

Effect of Pre-Adsorbed Proteins on Attachment, Proliferation, and Function of Endothelial Cells

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As certain proteins control cell adhesion, it has been hoped that cell transplantation and tissue engineering could be augmented by pre-adsorption of specific proteins to biological or synthetic surfaces. The questions that remain, however, are whether such proteins can affect cell production as well as adhesion, and if so, whether in a protein-specific manner. We examined the adhesion and the biochemical secretion of bovine aortic endothelial cells (BAEC) on tissue culture polystyrene (TCPS) discs coated with fibronectin (Fn), laminin (Ln), or gelatin. The three coating proteins nonspecifically promote sub-confluent and post-confluent endothelial cell production of total protein up to 2.5-fold of the reference value. Total soluble glycosaminoglycan (GAG) production slightly increased with the different coatings only at low cell density. In contrast, Ln and Fn, not gelatin, drastically enhanced post-confluent BAEC production of prostaglandin (PGI₂). However, antibody-blockage of the α_5 integrin, constituent of the Fn receptor in BAEC, appeared to inhibit the upregulation of PGI₂ production observed on Fn-coated surfaces. The results indicate that the cell adhesion mediators used as coating agents dictate cell biological production as well as adhesion and proliferation. *J. Cell. Physiol.* 191: 155–161, 2002. © 2002 Wiley-Liss, Inc.

The proliferation of vascular smooth muscle cells (SMC), central to pathology of vascular diseases including atherosclerosis, later arteriovenous graft occlusion, and restenosis after angioplasty or atherectomy, appears to be controlled by agents that mimic endothelial cell products (Gallagher et al., 1986; Segal et al., 2000). The endothelium releases factors that control vascular relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition (Davies and Hagen, 1993; Lüscher and Barton, 1997). Healthy endothelial cells (EC) are anticoagulant and anti-thrombotic because of the regulated secretion of anti-platelet agents such as prostacyclin (PGI₂) and glycosaminoglycans (GAG). The former compound is an unstable vinyl ether formed from the prostaglandin endoperoxide, and acts as a potent vasodilator and inhibitor of platelet aggregation and SMC proliferation. The latter are a complex and heterogeneous family of macromolecules composed of linear sulfated polysaccharide chains. Heparan sulfate is important for maintaining the non-thrombogenic properties of the endothelial surface, by virtue of its ability to bind anti-thrombin III and thrombin. Similarly, EC release heparan sulfate fragments that inhibit the growth of vascular SMC (Castellot et al., 1981; Etnanson et al., 2000). Synthetic vascular grafts bypass vascular obstructions, but do not spontaneously endothelialize in humans and require some type of anticoagulation to keep patency. Therefore, endothelialization of polymeric vascular implants has become a

primary goal in reducing thrombogenicity of both of the materials and restenosis (Absolom et al., 1988).

EC can be introduced in tissue engineered constructs as “living drug delivery systems”, but this effect relies on their ability to maintain PGI₂ and proteoglycan production (Nugent et al., 1999). Thus, we need to determine in advance which devices would optimally control vascular injury and choose potential therapies on the potency of the release of critical compounds. The question that arises is whether this release can be modulated in a predictable fashion. Adsorption of serum proteins present in the culture medium is the first event that occurs when cells are seeded on a material and the adsorbed protein layer influences cell adhesion, spreading, and proliferation (Balcells et al., 1999). As a consequence, coating of surfaces with cell-adhesion mediators like Fn, Ln, or other extracellular matrix (ECM) components has

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become a regular practice to enhance endothelial cell attachment (Kikuchi et al., 1996). Fn has been found to control capillary endothelial cell growth by modulating cell shape (Ingber, 1990). Matrix-driven cell size change has been described to modulate aortic endothelial cell proliferation and sheet migration (Madri et al., 1988). Dubose et al. (1986) found that the arrangement of F-actin regulates PGI₂ production. Nevertheless, little is known about the extent the adsorbed protein layer may regulate the cell phenotype. The purpose of this study was to evaluate whether the cell adhesion mediators fibronectin (Fn), laminin (Ln), and gelatin regulate the production of total protein, GAGs, and PGI₂ of BAEC in vitro, and if this regulation occurs specifically and/or in a surface concentration-dependent manner.

MATERIALS AND METHODS

Protein adsorption studies

Both cell experiments and protein adsorption studies were carried out on TCPS plates (10 cm diameter) (Corning Incorporated, Corning, NY). These plates were punched into 1-cm diameter discs for the adsorption studies with radiolabeled proteins. Bovine Fn, Ln, and gelatin (Sigma-Aldrich, St. Louis, MO) were dissolved in phosphate buffered saline (PBS, pH 7.4) (Gibco-BRL Products, Grand Island, NY) to obtain the appropriate coating concentration. TCPS plates were incubated for 2 h at room temperature with the coating solutions. Proteins radiolabeled with 2 mCi of ¹²⁵I (NEN Life Science Products, Boston, MA) following the iodo-bead method (Pierce, Rockford, IL) and purified by gel filtration, were used as tracers in the adsorption studies conducted in a custom designed chamber, which consisted of a perfused 24-well TCPS plate and two Plexiglass sheets sealed with two clamps and rubber rings. After incubation, the coating solution was rinsed and the TCPS discs placed in a gamma counter for quantification.

Kinetic studies were performed with incubation times ranging from 5 min to 4 h to determine the duration to reach equilibrium. Concentration-dependent experiments were carried out to quantify the amount of adsorbed protein as a function of the protein solution concentration, and the adsorption isotherms were established.

Cell culture, adhesion, and growth

EC were isolated from bovine aortas according to the collagenase dispersion method (Wong and Gotlieb, 1984; Rosenthal and Gotlieb, 1990). After isolation, cells were cultured to passage 5 in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL Products) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% of penicillin-streptomycin (PS) and 1% L-glutamine (G) (Gibco-BRL Products). For adhesion studies, cells were seeded in serum-free medium with 1% PS and 1% G at a density of 4×10^5 cells/well. After 24 h, the cells were rinsed with PBS and removed for counting or refilled with medium containing 10% FBS for further proliferation analysis. Detachment of cells was performed by trypsinization (3 ml 0.05% trypsin-EDTA) (Gibco-BRL Products). Cell numbers were measured by means of a Beckman Coulter counter (Z1 D/T, Beckman, Miami, FL). For cell proliferation studies, cells were seeded (4×10^5 cells/well) in serum-free medium for 24 h and

then grown in DMEM 10% FBS. The culture medium was changed every 48 h with fresh DMEM 10% FBS, and the cell number was determined on various days. Cell confluence was achieved after 5 days of culture.

Cell function

Cell function was evaluated based on the production of total protein, GAG, and PGI₂ in the conditioned medium (CM) collected after 24 h incubation with serum-free DMEM (without L-glutamine, and without phenol red) (Gibco-BRL Products) with 1% PS and 2% G. Cells attached on coated or uncoated TCPS plates were cultured until 3 days post-confluence. Three days after seeding and 3 days post-confluence, the culture medium was removed and the plates were rinsed twice with serum-free DMEM. TCPS plates were incubated for 30 min in that serum-free medium and then rinsed and incubated with 10 ml serum-free medium for 24 h. The CM was collected and centrifuged for 10 min to remove any cellular debris. Total collected volume was measured and 5 ml of collected medium were concentrated (two-fold) using Centriplus concentrators (Amicon Inc, Beverly, MA) with 3,000 MWCO to quantify total protein and GAG production. Total protein production was determined by means of the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA). Total GAG production was quantified by means of the dimethylmethylene blue (DMB) assay (Farndale et al., 1986). Heparan sulfate levels were also determined by the DMB assay by pre-treating the CM for 3 h at 37°C with 0.1 U/sample of the enzyme chondroitinase ABC (Seikagaku America, Falmouth, MA) to eliminate chondroitin sulfate and dermatin sulfate. PGI₂ present in the CM was determined by a 6-keto-prostaglandin F_{1 α} enzymeimmunoassay (EIA) system (Amersham, Piscataway, NJ). Purified mouse anti-human monoclonal antibody CD49e, and purified mouse IgG_{2a} monoclonal immunoglobulin as the isotype control, were purchased from Pharmingen (San Diego, CA). Blockage of the cell receptor was carried out in suspension, at 37°C for 20 min. Samples were centrifuged, and the excess of antibody was aspirated off before cell seeding.

All experiments were carried out in triplicate. All values are reported as the mean \pm SD. Statistical analysis used a non-paired t-test. Values of $P < 0.05$ (two-tailed analysis) were considered significant.

RESULTS

Protein adsorption studies

Elucidation of the kinetics of adsorption helped identifying the minimum coating time necessary to obtain a constant amount of adsorbed protein on the TCPS surfaces. Time-dependent experiments were carried out at a high (100 μ g/ml for Fn and Ln, and 2,000 μ g/ml for gelatin) and a low (1 μ g/ml for Fn and Ln, and 20 μ g/ml for gelatin) solution concentration. The significantly higher gelatin solution concentration values were chosen in accordance to those commonly used in our laboratory and in the literature (Marino et al., 1996; Zimrin, 1996). Though protein adsorption occurs within the first minutes after contact with surfaces (Kurrat et al., 1997), we observed that for all compounds examined, equilibrium was only achieved after 1.5–2 h of incubation. Fn and Ln adsorption isotherms (Table 1

TABLE 1. Parameters obtained by curve analysis of the adsorption isotherms

$c_{ad} = \frac{m_1 c_s}{c_s + m_2}$	Fn	Ln
m_1	950	625
m_2	24000	27000
r^2	0.9867	0.9981

c_{ad} , adsorbed surface density; c_s , the solution concentration; r , correlation factor. $m_1/m_2 = n \cdot K$ and $m_2 = 1/K$, where K is the binding constant and n is the equivalent binding sites of the adsorption.

and Fig. 1) appeared to follow a Langmuir isotherm (Young et al., 1988) with *plateau* values of 950 ± 9 and 625 ± 8 ng/cm², respectively. Gelatin adsorption at high solution concentration peaked after 15 min of incubation and then decayed to a *plateau* equilibrium value at 2 h, indicating desorption of initially loosely bound molecules (Mc Farland et al., 1998). Gelatin presented a very different adsorption isotherm than Fn and Ln, and no *plateau* was achieved within the concentration range examined (Fig. 2). The amount of adsorbed protein reached 1.5 µg/cm² for a solution concentration of 2,000 µg/ml.

Influence of coating proteins on cell attachment and growth

Fn, Ln, and gelatin are well-known cell adhesion mediators, and their ability to increase cell attachment has been previously described (Mueller et al., 1996). A preliminary cell-attachment experiment was performed to validate our method and to investigate the degree of specificity of the proteins for cell adhesion. Solutions of 50 µg/ml of Fn, 100 µg/ml of Ln, and 500 µg/ml of gelatin were used to coat TCPS plates. These solution concentrations resulted in a surface density of adsorbed protein of 500 ng/cm² (cf. Figs. 1 and 2). Uncoated TCPS plates, hydrated with PBS, were carried out in parallel as negative (PBS⁽⁻⁾) and positive (PBS⁽⁺⁾) controls, for BAEC being seeded in serum-free or in 10%FBS-containing medium, respectively. Cell number was increased by 25, 19, and 20% for Fn-, Ln-, and gelatin-coated TCPS, respectively (cf. Fig. 3) compared to PBS⁽⁻⁾.

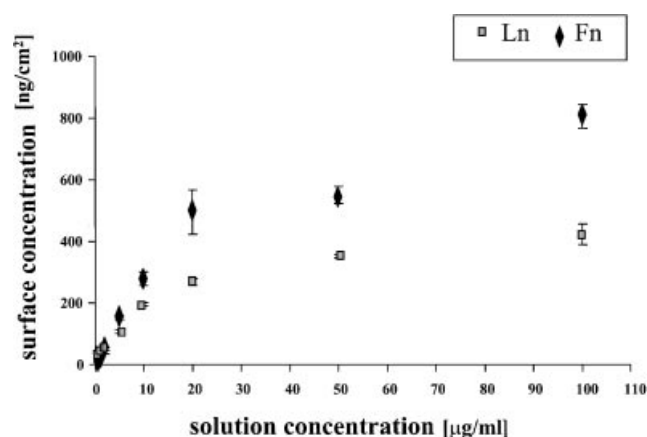


Fig. 1. Adsorption isotherms of Fn and Ln on TCPS determined using radiolabeled proteins as tracers after 2 h incubation time at room temperature.

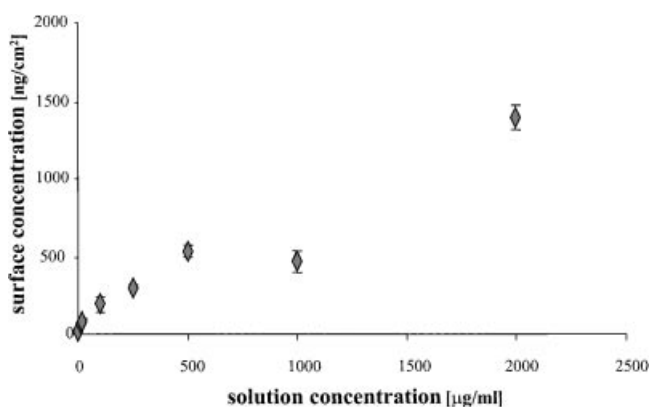


Fig. 2. Adsorption isotherm of gelatin on TCPS determined using ¹²⁵I-gelatin as tracer after 2 h incubation time at room temperature.

To compare cell proliferation on the coated and uncoated TCPS surfaces, cell numbers were determined in several days till confluence was achieved (data not shown). Uncoated TCPS supported greatest cell proliferation as these samples produced the highest cell density in both measured days. Similar to the previous cell attachment experiment, no significant differences were observed among TCPS coated with Fn, Ln, or gelatin.

Influence of coating proteins on biochemical production of BAEC

To study the influence of pre-adsorbed Fn, Ln, and gelatin on cell behavior, BAEC on pre-coated TCPS were incubated for 24 h in serum-free medium. The collected CM was analyzed for total protein, soluble GAG, and PGI₂ production (Figs. 4–6) on 3 days pre-confluence and 3 days post-confluence.

The total amount of protein produced by BAEC on the hydrated TCPS remained below 50% of the production of both sub-confluent and post-confluent BAEC seeded on TCPS pre-coated with Fn, Ln, or gelatin. Total protein production was independent of the cell density. In contrast, total GAG production by BAEC seeded on

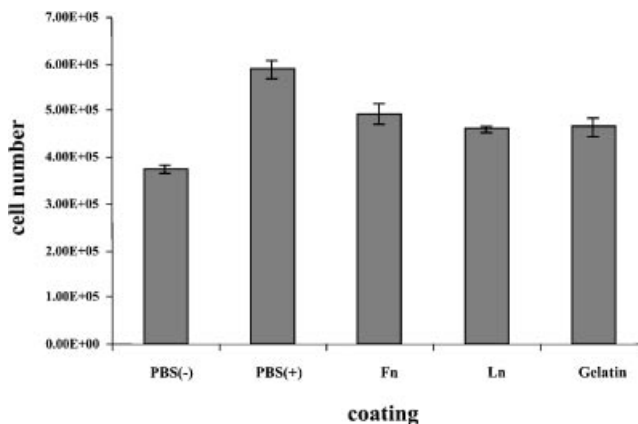


Fig. 3. Influence of pre-adsorbed protein on initial cell attachment (cell number on Day 0).

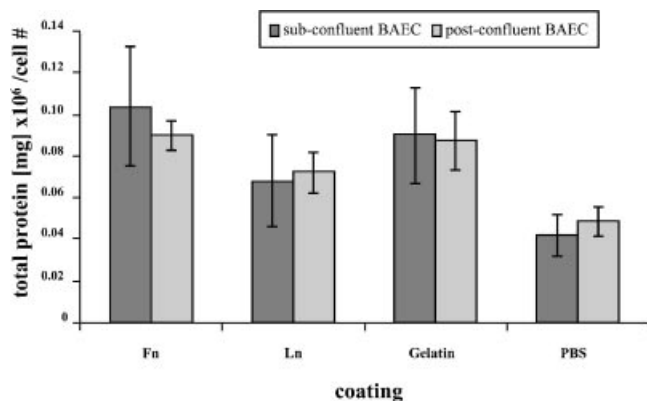


Fig. 4. Total protein production by sub-confluent and post-confluent BAEC seeded on TCPS pre-coated with 500 ng/cm² Fn, Ln, and gelatin.

pre-coated TCPS was 1.4-fold higher than the reference only in the sub-confluent state (Fig. 5).

In contrast, PGI₂ levels in the post-confluent CM from BAEC seeded on TCPS pre-coated with Fn and Ln, but not gelatin, were 3- and 3.8-fold, respectively, higher than those from sub-confluent medium (Fig. 6).

It has also been suggested that cell attachment, proliferation, and function depends on the surface concentration of adsorbed protein (Nikolovski and Mooney, 2000). As surface concentration increases, the conformation and orientation of the adsorbed protein layer are susceptible to change, and therefore, the regulation of cell attachment and proliferation previously observed may be also altered. Though both Fn and Ln enhanced PGI₂ production in post-confluent BAEC, we chose to examine concentration effects in cellular PGI₂ production with Ln as its solubility in PBS is substantially higher than that of Fn. Several concentrations of Ln (0.5, 5, 10, and 50 µg/ml) were used to coat TCPS plates providing surface concentrations of adsorbed Ln from 5 to 200 ng/cm². TCPS hydrated with PBS was carried out in parallel as a reference.

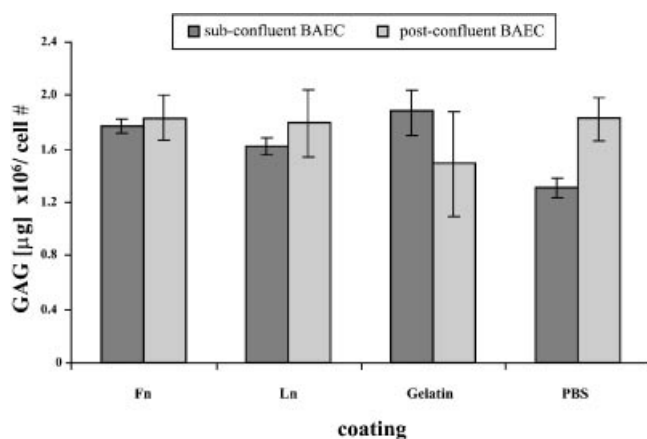


Fig. 5. Total GAG production by sub-confluent and post-confluent BAEC seeded on TCPS pre-coated with 500 ng/cm² Fn, Ln, and gelatin.

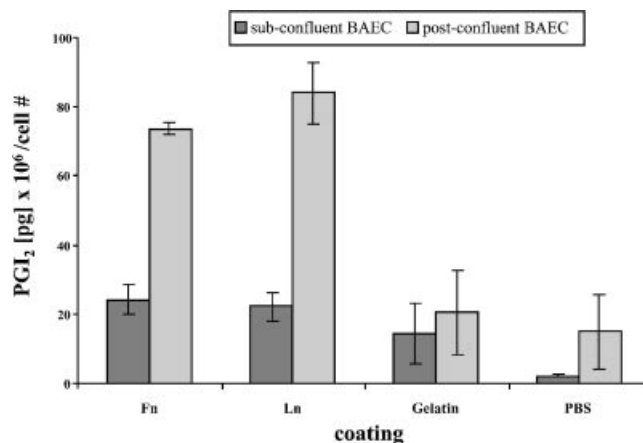


Fig. 6. PGI₂ production by sub-confluent and post-confluent BAEC seeded on TCPS pre-coated with 500 ng/cm² Fn, Ln, and gelatin.

Regulatory signals conveyed by immobilized ECM molecules depend on the density at which they are presented, and thus, on their ability to either prohibit or support cell spreading (Dexter et al., 2001).

The analysis of the CM showed that adsorbed Ln enhanced the cellular production of PGI₂ depending on the concentration of adsorbed Ln on the surface (data not shown). The surface concentration of Ln determined the production of PGI₂ on both sub-confluent and post-confluent BAEC. At surface concentrations below 70 ng/cm², the amount of PGI₂ measured in the CM was independent of the cell density, but at higher surface concentrations of Ln, the production of PGI₂ by post-confluent BAEC almost doubled the one of sub-confluent cells.

Integrin role in metabolite production

The specificity of PGI₂ production vs. the non-specificity of GAG production observed from BAEC seeded on different protein coatings may be an indicator that there is a difference of type and/or affinity of the integrin that regulates the production of the above mentioned cellular products. The use of monoclonal antibodies as blocking agents is a reliable technique to assess cellular function pre- and post-blockage of specific cell receptors. Antibody specificity is critical in maintaining the cell response to stimulus without alteration, and it is the key in determining the role of the receptor. We have shown that Fn- and Ln-coated TCPS stimulated PGI₂ production by BAEC. Therefore, to investigate the mechanism behind the observed stimulation and to test the hypothesis that the adhesion of BAEC to Fn or Ln leads to the activation of different signaling pathways than the adhesion of cells to gelatin, the following three-steps strategy was carried out: (1) incubation of BAEC with different concentrations of a specific monoclonal antibody to achieve complete saturation of the Fn receptor; (2) study the effect of the blockage on cell attachment to Fn-coated TCPS; and (3) determination of PGI₂ production by the selectively blocked cells.

A monoclonal antibody (CD49e), which reacts with the α₅ integrin, a dimer of 135/25 kDa, that associates with β₁ integrin (CD29) to form VLA-5, a well-established Fn receptor, was used to block its receptor. Parallel

studies with an isotype control antibody, as well as, with untreated cells on Fn-coated and uncoated TCPS were carried out as reference. BAEC with increasing level of receptor blockage were seeded on Fn-coated TCPS with two main purposes: to assess the appropriate antibody concentration to obtain complete blockage of the receptor and to determine if BAEC having a completely-blocked Fn receptor would still attached to Fn-coated surfaces through other receptors or unspecific binding. An antibody concentration of 1 $\mu\text{g}/\text{ml}$ was sufficient to block the receptor. This blockage decreased, but did not completely prevent cell attachment (Fig. 7).

Subsequently, Fn receptor-blocked BAEC were allowed to attach and proliferate till confluence. Confluence was achieved by the Fn receptor-blocked cells after 12 days of culture, while unblocked cells reached it 7 days after seeding, but there was no difference in cell morphology and measured cell numbers between Fn receptor-blocked and unblocked confluent BAEC. The analysis of the CM of post-confluent cells revealed that PGI_2 production by the Fn receptor-blocked cells was decreased by 43% in comparison with the unblocked ones (Fig. 8).

DISCUSSION

It is generally accepted that the physicochemical properties of a biomaterial are key determinants for the adsorption of proteins, whose quantities and conformation, in turn, mediate cell attachment to the material surface. Previously, we applied X-ray electron microscopy, atomic force microscopy, an enzyme-linked immunosorbent assay, and radiolabeling techniques to assess the activity of adsorbed protein on biomaterials (Balcells et al., 1999). The present study was, therefore, initiated in an attempt to determine if certain adsorbed proteins were applicable as regulators of cell function. Theoretical values for monolayer coverage of most proteins lay in the order of 200 and 700 ng/cm^2 for the side-on and end-on orientation, respectively (Mueller et al., 1996). Accordingly, it was found that Fn and gelatin adsorbed on TCPS as a multilayer. It is important to carefully analyze adsorption data to extract the optimal amount of reliable information. Scatchard plots of the isotherms (Fig. 9) confirmed that Ln adsorption may be treated by the classical Langmuir model ($n = 552$, $k = 4.31\text{E}-5$), but not for Fn and gelatin, where

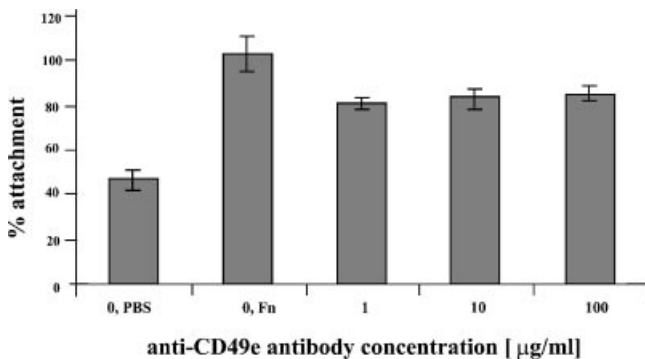


Fig. 7. Effect of antibody concentration on cell attachment assessed by cell number determination after 24 h of seeding (Day 0).

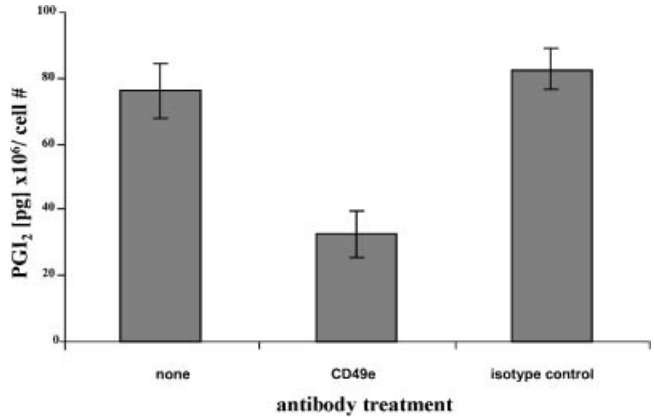


Fig. 8. PGI_2 production by post-confluent BAEC seeded on TCPS pre-coated with 500 ng/cm^2 Fn after blockage of their Fn receptor with rat anti-human monoclonal antibody CD49e, compared to unblocked cells and cells treated with rat IgG2a monoclonal immunoglobulin as the isotype control.

Scatchard plots revealed concave up and downward shape, respectively. It has been suggested that these curve shapes may be attributed to negative cooperativity or heterogeneous binding sites (concave up) and positive cooperativity (concave down).

The use of classical Langmuir theory for gas adsorption assumes the ideal case, where only one molecule can be adsorbed per site, only one type of site is present, no lateral interactions or cooperativity occur, only one adsorbing species is present in a dilute solution, and the adsorption process is reversible. This theory was originally and primarily espoused for the interaction of proteins with ions, i.e., small, spherically symmetric rigid solutes. Assuming an adsorption process of a protein with n identical binding sites, the isotherm data can be expressed in terms of multiple equilibria states, where the adhesion surface is considered to consist of multiple potential bindings sites of approximately the

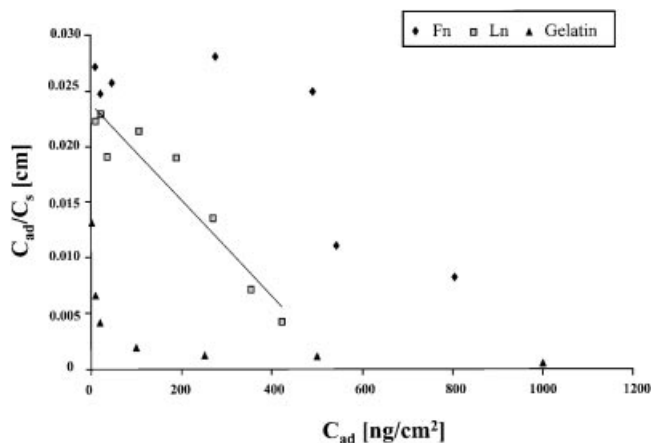


Fig. 9. Scatchard plots of Fn, Ln, and gelatin isotherms, where C_{ad} is the surface concentration of adsorbed protein in ng/cm^2 measured using radiolabeled proteins as tracers and C_s is the concentration of the protein solution in ng/ml .

same area as the projected area of the protein of interest. Adaptation of this model to understand protein interaction with the symmetric and rigid plane that is the adhesion surface requires that we match boundary conditions and initial assumptions to the Langmuir process. The validity of extrapolation from ions to planar surfaces is increased when experiments are conducted on surfaces of low binding site density so that the interactions can be considered individually, to be reversible and appropriately analyzed by multiple equilibria and related models.

The results confirm that cell adhesion is increased by the pre-adsorbed protein (25% for Fn, 19% for Ln, and 20% for gelatin), however, the biological effects of the three studied protein coatings were not significantly different from each other. This finding is in full agreement with Mooney et al. (1992), who described comparable levels of hepatocyte attachment to pre-coated plastic dishes regardless of the ECM molecule used. The enhancement of cell adhesion on TCPS was non-specific and independent of the type of coating protein. Such an observation may arise from the common means by which cells adhere to surfaces, namely through the interaction with short amino acid sequences (e.g., arginine-glycine-asparagine-serine) present in the primary structure of all of adsorbed ECM proteins under study (Mardon and Grant, 1994). The TCPS sample taken as positive reference allowed for the maximum cell adhesion, as 10% FBS added to the cell suspension in the seeding protocol promotes the adsorption of numerous proteins and growth factors in serum prior to the cell attachment to the surface.

In our experiments, we chose a surface concentration of adsorbed protein of 500 ng/cm^2 . This value of protein density may not specifically affect GAG production, but it does influence PGI₂ production. The specificity of PGI₂ production by post-confluent BAEC is a crucial point of this investigation and provokes further questions for future research regarding the nature of this specificity and its applicability to the design of novel materials that require regulation of cell function. Critical to this issue is whether GAG and PGI₂ synthesis and secretion are differentially regulated. Different ECM proteins interact with distinct integrins on cells. For example, SMC's adhere to Fn primarily through the integrin $\alpha_5\beta_1$, while they adhere to Ln through the integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ (Dexter et al., 2001). In addition, a single protein presents a repertoire of binding integrins expressed by different cell types. EC, for example, rely on $\alpha_2\beta_1$ and $\alpha_6\beta_1$ for binding Ln, whereas SMC interact with Ln through $\alpha_1\beta_1$ (Morla and Mogford, 2000). The signaling pathways activated by the adhesion of EC to Fn, Ln, or gelatin have not been thoroughly investigated and the roles of these signaling pathways in the regulation of BAEC proliferation and phenotype are not well-known. The binding of fibrin to integrin $\alpha_5\beta_3$ has been found to regulate PGI₂ formation of EC (Chang et al., 1996). Specific protein-material interactions origin conformational or orientational differences among the adsorbed protein molecules adding a new variable to these complex phenomena. The existence of a common integrin in the cellular membrane that regulates GAG production by BAEC, while several integrin-type molecules highly specific for every type of ECM component being

responsible for the production of PGI₂ may be an explanation of the distinctive behavior of BAEC on the investigated coatings.

CONCLUSIONS

Ln adsorption on TCPS followed the Langmuir isotherm, while Fn and gelatin adsorption analyzed by Scatchard plots showed negative and positive cooperativity, respectively. Pre-adsorbed Fn, Ln, and gelatin regulated BAEC attachment, proliferation, and function. While pre-adsorbed Fn, Ln, and gelatin enhanced cell attachment on TCPS, the rate of cell proliferation was reduced by the pre-adsorbed proteins. Both cell density and pre-adsorbed protein have been identified as key parameters regarding cellular production of protein, e.g., total GAG, and PGI₂. Total protein production was non-specifically enhanced by the coating proteins in both sub-confluent and post-confluent state. GAG production was only slightly nonspecifically increased by the interaction of sub-confluent BAEC with the adsorbed protein layer. In contrast PGI₂ release from post-confluent BAEC was specifically increased by Fn and Ln as coating agents.

The finding that PGI₂ production by BAEC was strongly dependent on the different protein coatings, while both GAG and total protein production were not substrate-specific processes raised the hypothesis that there is a difference of type and/or affinity of the integrin that regulates the production of the above mentioned cell products. Specific blockage of the Fn receptor of BAEC seeded on Fn-coated TCPS provoked a reduction of PGI₂ production and corroborated this hypothesis. The possibility of controlling the levels of PGI₂ produced by BAEC using surfaces pre-coated with Ln or Fn is of crucial relevance for future applications in tissue-engineered constructs. The controlled release of PGI₂, a powerful vasodilator, is key in the inhibition of events such as SMC proliferation and platelet aggregation, leading causes of pathologies such as restenosis and acute thrombosis.

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