to that described above for single protein adsorption.

**Cell Culture**

L cell, an established cell line of mice fibroblasts, was employed for the cell attachment study. Cultures were maintained in a 37°C water-jacketed incubator equilibrated with 5% \( CO_2 \) and kept at approximately 99% relative humidity. The cells were incubated in Eagle’s MEM supplemented with 10 v/v% fetal calf serum (FCS, M. A. Bioproducts, Maryland, USA) and 60 mg/liter kanamycin on a 250-ml plastic culture flask (Nunc, Denmark). The subculturing of cells was conducted at 5- to 7-day intervals with trypsin–EDTA (Gibco).

**Cell Adhesion**

Each of the polymer films or the glass plate was placed in a well of a Nunc multidish (24 wells, 15 mm diameter) and then PBS solution was added to each well, followed by preincubation for 2 to 3 h at room temperature. Finally, the wells were given the cell suspensions prepared as follows. The cultured L cells were trypsinized from the culture flasks, washed once in the medium with 10% FCS and once in the medium...
without serum, and then collected by centrifugation at 1000 rpm for 5 min. The pellet containing the cells was suspended in the medium without serum and the cell density was adjusted to 1.76 × 10^5 cells/ml with serumless medium.

In the case of cell adhesion onto protein-preadsorbed surfaces, single or mixed protein solutions were first added to each well and incubated for 3 h at 37°C. The protein concentrations used were 1 and 0.1 mg/ml for Alb and FN, respectively, in the case of single protein adsorption, and 40, 0.2, and 12.5 mg/ml for Alb, FN, and IgG, respectively, in the case of competitive adsorption. After that, the protein solutions were removed by aspiration without exposing the films to air, and PBS solution was added twice for rinsing. Immediately after aspiration of the PBS solution, 1 ml of cell suspension was applied to each well and incubated for 60 min at 37°C. After incubation, the films were removed from the wells with forceps and dip-rinsed twice in each of three different beakers containing PBS in order to remove the unattached cells and then put in a test tube for counting the number of cells attached. Two milliliters of 0.5 wt/wt% Triton-X100 in PBS solution was added to each test tube in order to dissolve the cells attached to the surfaces, and then the lactate–dehydrogenase (LDH) activity in each test tube was measured to determine the cell number using the method described in a previous paper (16).

RESULTS

Cell Adhesion to Substrates in Medium Containing Serum

L-cell adhesion to various substrates in the culture medium containing 10% serum is shown in Fig. 1. The surface property employed here is the water contact angle, which is directly related to the water wettability of the substrates. A larger contact angle means that the substrate is less wettable. As is seen, L cells preferentially adhere to the moderately wettable substrates having 60° to 80° water contact angles.

Cell adhesion takes place to a lesser extent if a substrate surface becomes either more hydrophilic or more hydrophobic than those having a 60° to 80° water contact angle. This is similar to cell adhesion in a serum-free medium as described in a previous paper (16). These results suggest that cell adhesion is independent of proteins present in the serum. Figure 1 gives also the result of cell adhesion in a serum-free medium onto surfaces preexposed to serum. Obviously, the result is similar to cell adhesion in the presence of serum.

Adsorption of Serum Proteins to Various Surfaces

Adsorption of Alb and FN in PBS was studied using various substrates and the results are shown in Fig. 2. It is apparent that protein adsorption preferentially occurs on surfaces with water contact angles ranging from 60° to 80° for the two proteins, quite similar to the cell adhesion results. The difference in protein adsorption between Alb and FN may be caused by the differences in molecular weights and shapes between the two proteins (33).

Cell Adhesion in Serum-Free Medium onto Surfaces Preadsorbed with Serum Proteins

Adhesion of L cells to substrate surfaces was measured after exposure to solutions containing different serum proteins for 60 min. In order to prevent an exchange of the adsorbed proteins with the proteins in the culture medium, L cells were seeded in the serum-free medium. The results are given in Fig. 3 for preadsorption with Alb and FN. It is interesting to note that Alb preadsorption entirely inhibited L cell adsorption, independent of the water wettability of the substrate surfaces. IgG preadsorption gives a result similar to that for Alb (data not shown). On the contrary, when FN was preadsorbed on the surfaces, L cells could adhere to all the surfaces except for PVA and the AAm-grafted PE, independent of their wettability. The number of adherent cells is 8 × 10^4 cells/cm^2, which virtually corresponds to the highest values observed in Fig. 1. On the other hand, PVA and the AAm-grafted PE did not cause any cell adhesion even when FN was precoated, because the FN adsorption was minimal on PVA, as shown in Fig. 2. The surface density FN adsorbed onto cellulose was as small as 0.7 μg/cm^2, but the cell adhesion was promoted by preadsorption of FN. This finding indicates that FN preadsorption of 0.7 μg/cm^2 is sufficient for promoting cell adhesion. A similar result was reported by Hughes et al. (34).

Competitive Adsorption of Serum Proteins onto Various Surfaces

It seems probable that cell adhesion in the presence of serum would occur following the competitive adsorption of
serum proteins onto the surfaces. To get a deeper insight into this competitive adsorption, protein adsorption was studied using mixed protein solutions of Alb, IgG, and FN, the concentration ratio of each protein being kept almost equal to that in serum. Alb and IgG adsorption was not influenced by the presence of other proteins (data not shown). In contrast to Alb and IgG adsorption, FN adsorption was greatly reduced in comparison with that from the single fibronectin solution, but this was not accompanied by any significant change of the contact angle–adsorption relation.

The result of competitive adsorption of FN from an Alb–IgG–FN mixed solution with the same concentration ratio as serum is shown in Fig. 4. It is apparent that the relationship between the FN adsorption and the contact angle of the substrates is similar to that in Fig. 2 except for glass and the collagen-immobilized surface, although the amount of FN adsorbed is roughly 1/25 of that in Fig. 2. This is because Alb and IgG coexisting at high concentrations prevented the FN adsorption as reported before (33).

**Effect of Serum Passing through a Gelatin Column (SPTG)**

To obtain a serum (SPTG) free of components which interact with gelatin (FN and vitronectin, cell adhesion proteins, have been reported to bind gelatin (35)), the normal serum was allowed to pass once or twice through a gelatin–Sepharose column. The substrate surfaces were exposed for 3 h to the serum before and after the column treatment and then cell adhesion was carried out for 1 h in the serum-free culture medium. The number of adherent L cells is plotted against the contact angle of substrates in Fig. 5. A decrease in cell adhesion using SPTG is obvious but the relationship between the cell number and the surface contact angle still remains unchanged. The decreased cell adhesion is undoubtedly due to the preferential adsorption of non-cell-adhesion proteins such as Alb and IgG because only the concentration of other cell adhesion proteins was lowered by the column treatment. The glass, which has a very low water contact angle but an exceptionally high cell-adhesive surface, showed a decrease in cell adhesion at the same rate as other surfaces did, but other highly cell-adhesive surfaces, that is, collagen- and FN-immobilized ones, were differently influenced by this treatment. By preadsorption of SPTG, the collagen-immobilized surface showed a large decrease in cell adhesion, whereas the FN-immobilized surface maintained a high level of cell adhesion.
serum. Without any other proteins. Preadsorption was carried out at 37°C for 3 h. L cells (1.76 x 10^5 cells/ml/well (1.76 cm^2)) were allowed to attach for 60 min at 37°C. Medium was Eagle MEM without serum. (See Table 1 for sample numbers.) (O) serum once subjected to gelatin-Sepharose column, (S) serum twice subjected to gelatin-Sepharose column, and (△) intact serum.

These results indicate that the collagen-immobilized surface needs some cell-adhesive proteins in serum for cell adhesion, but cells can attach to the FN-immobilized surface without any other proteins.

DISCUSSION

As is seen in Fig. 1, the relationship between cell adhesion and water wettability of substrate surfaces observed in the absence of serum is the same as that observed in the presence of serum. Also, as is seen in Fig. 2, the maximal protein adsorption occurred on the surfaces with water contact angles ranging from 60° to 80°. However, there was a remarkable difference in cell adhesion between the substrates with and without preexposure to each protein solution. When the substrates were precoated with Alb or IgG, cell adhesion was inhibited on all surfaces, independent of their water contact angles. All surfaces acquired a high cell affinity upon preadsorption of FN. In addition, the characteristic relationship between cell adhesion and contact angle as mentioned above disappeared. It appears that preadsorption of Alb and IgG makes the substrate surfaces so nonartificial as to prevent cell adhesion, while preadsorption of FN provides the surfaces with ligands for the receptor sites of cells. As FN hardly adsorbs onto PVA and the AAm-grafted surface because of their diffuse structures (36), one cannot observe any cell adhesion on them. We have already derived an equation which explains the maximal protein adsorption onto artificial surfaces with moderate water wettability (37).

When a substrate is in contact with serum containing various proteins with different concentrations, competitive protein adsorption may occur among the proteins. As seen in Fig. 4, the maximal amount of FN adsorbed on surfaces with contact angles of 60° to 80°, but FN adsorption decreased to 0.03 to 0.07 µg/cm². FN adsorption of more than 0.07 µg/cm² results in high cell adhesion and, in the case of less than 0.07 µg/cm², the number of adherent cells is determined by the relative amount of FN on each substrate. As reported by Hughes et al. (34), the minimal amount of FN required for cell adhesion was 0.05 µg/cm² which is in good agreement with our results. Thus, it seems likely that cell adhesion in the presence of serum to substrates with different wettabilities is governed by competitive adsorption of FN to the substrates.

In this study, we selected FN as the cell-adhesive protein, but, as reported recently by many researchers (38, 39), there are other proteins in serum that promote cell adhesion, such as vitronectin. Therefore, it may be necessary to study in more detail the effect of cell-adhesive proteins in cell adhesion on substrates, but their role in cell adhesion is probably similar to that of FN.

REFERENCES