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THE EFFECT OF VITRONECTIN AND OTHER EXTRACELLULAR MATRIX MOLECULES ON ENDOTHELIAL EXPANSION AND PLASMINOGEN ACTIVATION

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Abstract

Endothelial recovery following procedures used to alleviate blood vessel occlusion is modulated by the local extracellular matrix upon which it has to migrate and proliferate. This extracellular material is derived from vessel wall cells, and plasma proteins which bind to the exposed surfaces. We have demonstrated that vitronectin adsorbs efficiently to tissue culture polystyrene in competition with other plasma proteins, which suggests that it may adsorb to biomaterial surfaces *in vivo*. We have compared the adhesion, migration and proliferation of human umbilical artery endothelial cells on surface-coated vitronectin, with other extracellular matrix molecules encountered in this environment, namely fibronectin, laminin and collagen types I and IV. Endothelial proliferation was significantly reduced on the vitronectin surface. This was correlated with an increase in the ratio of plasminogen inhibitor-1 to urokinase in the cell/matrix layer. Laminin coated surfaces limited increases in endothelial culture area, due to poorer cell spreading on this surface. Such combination of cellular responses to vitronectin and laminin would discourage endothelial recovery, and encourage smooth muscle hyperplasia *in vivo*. These considerations are important in the design of biomaterial surfaces to optimise endothelial recovery.

Key Words: Endothelium, extracellular matrix, vitronectin, expansion assay, adhesion, migration, plasminogen activator.

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Introduction

The endothelium forms an important barrier to contact of the plasma and blood cells with underlying tissue, as well as presenting a non-thrombogenic surface to circulating blood. Procedures used to alleviate blood vessel occlusion such as balloon angioplasty, endarterectomy, laser ablation and vascular grafting result in varying degrees of endothelial damage and denudation. Such denuded areas are prone to the development of intimal thickening involving migration and proliferation of vascular smooth muscle cells, which frequently results in restenosis (Casscells, 1992). More recently stents have been introduced as a mechanism of maintaining lumen size against the forces of elastic recoil and narrowing due to vessel remodeling. Although these devices have reduced the incidence of restenosis somewhat (Goldberg *et al.*, 1995), they actually increase vascular smooth muscle cell hyperplasia (Rogers and Edelman, 1995). The rate and extent of endothelial recovery is thought to be a controlling factor of the magnitude of the smooth muscle cell response (Schwartz *et al.*, 1980; Reidy *et al.*, 1982; Clowes *et al.*, 1983; Doornekamp *et al.* 1996). Recovering endothelium has to attach, migrate and proliferate over the damaged surface and is likely to be profoundly affected by the nature of the local extracellular matrix (ECM) with which it makes contact (Madri *et al.*, 1989; Madri and Marx, 1992). The source of this ECM is damaged basement membrane and underlying connective tissue, material secreted by vascular smooth muscle cells, and plasma protein molecules such as vitronectin and fibronectin, which readily bind to ECM components of exposed connective tissue (Tomasini and Mosher, 1991; Preissner and Potzsch, 1995; Yamada, 1991). Vitronectin also binds competitively to certain biomaterial surfaces in the presence of other plasma proteins (Bale *et al.* 1989; Horbett, 1994).

The effects of the abundant plasma protein vitronectin upon endothelial cell behaviour have been little studied to date. This contrasts with the extensive *in vitro* studies on the effects of a number of ECM

molecules (such as fibronectin, laminin and collagens) upon endothelial cell adhesion, spreading, migration and proliferation (reviewed by Madri *et al.*, 1992; Herman, 1987; Ingber, 1991). When tissue culture polystyrene is exposed to growth media containing serum, vitronectin adsorbs to the surface in significant concentrations while fibronectin does not (Steele *et al.*, 1993). We have previously shown that bovine endothelial cells are dependent on this adsorbed vitronectin for attachment to and growth on the culture surface (Underwood and Bennett, 1989). In contrast, it is well known that most human endothelial cells will not grow well on tissue culture polystyrene unless it is precoated with fibronectin or gelatin. By inference these cells are therefore refractory to a vitronectin coated surface, unlike the bovine endothelial cells. Vitronectin has been localised in atherosclerotic lesions (Nicolescu *et al.*, 1987,1989; Guettier *et al.*, 1989) and binds avidly to biomaterial surfaces and to several ECM components (Horbett, 1994; Tomasini and Mosher, 1991). The biological consequences of this adsorption for endothelial cell recoverage are currently unknown. At sites of angioplasty, stent placement, or vascular graft anastomosis, resulting endothelial denudation, platelet activation and local thrombus formation are likely to activate plasma vitronectin (Tomasini and Mosher, 1991; Preissner *et al.*, 1993), with resultant localised binding to exposed ECM and biomaterial surfaces. This would present a potentially unfavourable surface for endothelial recovery.

The purpose of the studies reported here was to investigate the effects of surface coated vitronectin upon human arterial endothelial cell recovery, in a tissue culture model of wound repair. Fibronectin, laminin, collagen types I and IV, which the cells would also be likely to encounter in the wounded vessel environment, were included for comparison. For these studies fibronectin and vitronectin were specifically removed from serum used in the culture media, to avoid confounding effects. Adsorption of the plasma proteins vitronectin and fibronectin to tissue culture polystyrene was determined in competition with other proteins, and stability of the surface-coated proteins over time was measured. Endothelial cell adhesion, spreading and localisation of urokinase and plasminogen activator inhibitor-1 was determined on each substratum as well as expansion of the endothelial cell sheet. In this latter model endothelial cells were seeded in a restricted area to produce a confluent sheet, so that we were able to measure sheet migration and proliferation of the endothelium. These are vital facets of endothelial recovery and a determination of the relative effects of different ECM molecules upon them is a necessary prerequisite to both understanding the processes of normal wound healing and to optimise design of biomaterial surfaces.

Materials and Methods

Materials

The following ECM molecules were used to coat surfaces. Bovine fibronectin (FN), rat type I collagen (col I), mouse Engelbreth HolmSwarm sarcoma (EHS) type IV collagen (col IV), EHS laminin (LM) and gelatin type B solution were obtained from Sigma (St. Louis, MO, USA). Bovine vitronectin (VN) was purified from bovine serum using a monoclonal antibody affinity column as previously described (Underwood and Bennett, 1989). Chicken FN was purified from fresh plasma by affinity chromatography on gelatin Sepharose (Pharmacia, Uppsala, Sweden) as described (Ruoslahti *et al.*, 1982). All ECM molecules were stored in aliquots at -70°C. All other chemicals were of analytical grade. ECM molecules were tested for purity by a combination of SDS electrophoresis with protein staining and Western blotting and sensitive ELISA assays using monoclonal antibodies (mAbs) or polyclonal antisera which displayed cross-species reactivity. No cross contamination of ECM molecules was found except for a trace of col IV present in the col I preparation. All solutions used were prepared using pyrogen free water (Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia) and small aliquots were filtered through sterile Zetapore 0.2 μm pore nylon membranes (Cuno Pacific Pty Ltd, Blacktown, NSW, Australia). All reusable glassware was treated with E-Toxa Clean (Sigma) to remove pyrogens, and following several washes in tap and distilled water, was rinsed in pyrogen free water before sterilization by autoclaving at 120°C for 1 h followed by dry heat at 170°C for 3 h. All handling of materials was done using surgical gloves. Tissue culture polystyrene (TCPS) and non-tissue culture treated polystyrene (PS) were from Nunc (Roskilde, Denmark). Flexible polyvinyl (PV) ELISA plates were from Dynatec (Alexandria, VA, USA).

Cell culture

Primary cultures of human umbilical arterial endothelial cells (HUAECs) were prepared from fresh umbilical cords delivered by Caesarian section at Royal North Shore Hospital, Sydney, as described (Weis *et al.*, 1991) using 0.1% collagenase (Sigma C6885). Cells were routinely grown on TCPS precoated for 2 h at 37°C with chicken FN at 10 μg/ml (5 ml per 75 cm^2 flask). The culture medium was Medium 199 with Earle's Salts (Gibco, Life Technologies Inc., Grand Island, NY, USA) containing 20% pyrogen free foetal calf serum (FCS, P.A. Biologicals, Sydney, Australia),

100 units/ml penicillin, 100 μg/ml streptomycin sulphate, 100 μg/ml heparin (Sigma H3149) and 2% bovine brain extract (prepared according to Maciag *et al.*, 1979). Cells were passaged at a 1:3 split ratio after disaggregation with 0.125% trypsin, 0.02% EDTA (T/EDTA), and used between passages 6 and 10. In some experiments cells were cultured in medium containing FCS depleted of VN and FN. Depletion was sequential using mAb and gelatin Sepharose affinity columns as described above for the purification of these molecules. The depleted serum (DDS) was tested for residual VN and FN using dot blot ELISAs. Remaining activity was less than 1% of the starting level, and was considered negligible for the present work.

Cell adhesion

Dilutions of ECM molecules in phosphate buffered saline (PBS) were coated at 50 μl per well in 96-well TCPS or PS plates for 2h at 37°C or overnight at 4°C. Wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hat 37°C. Adhesion of HUAECs was estimated colourimetrically as previously described (Underwood *et al.*, 1992), having validated this method for HUAECs by comparison with directly counted trypsinised cells. HUAECs were seeded at $3x10^4$ per well. After 2 h incubation at 37°C, adherent cells were fixed with 4% formaldehyde in PBS (formol saline) and stained with methylene blue (C.I. 52015, BDH-Merck, Darmstadt, Germany) as previously described (Oliver *et al.*, 1989). Absorbances were read on a BioRad 3550 Plate Reader (Biorad Labs. Pty. Ltd., Regents Park, NSW, Australia), T 655 nm, R 450 nm.

Expansion of endothelium on ECM substrates

Endothelial expansion was measured using a method based on that described by Fischer *et al.* (1990). Wells of a 24-well TCPS plate were coated with sterile solutions of ECM molecules in PBS, 1 ml per well at 37°C for 2 h. Coating concentrations were as follows: bovine FN 5.0 μg/ml, VN 10 μg/ml, Col I 2.5 μg/ml, Col IV 10 μg/ml and LM 40 μg/ml. Surfaces were washed with Hanks balanced salt solution (HBSS, Gibco) before addition of cells. Pyrogen free sterilised silicone fences (obtained from E. Fischer, Aachen, Germany), were inserted gently into the wells. 500 μl of 1% BSA in serum free medium (BSA/SFM) were loaded around the outside of each fence and 100 μl of HUAEC cell suspension of varying cell concentration in BSA/SFM, inside the fence. The internal diameter of the fences was 0.63 cm giving a seeding area of 0.312 cm^2. This is equal to the culture area of wells on a 96-well plate and 1/6 of the culture area of the wells on a 24-well plate. Replicate plates were set up with the same cell loading in the absence of fences. Plates were incubated at 37°C for 4 h in a humidified CO_2 incubator to allow maximal cell adhesion. Fences were then removed and all wells were washed gently with SFM to remove non-adherent cells. One ml of growth medium containing 10% DDS was added per well and incubation continued at 37°C. At 24 h control wells were rinsed twice with PBS, fixed in formol saline for 20 min, rinsed with PBS and stored at 4°C in a further PBS wash. This process was repeated on subsequent days for test wells. Taking the 24 h time point as day zero, wells were fed with fresh medium on day 3 and the expansion was terminated on day 6.

For wells with fences, culture diameters were measured in two directions at right angles on methylene blue stained cells using a dissecting microscope with an eye piece graticule. Cell numbers in all wells at each time point were estimated from optical densities of solubilised dye as for cell adhesion. Optical densities were corrected for the ECM molecules coated. ECM secreted by the HUAECs over the culture period did not contribute further to this background optical density. Percent increases in culture area or optical density were calculated as $(x_t/x_0-1) \times 100$, where x_t and x_0 represent area or corrected optical density at time t and time 0 (24 h post seeding) respectively. The variance of this measurement was calculated as the (variance of the ratio x_t/x_0) x 100, considering x_t and x_0 as independent variables (Colquhoun, 1971). Increases in culture area represent combined effects of cell migration and proliferation, while increases in optical density are due solely to cell proliferation.

In order to seed the correct number of HUAECs inside the fence to give a confluent cover, a variety of seeding concentrations were tested using a bovine FN substrate coated at 5 μg/ml. Culture areas and optical densities of stained cells were measured at 4 h and 24 h post seeding. The cell loading which resulted in maximal cell density at 24 h, with no change in culture area from 4 h to 24 h, was 1.5×10^4 cells per fence and this seeding concentration was used in all subsequent expansion assays. When different ECM substrates were compared the cell density at 24 h post seeding was compared across substrates using the Analysis of Variance. If there were any significant differences between the substrates, the expansion experiments using fences were not done, as the comparison of increase in culture area is only valid if the starting cell densities are uniform.

Measurement of areas of individual spread cells

35 mm TCPS dishes were coated in duplicate with ECM molecules as for HUAEC expansion. 1×10^4 HUAECs were added per dish in medium containing 10% DDS and incubated at 37°C for 24 h. The cells were then fixed and stained with methylene blue as for

cell adhesion. The cells were viewed under phase contrast at 20 x magnification. Images were transferred to a Quantimet 570 image analysis system (Cambridge Instruments, Cambridge, UK) and the areas of at least 50 randomly selected cells per ECM coating were determined.

Estimation of adsorption of ECM molecules to TCPS surfaces

ECM molecules were labelled with NHS biotin (BioRad) at 1 μl per 100-200 μg of ECM molecule as described (Underwood and Steele, 1991). We have demonstrated that biotin labelling of these proteins does not alter their surface adsorption characteristics (Underwood and Steele, 1991). The amount of each ECM molecule bound to the surface was measured in two ways as shown in figure 1.

Method A. Coating solutions of ECM molecules at the concentrations used above for endothelial expansion, were prepared, spiked with biotinylated molecules of the same species. Aliquots of the 'spiked' coating solutions were serially diluted (3 in 4 or 2 in 3) in PBS and 50 μl samples of the dilution series were added to the wells of PV ELISA plates in duplicate. At the same time, 96- or 24-well TCPS plates were incubated with 50 μl or 1 ml per well respectively of the undiluted spiked coating solutions and incubated at 37°C for 2h. At the end of the 2h incubation, samples of the unbound material from the coating mixtures were serially diluted as above and 50 μl samples added to PV ELISA wells in duplicate. The ELISA plates containing the three parallel dilution series (1 - fresh coating solution, 2 and 3 -incubated for 2h in small or large wells) were sealed and refrigerated for three days to allow maximal surface adsorption of proteins (Underwood and Steele, 1991). Wells were then blocked with 1% BSA in PBS and surface-bound biotin was detected using a standard ELISA (Morris *et al.*, 1994), with peroxidase conjugated Streptavidin (Amersham, Amersham, Bucks., UK) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Sigma). Absorbance was read at 405 nm (490 nm reference) on a BioRad 3550 ELISA plate reader.

For each ECM molecule, dose response curves were constructed for the fresh coating solution and for the 2h-adsorbed material on each well type. The proportion of protein bound in the latter was calculated from the displacement of the 2h-incubated curves from the fresh-solution curve.

Method B. 96- or 24-well TCPS plates were incubated with biotin-spiked ECM molecules for 2h at 37°C as for method A above. At the start of the experiment, solutions containing biotin labelled protein only (at the same concentration used for 'spiking') were serially diluted (3 in 4 or 2 in 3) in PBS, and 50 μl or 1 ml samples were loaded into 96- or 24-well TCPS plates, respectively, in duplicate. These were incubated refrigerated for three days as for method A. After the 2h incubation at 37°C of the coating solutions, the wells were drained of coating solution to arrest adsorption and the coating solution was replaced with PBS. These wells were then refrigerated for three days with the others. Very little desorption takes place under these conditions (Underwood and Steele, 1991). These wells, and the dilution series of the 'spiking' solution, were blocked and analysed by ELISA as in A. For the 24-well plates 1 ml reaction volumes were used and 2 ml volumes for blocking and washing. After termination of colour development 100 μl aliquots were removed to empty 96-well TCPS plates to read the absorbance.

96- and 24-well dose response curves were constructed for each biotin labelled ECM molecule. The linear portions of these curves represented the region of independence for each molecule resulting from close to 100% binding of protein to the surface (Underwood and Steele, 1991). By marking off the absorbance of the 2h incubated coating mixture on the appropriate binding curve,the proportion of biotin labelled protein adsorbed in this time could be estimated and therefore the concentration of protein adsorbed from the coating mixture.

The adsorption of some proteins was measured using method A, some with method B and some using both. The use of 3/4 and 2/3 dilution curves allowed accurate determination of surface concentrations. Surface densities of adsorbed proteins in ng/cm^2 were calculated using a surface area estimate of 0.634 cm^2 for fluid contact in the 96-well TCPS plates and 4.55 cm^2 in the 24-well plates.

Estimation of surface leaching of ECM molecules with incubation time

24-well TCPS plates were coated for 2h at 37°C with solutions of ECM molecules 'spiked' with biotin label to give final coating concentrations as used above for endothelial expansion. Coated wells were treated exactly as those in the expansion assay except that cells were omitted. After the initial coating step, the 4h mock cell adhesion step, and subsequent incubation in culture medium, sample wells were washed in PBS and stored in PBS at 4°C until recovery of the last samples. Biotin label retained on the surface was estimated by ELISA as above. Percent leaching was calculated using the ELISA absorbance of the initial 2h coated wells (without further incubation) as 100%.

Comparison of adsorption of VN and FN onto TCPS from plasma

Fresh bovine plasma was prepared from blood containing 15% volume of acid citrate dextrose (ACD)

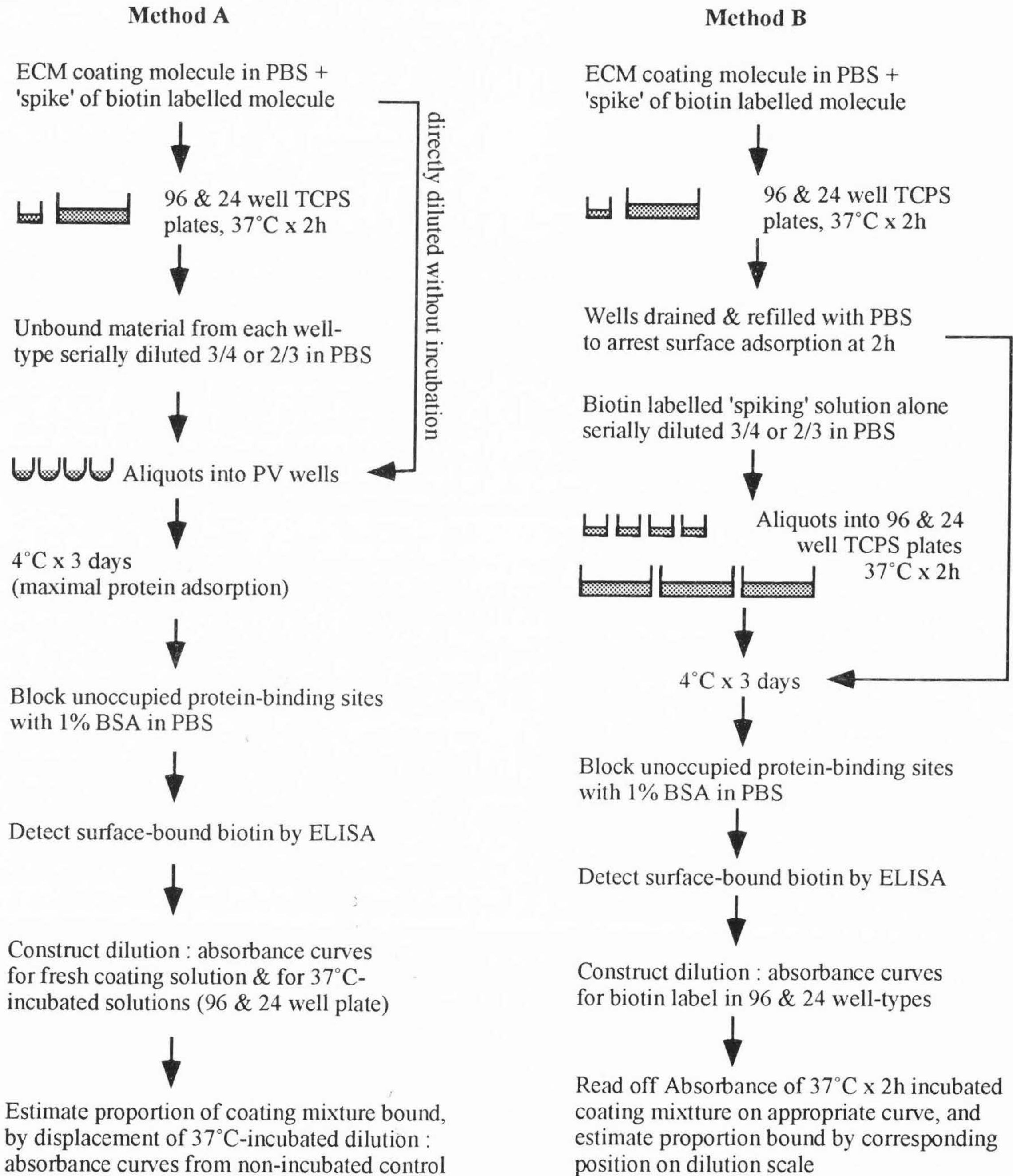


Figure 1. Methods of estimating surface concentration of coated ECM molecules.

buffer, collected from the local abattoir. Aliquots of a dilution series in PBS (containing 1% ACD buffer) were incubated in 96-well TCPS plates for 3h at room temperature on a plate shaker. Dilution series of purified bovine FN and VN in PBS/ACD buffer were similarly treated and the wells subsequently blocked with 1% BSA in PBS for 1h at room temperature. The plasma dilutions were also incubated in wells which had been precoated with either PBS or collagen type I at 10 μg/ml, for 2h at 37°C, and pre-blocked with 1% BSA in PBS for 1h at room temperature. After incubations and blocking, wells were washed in PBS and stored with PBS at 4°C overnight. Presence of VN or FN adsorbed on the surface was detected by ELISA using the

monoclonal antibodies A27 and A22 as described by Underwood *et al.* (1993).

Measurement of activity of the plasminogen system

96-well TCPS plates were coated with ECM molecules at the concentrations used for endothelial expansion above, at 37°C for 2h. Wells were rinsed with SFM. HUAECs were seeded at 1×10^4 per well in BSA/SFM and allowed to adhere at 37°C for 4 hours. Non-adherent cells were washed away with SFM and the plates incubated in growth medium with 10% DDS for 24 h. Wells were rinsed once with PBS containing 1 mM $CaCl_2$ and $MgCl_2$, once with PBS, then either fixed with formol saline for 20min, fixed with 1:1 methanol/ethanol for 10 min, or treated with hypotonic NH_4OH to prepare ECM as described by Gospodarowicz *et al.* (1981). Wells were rinsed twice with PBS and stored short term with PBS, 50μl per well, at 4°C. Urokinase (uPA) and plasminogen activator inhibitor-1 (PAI-1) were detected on the cell surface (formalin fixation), in the ECM (NH_4OH treatment), or at all levels in the complete cell layer, including the ECM (methanol/ethanol fixation), by ELISA. The following primary antibodies were used: Anti PAI-1 mAb (American Diagnostica Inc., Greenwich, CT, USA, no. 3785) and anti uPA mAb (American Diagnostica no. 3689). Secondary antibody was biotinylated anti-mouse Ig (Amersham), 1/1000 + 10% FCS for 1 h, followed by peroxidase conjugated streptavidin (Amersham), 1/500 for 30 min, and ABTS as the substrate. Other conditions for the ELISA were as previously described (Underwood *et al.*, 1992).

Statistical analysis

Each experiment was done a minimum of three times. Statistical significance was estimated using the Analysis of Variance and Student Newman Keul's test where appropriate. In some cases the means of several independent experiments could be compared directly. In others the statistical significance of each individual experiment had to be analysed separately. In these latter cases, for estimates of increases in area and cell mass, the variance of the ratios (time t/time 0, see expansion of endothelium on ECM substrates, above), were calculated and used in the Analysis of Variance.

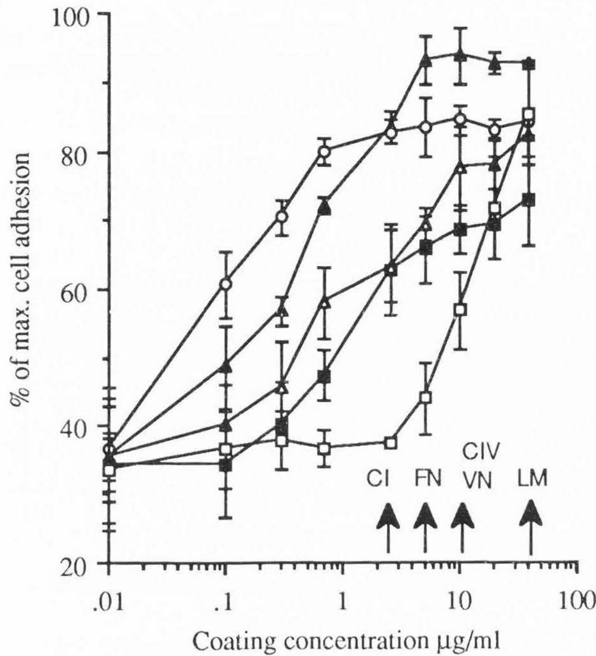
Results

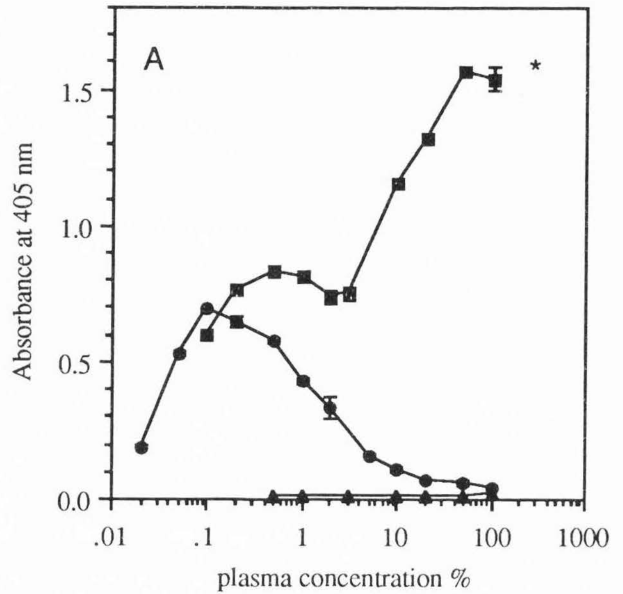
Determination of surface densities of coated ECM molecules for HUAEC adhesion

2h HUAEC adhesion was measured on the five different ECM molecules coated on TCPS or PS for 2 h at 37°C or 4°C overnight. Greatest sensitivity was exhibited by ECM molecules coated on TCPS for 2 h at 37°C. These results are shown in Fig. 2 and demonstrate that similar levels of HUAEC adhesion can be achieved on each ECM substratum, but at different coating concentrations. The minimum coating concentrations required to achieve maximum cell adhesion were 2.5 μg/ml for col I, 5 μg/ml for FN, 10 μg/ml for each of VN and col IV and 40μg/ml for LM.

The surface densities of the ECM molecules corresponding to the minimal coating concentration for maximal cell adhesion in 96-well plates (listed above) and the same coating concentration in 24-well plates, are shown in Table 1. For each individual ECM molecule there was no significant difference between the surface density on the 96-well TCPS compared with the 24-well TCPS ($p > 0.05$, Student's t-test). Surface densities of VN and col I were similar but there were significant differences between these and the remaining ECM molecules. Significantly different surface densities were also observed between FN, col IV and LM ($p < 0.01$, Analysis of Variance and Student Newman Keul's test). The very high surface density of coated LM suggests either an end-on orientation of this molecule, or multilayering. That different surface densities of these ECM molecules are required to achieve the same degree of cell adhesion, indicates that either, 1) the integrins which recognise the different molecules show different adhesion efficiencies, or 2) the different ECM molecules are clustered differently on the TCPS surface, or 3) the different molecules show different degrees of denaturation, resulting from different isolation procedures.

The adsorption of FN and VN to TCPS from plasma is shown in Fig. 3. FN exhibited a Vroman effect at low plasma concentrations. Precoating with BSA eliminated all FN binding. The only conditions under which FN bound to the surface from high plasma concentrations were when collagen I, to which it specifically attaches, was precoated (Fig. 3A). In contrast VN bound to the surface in the presence of plasma proteins, whether or not the surface had been precoated with other proteins (Fig. 3B). Unlike FN, plasma VN does not bind to collagen type I. Such precoated molecules would be expected to inhibit the surface adsorption of VN by competition for protein binding sites but this did not occur to any significant extent. VN also exhibited a Vroman effect at low plasma concentrations. The amounts of FN bound to coated col I, and VN adsorbed to the surface, (both from undiluted plasma), were significant. Although the comparative ELISA absorbances of FN coated from PBS at 5μg/ml and VN coated from PBS at 10 μg/ml, (asterisks in Fig. 3), suggest that FN and VN coated on these surfaces from plasma may be sufficient to support cell adhesion, such results need to be interpreted with caution. It is possible that the monoclonal antibodies displayed different binding characteristics to FN and VN





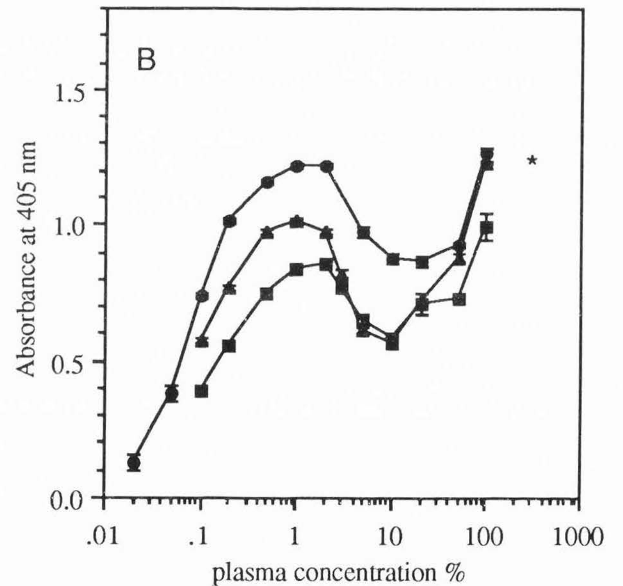


Figure 2. HUAEC adhesion to ECM molecules coated on tissue culture polystyrene. Dilutions of ECM molecules in PBS were incubated, 50 µl per well in 96-well TCPS plates, for 2h at 37°C. Following blocking with 1% BSA in PBS HUAECs were added in SFM, $3x10^4$ per well and incubated at 37°C for 2h. Cell adhesion, expressed as a percentage of the maximum value observed in a particular experiment, was estimated from the optical density of stained adherent cells. VN ■; FN ▲; LM □; col I ○; col IV △. Points are means and s.e.m. of three separate experiments. Arrows denote the minimal coating concentration giving a maximal adhesion response for each molecule and are referred to as 'medium' concen trations in subsequent figures.

Figure 3. (*at right*) Adsorption of VN and FN to surfaces from plasma. Wells of 96-well TCPS plates were coated with PBS or type I collagen as for Fig. 2. Following blocking with BSA, 50 µl volumes of citrated bovine plasma, or plasma dilutions in citrated PBS, were incubated per well for 3h at room temperature on a plate shaker. Similar plasma preparations were added to untreated wells and blocked with BSA after the 3h incubation. Dilutions of purified VN or FN in citrated PBS were similarly incubated in untreated wells and subsequently blocked. Bound VN and FN were detected by ELISA using monoclonal antibodies. Results from one experiment are shown, means and s.e.m. of 3 replicates. **A**: FN bound from plasma to ● untreated TCPS; ■ precoated col I; ▲ precoated BSA. * FN bound to untreated TCPS from 5 µg/ml in PBS. **B**: VN bound from plasma to ● untreated TCPS; ■ precoated col I; ▲ precoated BSA. * VN bound to untreated TCPS from 10 µg/ml in PBS.

adsorbed from plasma in the company of other plasma proteins, compared to purified molecules adsorbed from PBS, due to differences in protein conformation, spatial distribution and steric hindrance. We trust that such differences are not over-large. The two monoclonal antibodies used are fairly robust in their responses to FN and VN coated on different types of polystyrene and denatured vs native conformations.

Endothelial monolayer expansion and proliferation

In order to measure the expansion and proliferation of HUAECs from the same starting point of cell adhesion on each ECM surface, 24-well plates were coated with ECM molecules at the minimum concentrations

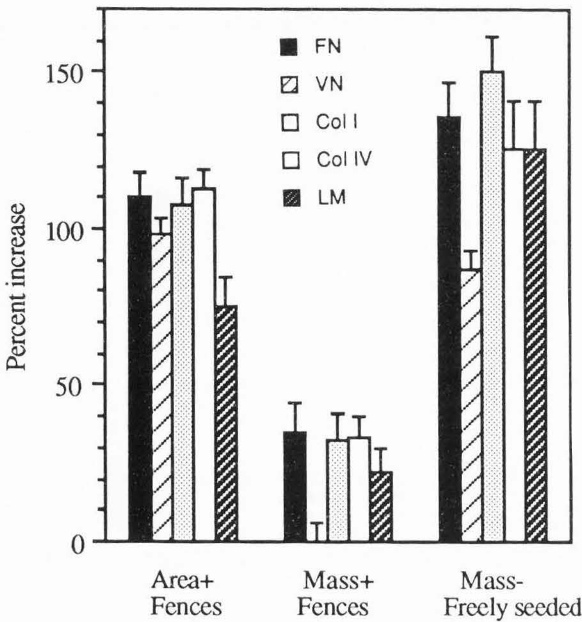


Figure 4. Effects of coated ECM on HUAEC expansion and proliferation. Wells of 24-well TCPS plates were coated with concentrations of ECM molecules as identified by arrows in Fig. 2. 1.5x10^4 HUAECs were seeded per well either inside fences or freely in the wells. Percent increase in culture area and cell mass were estimated after 6 days culture as described in Materials and Methods. The + signs on the abscissa represent cells seeded within fences and the minus sign represents freely seeded cells. Means and s.e.m. of eight separate experiments.

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required for maximal cell adhesion (see above). Experiments were also done with superoptimal coating concentrations. HUAECs were seeded either within fences to give information on sheet expansion and proliferation, or freely in the well to investigate the effects of removal of contact inhibition on cell proliferation. The results with either ECM coating concentration were essentially similar. There was no significant difference between 24 h adhesion of HUAECs to wells coated with any of the five ECM molecules ($p > 0.05$, Analysis of Variance), so the expansion assay was valid, as the starting point of equal cell adhesion on the different ECM molecules had been achieved.

Fig. 4 shows the combined expansion and proliferation results of eight separate experiments. LM yielded significantly lower increases in culture area than the other four ECM molecules ($p < 0.01$, Analysis of Variance and Student Newman Keul's test). In contrast, increase in cell mass of HUAECs seeded within fences

Table 1. Surface concentration in ng/cm^2 of ECM molecules coated on 96 or 24-well TCPS culture plates.

96 well plate: coating volume 0.05 ml

Coated molecule	FN[2]	VN[3]	LM[2]	colI[4]	col IV[2]
ng/cm^2 incubated	394	788	3152	197	788
ng/cm^2 bound[1]	**246**	**112**	**669**	**120**	**341**
SEM	24.9	12.5	64.8	7.7	19.2
N[5]	4	4	4	5	3

24-well plate: coating volume 1.00 ml

Coated molecule	FN[2]	VN[3]	LM[2]	colI[4]	col IV[2]
ng/ml incubated	1099	2198	8792	550	2198
ng/cm^2 bound[1]	**204**	**122**	**589**	**144**	**352**
SEM	14.6	4.1	57.6	10.1	19.1
N[5]	4	3	4	4	3

[1]Entries in bold are the means of three to five determinations estimated by method A[2], or method B[3] in Fig.1, or by a combination of both methods[4].
[5]N = number of determinations.

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was similar on all substrata except VN, on which there was very little growth at all. Microscopic examination of the cultures after monolayer expansion revealed that on all substrata except VN the monolayer was still confluent, with cells migrating outward at the edges. On VN, however, although the area of overall coverage was the same as on FN or collagens, the monolayer was no longer intact, due to cell detachment.

When the same number of HUAECs were seeded freely in the wells of the 24-well plates, in the absence of fences, cell growth was markedly increased over that observed in the fenced wells. The inhibitory effect of VN was still significant ($p < 0.05$, Analysis of Variance and Student Newman Keul's test). The dramatically greater proliferative response of HUAECs seeded freely in the well compared to cells seeded within fences suggested that cell : cell adhesion molecules in the latter may be contributing to reduced proliferative responses.

To test whether the effects of LM and VN, as compared to the other ECM molecules, were due to differential leaching of these ECM molecules from the TCPS surface, the rates of leaching of the individual molecules over a four day incubation, under the same conditions as the HUAEC expansion, were determined using biotin labelled molecules. The results are shown in Fig. 5. Significant leaching was only observed with col I and col IV (50% and 40% respectively), neither of which showed any tendency towards reduced migration or proliferation.

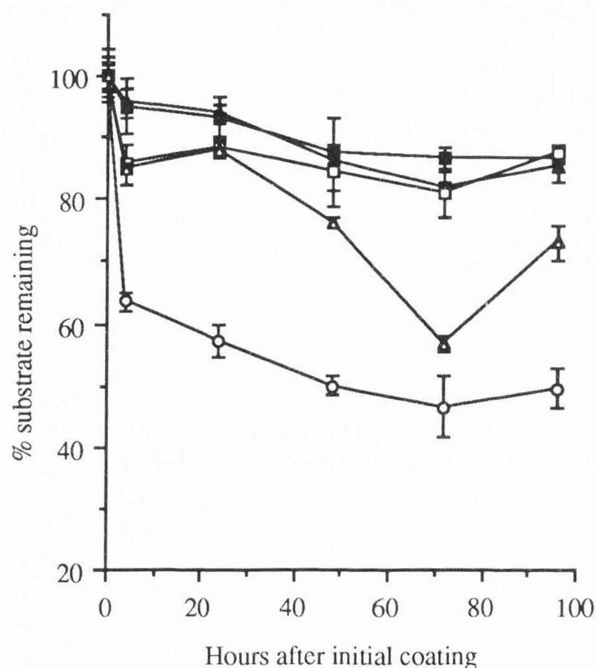

Figure 5. Persistence of adsorbed biotinylated ECM molecules over time. Wells of 96-well TCPS plates were coated as for cell adhesion, with concentrations of ECM molecules (as identified by arrows in Fig. 2) spiked with biotin labelled molecules. The plates were fed with culture medium and treated exactly as for HUAEC expansion assays, without the addition of cells. At various time points, biotin label remaining adsorbed to the wells was determined by ELISA. For each ECM molecule the percent remaining was determined at each time point, taking the ELISA OD at the end of the coating period as 100% (corrected for unlabelled negative controls). VN ■; FN ▲; LM □; col I ○; col IV △. Points are means and standard errors of four replicates from one experiment.

Alternatively the removal of essential factors from serum during the depletion of FN and VN could be involved in differential ECM responses. To test for the existence of a critical serum factor, fenced and unfenced cultures were set up on each substratum using DDS or complete FCS as medium supplement. There was no difference in cell proliferation between these two media on any substratum (data not shown). These results indicate that the observed effects on the VN and LM substrata are substratum-mediated rather than due to differences in trophic support by medium factors.

Effect of substratum on spread cell areas

Since proliferation of HUAECs was similar on LM compared with that on FN or collagens, the observed reduced increase in culture area on LM could have been due to reduced cell spreading on this surface. The morphology of cells seeded on LM, FN or VN at low density, and cells which had reached confluence is shown in Fig. 6. Individual cell areas were measured on the low density cells. The mean results were 3237 μm^2 (standard error of the mean, s.e.m. 344) for LM, 4292 μm^2 (s.e.m. 337) for FN and 4825 μm^2 (s.e.m. 319) for VN. The areas on LM were significantly lower than on the other two substrata ($p < 0.05$, Analysis of Variance and Student Newman Keul's test), but the difference between FN and VN was not significant. Calculations of individual cell areas in the confluent cultures, based on the number of cells present per field in Fig. 6 D-F (60, 48, 42 for LM, FN, and VN respectively) yielded estimates of 2125 μm^2 for LM, 2656 μm^2 for FN and 3036 μm^2 for VN. Although the cell areas were smaller when the cells were confluent, the relative cell areas on each subset were remarkably similar when normalised to the mean area across substrata. For LM, FN and VN these relative cell areas were 0.815, 1.019 and 1.165 for the dispersed cells and 0.786, 1.042 and 1.172 for the confluent cells. It is worthy of note that VN, having the lowest surface density, exhibited the greatest cell spreading (indicating the strongest cell-surface adhesion), while LM having the highest surface density, exhibited the lowest cell spreading.

Status of the plasminogen system on different ECM substrata

Proliferation of HUAECs on VN was minimal, while cell spreading on this surface was maximal. Reduced proliferation could have been due to poor release of focal contacts required for the changes in cell shape accompanying mitosis. To investigate this possibility, activity of the plasminogen system in the HUAEC cell layer was assessed by estimation of the levels of uPA and PAI-1 in the cell layer. The results are shown in Fig. 7. The expression of uPA on the cell surface (cells fixed in formol saline), was not significantly different between substrata ($p > 0.05$, Analysis of Variance). In the total cell layer (including the ECM, cells fixed in methanol/ethanol), however, expression of uPA was significantly lower on VN than on the other four substrata ($p < 0.01$, Analysis of Variance and Student Newman Keul's test). The amount of uPA in the total cell layer was considerably higher than on the apical cell surface on all substrata, suggesting a basolateral location. In contrast to uPA, the amount of PAI-1 both in the ECM and in the total cell layer was considerably higher on VN than on other substrata ($p < 0.01$, Analysis of Variance and Student Newman Keul's test). Similar amounts of PAI-1 were

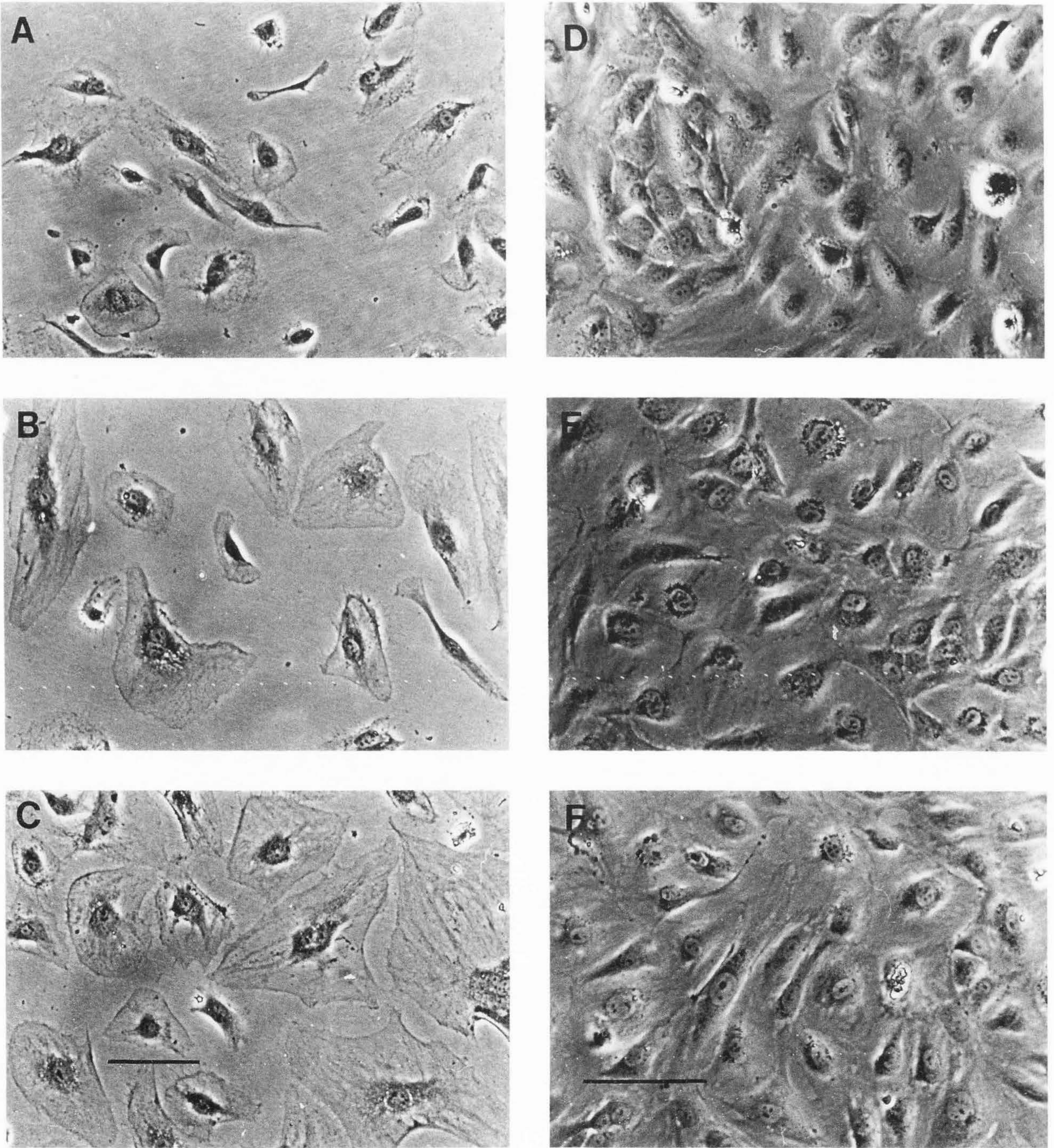

Figure 6. Phase contrast micrographs of HUAECs cultured on LM, FN or VN. 35 mm TCPS dishes were coated with medium concentrations of LM (A and D), FN (B and E) or VN (C and F) as for Fig. 4. HUAECs were seeded at $1x10^4$ (A-C) or $1x10^5$ (D-F) cells per dish and cultured for 24 h (A-C) or to confluence (4 days, D-F). Cultures A-C were formalin fixed and stained with methylene blue. Cultures D-F were photographed without fixation or staining. The bars represent 100 μm for (A-C) or (D-F), respectively.

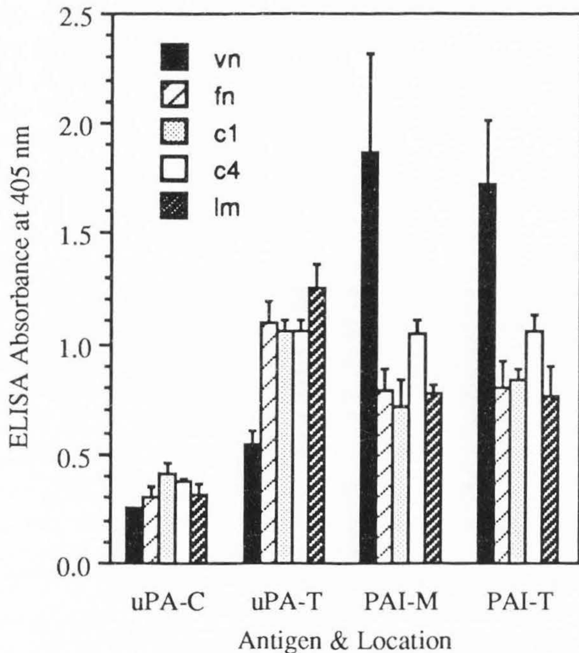

Figure 7. Status of the plasminogen system in the HUAEC cell layer. Wells of 96 well TCPS plates were coated with ECM molecules at concentrations identified from cell adhesion (arrowed in Fig. 2). HUAECs were seeded at $1x10^4$ per well and grown for 24 h as described in Materials and Methods. The presence of uPA and PAI-1 were detected by ELISA. Expression of uPA on cell surfaces (uPA-C) was estimated on formalin fixed cultures. uPA and PAI-1 in the total cell layer (uPA-T and PAI-T) were estimated from methanol: ethanol fixed cultures and PAI-1 in the ECM (PAI-M) was estimated from cell-free ECM. Means and s.e.m. of 3 separate experiments.

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detected in the ECM and in the total cell layer suggesting that most of the PAI-1 in the latter was in fact localised to the ECM.

Discussion

The experiments described in this paper compared the sheet expansion and proliferation of cultured HUAECs on VN with other coated ECM molecules. The results of this study are directly relevant to understanding the relationship between cell adhesion and spreading, and proliferation and migration responses, as is discussed below. Understanding these principles is fundamental to the design of surfaces for enhanced endothelialisation.

Similar levels of initial HUAEC adhesion to each of the five ECM molecules studied were obtainable, but required a range of coating concentrations. This is in agreement with our previous findings using bovine corneal endothelial cells (Underwood and Bennett, 1993). The use of ECM coating conditions which resulted in a similar biological response, i.e., cell adhesion, allowed us to compare sheet migration and proliferation from the same biological starting point on each coated ECM molecule, rather than the more common practice of using the same coating concentration of each.

Specific effects of vitronectin and laminin on endothelial expansion and proliferation

We have demonstrated that LM reduced the sheet migration of HUAECs while VN inhibited their proliferation (both with and without cell-cell contact). Many detailed analyses of proliferation and migration of subconfluent endothelial cells, (from both capillary and large vessels), have suggested that these responses are directly controlled by cell and nuclear area, regardless of the particular ECM molecule coated on the surface (Folkman and Moscona, 1978; Ingber *et al.*, 1987; Ingber and Folkman, 1989; Ingber, 1990). According to these authors increased surface concentration of ECM molecules increases cell spreading and increases DNA synthesis. On the other hand, cell migration is reduced by cell spreading and therefore optimal at lower surface ECM concentrations. According to this theory one would expect HUAECs, which occupy the smallest spread areas on LM, to exhibit the fastest migration on this surface, compared to FN or VN. Our results show that this is clearly not the case when cells are migrating as a monolayer. The outward expansion in area of the HUAECs was lowest on the LM surface. The gross area occupied by the cells was clearly limited by the degree to which the cells spread on the substratum, and outward cell migration was restricted by cell-cell contact. Cell-cell contact also reduced the proliferation rates of HUAECs by up to three-fold compared with those of cells seeded with few cell-cell contacts. This well known phenomenon of contact inhibition is thought to be mediated by specific cell-cell adhesion molecules such as endothelial cadherin and platelet/endothelial cell adhesion molecule (PECAM) (Lampugnani *et al.*, 1992; DeLisser *et al.*, 1994), although constriction of spread cell areas in the confluent culture may also play a part.

The paradigm of control of cell proliferation rates by cell/nuclear areas (Ingber *et al.*, 1987), breaks down in our system, with respect to HUAEC proliferation on both LM and VN coated surfaces. On LM the HUAEC proliferation rate was similar to that on FN, while the spread cell areas on LM were significantly lower. On VN, where spread cell areas were the largest, proliferation was the lowest. We suggest that these low

proliferation rates are due to increased strength of attachment of HUAECs to the VN coated surface, such that the cells are unable to complete the cell cycle. Similar findings have been reported by Madri *et al.* (1988), who used a sheet expansion assay to investigate the migration and proliferation of bovine aortal endothelial cells. In their system very high surface concentrations of FN yielded maximal spread cell areas and minimal proliferation rates. As we found with HUAECs, they observed similar cell proliferation rates on LM and collagens, while LM produced significantly smaller spread cell areas and sheet migration rates.

We suggest that the correlation between spread cell areas and cell proliferation or cell migration originally proposed, (Folkman and Moscona, 1978; Ingber *et al.*, 1987; Ingber and Folkman, 1989; Ingber, 1990), can be modified by the following factors. A) Cell-cell contact, as would exist at the endothelial wound edge, restricts outward migration such that surfaces which encourage individual cell spreading would support greater sheet migration, in opposition to effects on the migration of individual cells. B) Cell-cell contact limits cell proliferation, either by signalling from cell-cell adhesion molecules, or by reducing individual cell-surface contact areas, or a combination of both. C) Individual cell-ECM receptor interactions modulate the degree of control of cell area over proliferation, such that cells occupying smaller surface areas on LM proliferate at similar rates to cells occupying larger surface areas on FN. D) There is an optimum degree of cell adhesion to the ECM substratum, above which proliferation is inhibited. In the system described by Madri *et al.* (1988), high concentrations of surface FN may have resulted in inappropriate interaction of cellular integrins and consequent lack of correct proliferation signals. Our observations of poor proliferation of HUAECs on VN are unlikely to be explained by the same mechanism, as cell spreading on this surface, although greater than on FN, was not significantly so. We suggest that higher adhesive strength on VN was generated by lower plasminogen activation, as discussed below.

Role of plasminogen activation in cell substratum adhesion

We have demonstrated in this paper that HUAECs adhere and spread efficiently on a VN coated surface. We have also previously shown that human umbilical vein cells can attach efficiently to VN (Steele *et al.*, 1993). In the present work we have shown that with extended culture time on a VN substratum, HUAECs grew poorly and tended to detach from the surface. This is in contrast to bovine endothelial cells which can be cultured long term on VN and grow as efficiently as on other ECM substrata (Underwood and Bennett, 1993). We present evidence that the poor proliferation of human endothelial cells on VN may be related to the strength of cell adhesion to the culture surface, controlled by the plasminogen system. Previous reports have shown that proliferating and migrating endothelial cells increase expression of uPA and its receptor (Pepper *et al.*, 1992, 1993). On all substrata we found that the amount of HUAEC uPA was higher in the total cell layer than exposed on the cell surface, suggesting a basolateral distribution, in agreement with previous findings, and supporting the idea of a role of uPA in local proteolysis of the ECM (van Hinsbergh, 1992) . We found significantly reduced expression of uPA in the cell layer of HUAECs grown on VN, compared to other ECM substrata. This is in agreement with previous findings (Ciambrone and McKeown-Longo, 1992), which described increased synthesis of uPA by HT-1080 cells on FN compared to VN. In contrast to uPA, we found levels of its inhibitor, PAI-1, were much higher in the cell layer of HUAECs grown on VN compared with the other matrix molecules, and that virtually all of the PAI-1 was located in the ECM. Increased localisation of PAI-1 on VN substrata has also been reported for human umbilical vein endothelial cells (HUVECs) (Grulich-Henn *et al.*,1992) and HT-1080 cells (Ciambrone and McKeown-Longo, 1992), and is probably due to specific binding of secreted PAI-1 to VN rather than increased synthesis. Recent reports (Walz and Chapman, 1994; Wei *et al.*, 1994; Moser *et al.*, 1995) have demonstrated that VN can bind to both uPA and its receptor, and that binding to uPA and PAI-1 can occur concurrently. This provides a mechanism of efficient transfer of PAI-1 from VN to receptor-bound uPA. VN and PAI-1 have been found co-localised in atherosclerotic lesions (Lupu *et al.*, 1995), and Ciambrone and McKeown-Longo (1990, 1992) have shown that binding of PAI-1 to VN substrata results in a decrease in HT-1080 cell-associated plasmin activity and increased cell adhesion to the substratum. These reports, together with our finding of reduced immunolocalised uPA and increased PAI-1 in the cell layer of HUAECs on VN, suggest that the decreased proliferation of HUAECs on VN is due to their superoptimal surface contact with the substratum brought about by reduced localised plasminogen activation. Further inhibition of endothelial cell proliferation may result from increased production of transforming growth factor (TGF)β as a result of VN binding as recently suggested (Ribeiro *et al.*, 1995).

Importance of plasma proteins in responses to vascular injury

VN is present at high levels (200 to 300 μg/ml) in

circulating plasma and is secreted by activated platelets. In plasma it is normally found in a compact, relatively non-reactive state but readily unfolds into a highly reactive molecule when exposed to ECM or non-biological surfaces (Tomasini and Mosher, 1991; Preissner *et al.*, 1993). We have shown here that VN will adsorb to tissue culture polystyrene from plasma whether or not the surface has been precoated with other ECM molecules, and other reports have demonstrated competitive surface adsorption of VN from plasma (Bale *et al.* 1989; Horbett, 1994). It has been suggested that VN localisation at sites of vascular injury may significantly modulate endothelial recovery (Preissner *et al.* 1993). The high affinity of VN for binding to exposed surfaces gives a high likelihood of significant VN coating of implanted stents and vascular graft material, rendering them both thrombogenic due to platelet adhesion, and inhibitory to endothelial coverage. Human vascular smooth muscle cells, on the other hand, readily grow on VN coated surfaces (unpublished observations), and such a surface has been shown to cause modulation of primary cultured rabbit smooth muscle cells to a proliferative form more readily than other coated ECM molecules (Hayward *et al.*, 1995). Such a combination of responses would encourage vascular hyperplasia and ultimate restenosis.

When fabricating a device to control vascular lumen size, such as a stent or artificial vascular graft, it is desirable to provide a surface which will encourage endothelial recovery. It is therefore important to determine interactions of such surfaces with cell adhesive proteins in the blood such as VN and FN. It is also important to determine the effect of such surfaces on the deposition of ECM molecules by the endothelial cells themselves, as such deposited molecules, for example LM, may also influence outward migration (Madri and Stenn, 1982; Clyman *et al.*, 1994).

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Discussion with Reviewers

W. van Oeveren: In the present paper and in the available references describing vitronectin binding, various polymers have been used as artificial (test) surfaces. In the introduction and discussion the authors also mention stents. Since these permanent implants in narrowed arteries are used in an increasing number, their potential complications are of great importance. Despite their limited surface area, stents frequently induce thrombotic events and intimal hyperplasia, in part due to delayed recovery of endothelial coverage and function. In general, stents are made from titanium or other metals. Do the authors know if vitronectin binding to these materials is similar to polymers and/or is a specific polymer coating with reduced vitronectin binding required for stents?

Authors: In preliminary experiments we have found that VN binds to stainless steel and titanium. Endothelial responses *in vitro* to such coated materials have not yet been investigated and will be a topic of future study.

W. van Oeveren: Endothelial cell seeding is experimentally often used before implantation of smaller caliber vascular grafts. This method did not prove very successful after implantation, in part because endothelial cells are rapidly washed off the surface at high blood shear. Could this be due to insufficient vitronectin in the endothelial cell suspension or is this observation challenging your conclusion, which is based on experiments without a flow system?

Authors: To our knowledge vitronectin pre-coating has not been tried with endothelial cell seeding, although fibronectin pre-coating has. Attachment of endothelial cells seeded on an untreated graft surface would depend upon adsorption of vitronectin or fibronectin from the culture medium to the particular graft material. The strength of such cell adhesion has not been experimentally compared with pre-coated vitronectin in either static or flow conditions. Our experiments on culture of HUAECs on coated vitronectin show that although the cells exhibit maximal adhesion and spreading, they proliferate poorly and over time they detach from the surface. We assume that the increased adhesion strength, which we postulate interferes with cell replication, eventually leads to cell death and subsequent detachment. Pretreatment of the graft surface with a mixture of ECM cell-adhesive molecules may be optimal for maintaining seeded endothelial cells on the graft surface in flow conditions, whilst not compromising their normal function.