The Role of Cellular Fibronectin in the Interaction of Human Endothelial Cells with Polymers

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Abstract: During in-vitro adhesion, spreading and proliferation of human endothelial cells (HEC) on tissue culture polystyrene (TCPS), cellular fibronectin is deposited onto the surface of TCPS in spite of the fact that relatively large amounts of proteins have been adsorbed from the serum-containing culture medium to this surface. Evidence is presented that serum proteins, adsorbed to the TCPS surface, are displaced by cellular fibronectin. In addition, the interaction of HEC with polyethylene, precoated with monoclonal antibodies directed against HEC membrane antigens and against extracellular matrix compounds, was studied. F(ab')₂ fragments of two monoclonal antibodies were also included in this study. PreadSORption of these antibodies and F(ab')₂ fragments resulted in cell adhesion and spreading as well as moderate cell proliferation (or no proliferation) for several days. A good cell proliferation of HEC was only observed on polyethylene precoated with fibronectin or an antibody directed against fibronectin. The results indicate that the direct or indirect deposition of fibronectin is a prerequisite for the proliferation of HEC. It is suggested that fibronectin, bound to a solid substrate, provides a biochemical signal necessary for the proliferation of HEC.

INTRODUCTION

A systematic study on the interaction of human endothelial cells (HEC) with polymers may lead to the development of vascular grafts, which allow the formation of an endothelial lining on the luminal surface.

HEC, suspended in culture medium containing 20% human serum, adhere and spread well on moderately wettable polymers like tissue culture polystyrene (TCPS).¹ During adhesion and spreading, cellular fibronectin is deposited onto the TCPS surface in spite of the fact that proteins such as albumin, high-density lipoprotein and immunoglobulin G have been adsorbed from the serum-containing culture medium to this surface.^{2–4} These results suggest that the desorption of cellular fibro-

nectin is accompanied by desorption of adsorbed serum proteins. In order to test this hypothesis, the decrease of the amount of albumin, previously adsorbed to TCPS from (solutions) of serum-containing culture medium, was measured after incubation of the pretreated TCPS with a suspension of HEC. The amount of fibronectin, deposited by HEC on the pretreated TCPS, was also determined. The displacement of surface-adsorbed proteins by cellular fibronectin will occur more easily in the case of relatively hydrophilic surfaces compared to hydrophobic surfaces, because proteins are more strongly adsorbed to the latter surfaces.^{5,6} Most probably as a result of this phenomenon, HEC do not adhere, spread and proliferate on hydrophobic polymers like polyethylene and polytetrafluoroethylene (Teflon).^{7,8} However, hydrophobic polymers to which fibronectin has been preadsorbed, give rise to adhesion, spreading and proliferation of HEC.⁴

It has been reported that adhesion and spreading

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of baby-hamster kidney (BHK) cells can be achieved by a substratum-adsorbed antibody, which is directed against BHK cell membranes.⁹ We investigated the interaction of HEC with polyethylene, precoated with monclonal antibodies directed against cell membrane glycoproteins and against extracellular matrix compounds of HEC. In addition, F(ab')₂ fragments of a monoclonal antibody directed against an endothelial cell membrane glycoprotein, and F(ab')₂ fragments of a monoclonal antibody directed against cellular fibronectin, were included in this study. The results with respect to adhesion, spreading and proliferation of HEC on these surfaces were compared with the results of experiments in which polyethylene had been precoated with purified human plasma fibronectin.

MATERIALS AND METHODS

Coating of TCPS with (diluted) serum-containing culture medium

Twenty-four-well clusters of TCPS (Costar Europe) were used for EIA determinations of adsorbed proteins (see below). Forty-eight-well clusters of TCPS were used for experiments in which radio-iodinated human serum albumin was present as tracer protein. For these experiments, ¹³¹I-labeled human serum albumin (Medgenix, Fleurus, Belgium) was added to human serum-containing culture medium to a final concentration of 0.012 mg/ml.

The wells of the clusters were exposed to serum-containing culture medium or solutions of serum-containing culture medium in phosphate-buffered saline (PBS) for 1 h at room temperature. After incubation with (solutions of) serum-containing culture medium, the wells were rinsed three times with PBS.

Determination of the amounts of adsorbed human serum albumin and (cellular) fibronectin

The relative amounts of adsorbed albumin were determined either by means of a two-step enzyme-immunoassay (EIA)^{2,10} or by counting the amount of adsorbed ¹³¹I-labeled human serum albumin.¹¹ The adsorbed amounts of fibronectin were determined with the EIA.^{2,10} In order to make the adsorbed proteins beneath the layer of adherent HEC accessible to the first antibody solution of the EIA, the cells were lysed with 0.1 N ammonia for 10 min,² followed by rinsing three times with PBS.

Table 1. Monoclonal antibodies

Code	Directed Against
CLB-HEC-19	Membrane glycoprotein of HEC
CLB-HEC-347	Membrane glycoprotein of HEC
CLB-10G11	Glycoprotein Ia-IIa
CLB-HEC-65	Glycoprotein IIa* (CD31)
CLB-C17	Glycoprotein IIb-IIIa
CLB-Rag 38	von Willebrand factor
CLB-HEC-FN-140	Human fibronectin
Anti-erythrocyte	Erythrocytes; glycophorin A

Fibronectin purification

The purification of human plasma fibronectin has been described in Ref. 7.

Monoclonal antibodies and F(ab')₂ fragments

All monoclonal antibodies (see Table 1) were prepared and characterized in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. The purification of antibodies has been described in Ref. 7.

F(ab')₂ and Fc fragments were prepared by means of digestion of purified antibodies with Sepharose-immobilized pepsin (Pierce Rockford, Il). F(ab')₂ and Fc fragments were separated by chromatography on protein-A-Sepharose.

Coating of polyethylene with fibronectin, antibodies and F(ab')₂ fragments

Sheets of low-density polyethylene (Talas, Ommen, The Netherlands) were cleaned as described in Ref. 10 and mounted in test devices similar to those shown in Ref. 1 and 10. Thereafter, the sheets were exposed to protein solutions of 0.05 mg/ml at 4 °C for 15-17 h.

Cell adhesion and proliferation

HEC were isolated from human umbilical cord veins according to the method of Willems *et al.*¹² Cells were routinely cultured in TCPS flasks (Costar Europe, Badhoevedorp, The Netherlands) precoated with partially purified fibronectin.¹ Cells were harvested by means of trypsin (0.015%), after which trypsin was inactivated by addition of serum-containing culture medium. Subsequently, the iso-

Fig. 1. Relative amounts of adsorbed human serum albumin (HSA) and fibronectin (FN) on TCPS which had been exposed for 1 h to (a solution of) serum-containing culture medium ((a): 1% serum; (b): 20% serum), followed by incubation for 6 h with serum-free medium either with endothelial cells (200 000 per cm^2) or without cells.

Detection: enzyme-immunoassay. In each experiment the amount of HSA adsorbed to TCPS after incubation with serum-free medium lacking cells was taken as 100%, and the adsorbed amount of FN after incubation with a cell suspension in serum-free medium was taken as 100%. Values are means of four determinations ($\pm$ SD). Unpaired *t* test: **P* > 0.0005 compared to the amount of adsorbed protein after incubation with medium lacking cells.

lated cells were washed three times with culture medium lacking serum and resuspended in serum-free medium. HEC were seeded into the wells of the test devices mentioned above. The cell suspensions used for seeding contained 125 000 cells/ cm^2 and 31 000 cells/ cm^2 for adhesion and pro-

liferation experiments, respectively. Adhesion and proliferation experiments were carried out at 37°C. Cell adhesion was measured after 2 h and cell proliferation was determined after 2, 6 and 9 days. In the proliferation experiments the cells were allowed to adhere for 2 h, after which the serum-free medium was replaced by serum-containing medium.

The number of adherent cells was determined either by counting the nuclei of the cells with a flow cytometer (Technicon, Avelingen, The Netherlands) or by measuring the amount of DNA by means of Hoeschst dye 33258.^{13,14}

In the experiments in which the adsorbed amounts of human serum albumin and fibronectin were determined, HEC (200 000 cells/ cm^2 in serum-free medium) were allowed to adhere to TCPS for 6 h. Control experiments were carried out in the same way, using serum-free medium lacking cells.

RESULTS AND DISCUSSION

In order to investigate whether or not a displacement of adsorbed serum proteins by cellular fibronectin would occur, a HEC suspension in serum-free culture medium was pipetted into TCPS wells which had been pre-exposed to serum-containing culture medium or to solutions of this medium. In simultaneous experiments, pre-exposed TCPS wells were incubated with serum-free medium lacking cells. The relative amount of adsorbed fibronectin and human serum albumin (HSA) present on the TCPS surfaces after incubation with a HEC suspension as well as after incubation with serum-free medium were determined with an enzyme-immunoassay (EIA).² The results of the EIA determinations are shown in the graphs of Fig. 1(a) and (b). These graphs reveal that, irrespective of the serum concentration in the coating solution, the amount of fibronectin on the TCPS surface is much higher if the surface had been incubated with HEC suspension in culture medium instead of culture medium lacking cells. This is in agreement with the results obtained by Van Wachem *et al.*² The experiments prove that adhering and spreading HEC deposit cellular fibronectin onto the TCPS surface.

It was found that the amount of HSA adsorbed to TCPS which had been pre-exposed to a solution of culture medium containing 1% serum (or less), followed by incubation with a HEC suspension in serum-free medium, was decreased compared to

Fig. 2. Relative amounts of adsorbed human serum albumin (HSA) on TCPS, which had been exposed for 1 h to (a solution of) serum-containing culture medium (20% and 1% serum, respectively, supplemented with ^{131}I -labeled HSA), followed by incubation for 6 h with serum-free medium either with endothelial cells (200 000 per cm^2) or without cells.

Detection: γ -ray counting of TCPS wells. In each experiment, the amount of HSA adsorbed to TCPS after incubation with serum-free medium lacking cells was taken as 100%. Values are means of four determinations ($\pm\text{SD}$). Unpaired t test: * $P < 0.01$; $^+P < 0.025$ compared to the amount of adsorbed HSA after incubation with medium lacking cells.

the pretreated TCPS which had been incubated with serum-free medium lacking cells (Fig. 1(a)). However, if 20% serum was present in the precoating solution, no decrease of the amount of adsorbed HSA due to the presence of cells could be observed (Fig. 1(b)). Most probably, the EIA is not suitable to detect differences between different amounts of adsorbed HSA, when these amounts are large.²

A decrease in the amount of adsorbed HSA after adhesion and spreading of HEC on TCPS, which had been pre-exposed to (solutions of) serum-containing culture medium, was also determined with the aid of experiments in which ^{131}I -labeled HSA was added as tracer protein to the serum solutions. The concentrations of human serum in the solutions used for the pre-exposure of TCPS were taken as 1% and 20%. Figure 2 shows the results of experiments in which the relative amounts of adsorbed HSA were determined by measuring the amounts of ^{131}I -labeled HSA on the TCPS surfaces. From this figure, it may be concluded that a decrease in the amount of adsorbed HSA also occurred if TCPS had been pretreated with a solution containing 20% human serum.

The most plausible explanation for the results mentioned above is that previously adsorbed serum proteins were displaced by cellular fibronectin during adhesion and spreading of HEC on TCPS. It has been mentioned in the introduction

Fig. 3. Adhesion of endothelial cells onto uncoated polyethylene and polyethylene coated with various antibodies after 2 h of incubation. Cell adhesion is expressed as the percentage of the number of cells adhered to polyethylene coated with fibronectin. All values are means of four determinations ($\pm\text{SD}$).

that the displacement of adsorbed serum proteins by cellular fibronectin will occur more easily in the case of relatively hydrophilic surfaces (like TCPS) compared to hydrophobic surfaces. As a result, HEC do not adhere, spread and proliferate on hydrophobic polymers, unless these polymers have been precoated with fibronectin.⁴

Another method of binding cells to a solid surface was reported by Grinnell and Hays⁹ and involves the use of substratum-bound antibodies, which are directed against plasma membrane proteins of the cells. We investigated the adhesion, spreading and proliferation of HEC on polyethylene, precoated with monoclonal antibodies directed against cell membrane glycoproteins and against extracellular matrix compounds. In addition F(ab')_2 fragments of two of these monoclonal antibodies were included in this study.

Figure 3 shows the results with respect to the adhesion of HEC to uncoated polyethylene and polyethylene precoated with the various anti-HEC antibodies. The results of HEC adhesion to polyethylene precoated with fibronectin, mouse IgG and anti-erythrocyte are also shown in this figure. The largest numbers of adherent HEC were observed on surfaces coated with the monoclonal antibodies CLB-HEC-19 and CLB-HEC-347. The adhesion of HEC on polyethylene precoated with the antibodies CLB-10G11, CLB-HEC-65 and CLB-C17 was similar to the adhesion on the fibronectin-coated surface. Cell spreading on the polyethylene surfaces coated with CLB-HEC-19, CLB-HEC-347, CLB-10G11, CLB-HEC-65 and CLB-C17 was not quite as good as on the reference surface.

Fig. 4. Proliferation of endothelial cells after 2 h (= 0 days) and 6 days on uncoated polyethylene, on fibronectin-coated polyethylene, on polyethylene coated with antibodies and on polyethylene coated with mixtures of antibodies. Values are means of three determinations ($\pm$ SD).

Significantly less cell adhesion was observed on polyethylene coated with CLB-Rag-38 (directed against von Willebrand factor) and on uncoated polyethylene. HEC were not well spread on these surfaces. As expected, HEC hardly adhered to polyethylene coated with mouse IgG or anti-erythrocyte.

Polyethylene precoated with CLB-HEC-FN-140 (directed against fibronectin) was not included in the experiments from which the results are presented in Fig. 3, but the results of HEC adhesion (0 days = 2 h) and proliferation (6 days) on this surface are shown in Fig. 4, together with the results of HEC adhesion and proliferation on polyethylene precoated with the antibodies CLB-HEC-19, CLB-Rag-38 and mouse IgG. HEC adhesion and proliferation on uncoated polyethylene and polyethylene coated with fibronectin were also determined.

The numbers of HEC that adhered after 2 h to polyethylene coated with CLB-Rag-38 (Figs 3 and 4), and CLB-HEC-FN-140 (Fig. 4) were rather low compared with the number of cells that adhered to the fibronectin-coated surface. HEC showed a very good adhesion to the CLB-HEC-19 surface (Fig. 3) but HEC did not proliferate on this surface. Therefore, HEC adhesion and proliferation were also determined on polyethylene precoated

with mixtures of antibodies. The results of these experiments are also presented in Fig. 4. HEC did not proliferate on polyethylene coated with mixtures of antibodies in which relatively high concentrations of CLB-HEC-19 were present.⁷ For this reason, the concentration of CLB-HEC-19 in the coating solutions was kept rather low (5 μ g/ml).

Proliferation of HEC determined after 6 days was found to occur on polyethylene coated with fibronectin, CLB-HEC-FN-140, CLB-Rag-38, mixtures of CLB-HEC-19 and CLB-Rag-38, and mixtures of CLB-HEC-19 and CLB-HEC-FN-140 (Fig. 4).

It must be mentioned, however, that cell spreading on polyethylene precoated with CLB-Rag-38 (directed against von Willebrand factor) or the mixture of antibodies in which CLB-Rag-38 was present, was not very good. In these cases, the edges of many cells had been detached from the material surface. In agreement with earlier results from our laboratory,^{4,7} HEC did not proliferate on uncoated polyethylene and polyethylene coated with mouse IgG. HEC also did not proliferate on polyethylene treated with a solution containing a low concentration of CLB-HEC-19 (5 μ g/ml).

Earlier experiments¹¹ showed that proliferation of HEC did not occur or hardly occurred on polyethylene precoated with the antibodies CLB-HEC-19 (50 μ g/ml), CLB-HEC-347, CLB-10G11, CLB-HEC-65 and CLB-C17. These antibody-coated surfaces gave rise to good cell adhesion (Fig. 3). Evidently, attachment and spreading of HEC on a solid surface does not automatically lead to cell proliferation.

Intact monoclonal antibodies, adsorbed to a solid surface, may interact with blood platelets via their Fc part.¹⁵ By precoating of vascular grafts with $F(ab')_2$ fragments of monoclonal antibodies instead of intact antibodies, platelet adhesion may thus be prevented. For this reason, polyethylene coated with $F(ab')_2$ fragments was also included in the proliferation studies.

In Fig. 5 the results are shown with respect to the proliferation of HEC, determined after 2, 6 and 9 days, on polyethylene precoated with fibronectin and on polyethylene precoated with mixtures consisting of intact antibodies, mixtures of antibodies and $F(ab')_2$ fragments, and a mixture of $F(ab')_2$ fragments.

HEC proliferated well on polyethylene coated with a mixture of intact CLB-HEC-FN-140 and $F(ab')_2$ fragments of CLB-HEC-19. HEC showed a moderate-to-good proliferation on polyethylene coated with a mixture of the intact monoclonal anti-

Fig. 5. Proliferation of endothelial cells after 2, 6 and 9 days on fibronectin-coated polyethylene, on polyethylene coated with mixtures of antibodies, on polyethylene coated with mixtures of antibodies and F(ab')₂ fragments, and on polyethylene coated with a mixture of F(ab')₂ fragments. Values are means of three determinations ($\pm$ SD).

bodies CLB-HEC-19 and CLB-HEC-FN-140. This result is in agreement with the results presented in Fig. 4. A good cell spreading was observed in experiments in which HEC proliferated well.

The results obtained from our proliferation experiments carried out thus far suggest that a good proliferation of HEC only takes place on polyethylene which has been precoated with fibronectin or on polyethylene which can indirectly bind (cellular) fibronectin via CLB-HEC-FN-140.

Figure 5 shows that HEC hardly proliferated when F(ab')₂ fragments of CLN-HEC-FN-140 instead of intact antibody molecules were adsorbed to polyethylene. This may be explained by a reduced capacity of the adsorbed F(ab')₂ fragments to bind fibronectin ($\pm$ 40% compared to the capacity of the intact antibody) and is probably caused by the preparation procedure for obtaining (Fab')₂ fragments. It is also possible that the adsorption of the F(ab')₂ fragments onto polyethylene occurs in such a way that the F(ab')₂ fragments cannot properly bind (cellular) fibronectin. Hardly any proliferation of HEC was observed on polyethylene precoated with a mixture of the antibodies CLB-HEC-19 and mouse IgG. This is in agreement with the results mentioned before.

A dependence of HEC proliferation upon fibronectin, directly or indirectly bound to a substratum, explains why HEC do not proliferate on polyethylene precoated with antibodies or F(ab')₂ fragments which do not bind fibronectin. The hydro-

phobic polyethylene strongly adsorbs proteins; therefore, these antibodies or F(ab')₂ fragments are not easily displaced from the polyethylene surface by cellular fibronectin.

If substratum-bound fibronectin is really a prerequisite for the proliferation of HEC, it is conceivable that part of the bound fibronectin molecule provides a biochemical signal which initiates a series of events in the endothelial cell, leading to DNA replication. Probably HEC are also triggered by this biochemical signal to synthesize and secrete cellular fibronectin. More experiments have to be carried out, however, in order to come to conclusions about such a role of fibronectin.

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