

Objective: To investigate whether endothelial monolayer permeability changes induced by inflammatory mediators are affected by the extracellular matrix protein used for cell seeding.

Methods: Human umbilical venular endothelial cells (HUVEC) were grown to confluent monolayers on membranes coated with either collagen, fibronectin or gelatin. The permeability to albumin and dextran was then assessed, both under normal conditions and after treatment with tumor necrosis factor-alpha (TNF- α) and bacterial lipopolysaccharide (LPS).

Results: With any of the three protein coatings, tight junctions were formed all over the monolayers. The permeability of the coated membranes to albumin and dextran was reduced strongly by confluent monolayers; the relative reduction was similar for the three matrix proteins used. Pre-incubation of the monolayers with either TNF- α or LPS increased permeability dose dependently. However, the relative increase due to either treatment was independent of the protein used for membrane coating.

Conclusion: The extracellular matrix protein used for initial seeding of endothelial cultures plays a minor role in determining the permeability changes induced in HUVEC monolayers by inflammatory mediators.

Key words: Endothelium, Extracellular matrix (ECM), HUVEC, Inflammatory mediator, Tumor necrosis factor, endotoxin, monolayer permeability

Permeability characteristics of human endothelial monolayers seeded on different extracellular matrix proteins

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Introduction

The vascular endothelium acts as a dynamic barrier that selectively restricts the passage of plasma and cells from the blood into the adjacent tissues. Local and reversible alterations of barrier function typically occur as part of the immune response during which inflammatory mediators cause a transient increase of vascular permeability resulting in the formation of tissue oedema. However, during systemic inflammatory response syndrome (SIRS),¹ over-production of inflammatory mediators may irreversibly damage vascular integrity and cause excessive loss of fluid from the circulation. This hazardous event may lead to prolonged tissue hypoperfusion, organ dysfunction and death.² Therefore, prevention of vascular damage could contribute to improved survival of patients suffering from SIRS.^{3,4} Many have studied the role of the endothelium in vascular permeability using *in vitro* models of endothelial monolayers cultured on semi-permeable supports.⁵⁻⁷ Multiple mechanisms may be involved in increased endothelial permeability, such as cell contraction and retraction,⁸ enhanced transcellular vesicle transport,⁹ disruption of intercellular junctions¹⁰ and apoptosis.¹¹

Recently, it has been suggested that endothelial permeability may also depend on the composition

and structure of the underlying extracellular matrix (ECM).¹² For example, it has been shown that fibronectin significantly contributes to endothelial monolayer permeability by providing attachment sites for ECM proteins and endothelial cells.¹³ When monolayers cultured on fibronectin were treated with tumor necrosis factor- α (TNF- α), a permeability increase was observed which was accompanied by a loss of fibronectin from the ECM.¹⁴ TNF- α also affects the expression of other matrix receptors on endothelial cells, confirming a role for endothelial - ECM contacts in vascular permeability.¹⁵ Furthermore, stimulation of endothelial cells with TNF- α induced the expression of a 96-kDa matrix metalloproteinase,¹⁶ suggesting that matrix degradation may be involved in increased permeability.¹⁷

For the purpose of studying endothelial permeability *in vitro*, these findings seem to imply that endothelial cells should be cultured on (a mixture of) ECM proteins, preferably containing fibronectin, but other ECM coatings like gelatin and different types of collagen are used frequently.¹⁸ It remains unclear whether different coatings eventually result in different monolayer permeability changes under the influence of inflammatory mediators. A comparison of *in vitro* studies on endothelial permeability is hampered by the many differences in the origin and species of

the endothelial cells and, particularly, by the differences in the design of the monolayer system, including the matrix used for cell seeding.

Therefore, we have compared the permeability characteristics of human umbilical venular endothelial cells (HUVEC) seeded on membranes coated with either collagen, fibronectin or gelatin. For this purpose, we have selected a suitable *in vitro* model that supports the attachment and growth of HUVEC, and allows restricted diffusion of macromolecules, visual examination by both bright field microscopy and ultramicroscopy, and easy and simultaneous permeability determinations. The inflammatory mediators TNF- α and bacterial lipopolysaccharide (LPS), which are involved in the onset of SIRS, have been shown to enhance endothelial permeability both *in vivo* and *in vitro*.¹⁹⁻²² We measured the permeability for both albumin and dextran in untreated HUVEC monolayers and in cultures after treatment with either TNF- α or LPS.

Materials and methods

Materials

Culture medium M199, supplemented with 25 mmol/l HEPES, Earl's salts and L-glutamine, as well as heat-inactivated newborn calf serum (NCS), penicillin-streptomycin and trypsin/EDTA were obtained from Life Technologies (Paisley, Scotland). Normal human serum was supplied by a local transfusion service. A pool of serum of at least five donors was first heat inactivated (30 min at 56°C), and stored at -20°C before use. Transwell-well culture inserts, tissue culture plates and flasks were all from Costar (Cambridge, MA). Fluorescin-labelled bovine serum albumin (FITC-BSA), rhodamin-labelled dextran (RITC-DEX, mean molecular weight 70,000), collagenase type I (from *Clostridium histolyticum*), pig skin gelatin (type A) and LPS (*E. coli*, strain 055:B5) were purchased from Sigma Chemical (St Louis, MO). Human fibronectin was obtained from Boehringer Mannheim (Mannheim, Germany). A crude fraction of endothelial cell growth factors (ECGF) was kindly provided by the Department of Pediatrics, University Hospital of Nijmegen, The Netherlands. Heparin was obtained from Leo pharmaceutical products (Weesp, The Netherlands). Recombinant human TNF- α (6×10^7 U/mg, endotoxin content < 1 EU/mg) was obtained from Bender & Co, Vienna.

Isolation and culture of HUVEC

Endothelial cells were isolated according to a modification of the method described by Jaffe and co-workers.²³ Upon delivery the umbilical cords were placed in sterile, ice cold cord buffer (140 mmol/l NaCl, 4 mmol/l KCl, 11 mmol/l D-glucose, 10 mmol/l

HEPES, 100 IU penicillin, and 0,1 mg/ml streptomycin, pH 7,3) and stored at 4°C until use. In brief, the umbilical vein was rinsed with cord buffer to remove blood and debris from the lumen after which the vein was filled with medium M199 containing 0.1% (w/v) collagenase. Endothelial cells were detached by incubation for 20 min at 37°C and harvested by collecting the collagenase solution. After centrifugation (5 min at $300 \times g$), the cells were resuspended in complete medium (M199 with 10% NCS, 10% normal human serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, 150 μ g/ml crude ECGF and 5 U/ml heparin) and seeded onto 0.1% gelatin-coated 25 cm² culture flasks. The cultures were kept in a humidified incubator at 37°C under a 5% CO₂/95% air atmosphere. After one day, and then three times weekly, the culture medium was refreshed. Confluent cultures (typically yielding 10^5 cells/cm² or more) were passaged by detaching the cells with 0.5 mg/ml trypsin and 0.2 mg/ml EDTA, which was followed by centrifugation and resuspension in complete medium. For appropriate growth, a split ratio of 1:2 or 1:3 was used. All experiments were performed on cells that had been passaged less than four times.

Endothelial origin of the cultures was confirmed by phase contrast microscopy, which revealed confluent cultures displaying a typical cobblestone morphology, while immunocytochemical analysis revealed positive staining for von Willebrand Factor and CD31 (platelet endothelial cell adhesion molecule (PECAM)-1). Activation with TNF- α or LPS-induced expression of intercellular adhesion molecule (ICAM)-1 and E-selectin (data not shown).

Preparation of microporous membranes and cell seeding

For permeability determinations, two different culture inserts were used, one containing a polyester (PE) membrane (Transwell Clear) and the other containing a collagen pre-treated polyethylene terephthalate (PTFE) membrane (Transwell COL). Both types of membrane contained 0.4 μ m diameter pores and a growth area of 1 cm². However, differences existed in pore density and membrane thickness. While the PTFE membranes were obtained with a collagen (bovine, type I/III) coating in place, and thus were ready for culturing, the PE membranes were prepared for cell seeding by overnight incubation at room temperature with either fibronectin (10 μ g/cm²), or gelatin (10 μ g/cm²) in medium M199. No significant difference in permeability was observed between uncoated and coated PE membranes (data not shown).

The inserts were placed in 12-well culture plates, resulting in a two-compartment system separated by the membrane. Approximately 10^5 cells/cm² in 0.5 ml of complete medium were seeded at the upper side of the membrane, whereas 1.5 ml of complete medium

was added to the lower compartment. These volumes prevented hydrostatic fluid pressures across the membranes. Both compartments were frequently replenished with complete medium as described. Cultures were grown for six days, resulting in the formation of confluent monolayers, which was confirmed by phase contrast light microscopy.

In one experiment, the confluent cell layer was selectively removed from the membranes. This was achieved by briefly incubating the monolayers with a 25 mmol/l NH_4OH solution. Removal of attaching cells from the membrane was confirmed by transmission electron microscopy examination.

Transmission electron microscopy of HUVEC monolayers

Cultured cells on Transwell inserts were washed twice with serum-free medium prior to fixation with 2% glutaraldehyde in phosphate-buffered saline (PBS). After 60 min of fixation the Transwells were washed with PBS and treated with 1% OsO_4 for 30 min and washed again with PBS. After several steps of dehydration, with 30%-70%-90%-100% alcohol, followed by 1:1 mix, 1:2 mix and pure epon 812, the membranes were dissected from the Transwells with a small diamond knife and flat embedded in pure epon 812. Ultrathin sections were stained with uranyl acetate and lead nitrate. The sections were examined with a JEOL 1200 EX/II electron microscope (Tokyo, Japan) at 60 kV.

TNF- α and LPS treatment

Dilutions of TNF- α (50, 500 pg/ml and 10 ng/ml) or LPS (50,500 ng/ml and 10 $\mu\text{g}/\text{ml}$) were prepared in complete medium. In the LPS experiments, medium containing 20% normal human serum was used instead of heat-inactivated human and calf sera. At the start of the experiment 0.5 ml of TNF- α - or LPS-containing medium was added to the upper compartment of the chamber. After overnight incubation at 37°C, the permeability of the monolayers was determined.

Permeability studies

For the detection of macromolecular passage across the membranes, a tracer solution containing both FITC-labelled BSA (250 $\mu\text{g}/\text{ml}$) and RITC-labelled dextran (250 $\mu\text{g}/\text{ml}$) was prepared in complete medium. In the upper compartment, culture medium was replaced by 0.5 ml of the tracer solution, while the lower volume was refreshed with 1.5 ml complete medium. After 1, 6 and 24 h of diffusion, 250 μl samples were drawn from the lower compartment, which was replenished with an equal volume fresh medium each time. The collected samples were

diluted with 1 ml PBS and the concentrations of FITC-BSA and RITC-DEX were measured using a Hitachi 5000 fluorophotospectrometer, at emission/excitation wavelengths of 495/520 nm and 560/580 nm, respectively. The albumin and dextran concentrations in the samples were calculated using linear regression of a diluted series of the tracer.

Statistical analysis

Differences between permeability to albumin and dextran were calculated by paired Student t-test. In order to control for the different membrane types used in our studies, the permeability of untreated HUVEC monolayers was calculated as a ratio of the corresponding unseeded membranes. Similarly, the effect of treatment of monolayers on different membranes was compared to control monolayers cultured on corresponding membranes. Differences between permeability ratios were calculated by Kruskal-Wallis non-parametric ANOVA.

Results

Permeability of membranes with different protein coatings and without seeded cells

Membranes coated with collagen, fibronectin or gelatin showed significantly different permeability rates. The diffusion of both albumin and dextran occurred faster across the collagen-coated membranes than across the fibronectin or gelatin-coated membranes (Fig. 1). Permeability to albumin and dextran appeared to be essentially the same, although average dextran concentrations in the lower compartment were almost invariably slightly less than albumin concentrations. However, during the permeability assay this difference never reached statistical significance.

After 1 h of diffusion, the concentrations of albumin and dextran in the lower compartment of the collagen-coated membrane inserts were $11.2 \pm 4.5 \mu\text{g}/\text{ml}$ and $10.1 \pm 4.4 \mu\text{g}/\text{ml}$, respectively. This accounted for approximately 17% of the calculated equilibrium concentration of 62.5 $\mu\text{g}/\text{ml}$. Significantly less albumin and dextran accumulated in the lower compartment of the gelatin- or fibronectin-coated membranes, both allowing less than 2 $\mu\text{g}/\text{ml}$ during the first hour, or about 5% of the calculated equilibrium concentration. At the end of the assay, after 24 h, these differences were still significant, although less explicit: the tracer concentration in the lower compartment of the collagen-coated membrane inserts were $49.4 \pm 4.3 \mu\text{g}/\text{ml}$ (for albumin) and $46.4 \pm 4.9 \mu\text{g}/\text{ml}$ (for dextran), while less than 34 $\mu\text{g}/\text{ml}$ of either molecule had accumulated in the lower compartment of the fibronectin or gelatin-coated membrane inserts.

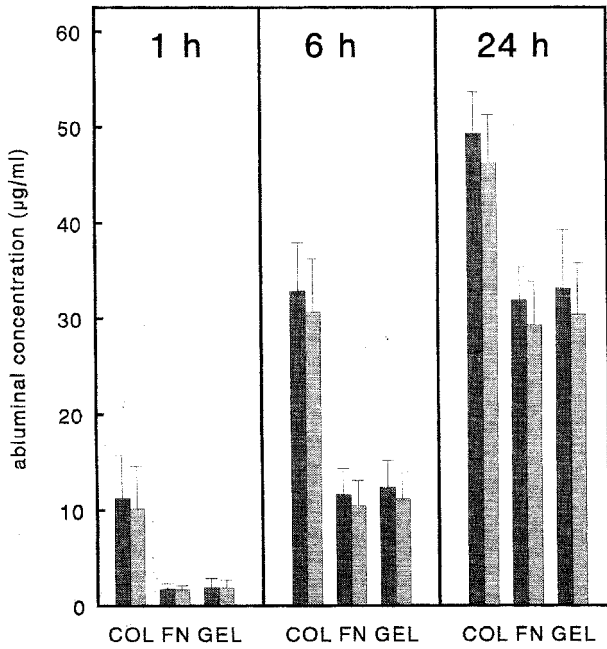


FIG. 1. Permeability of Transwell culture inserts for FITC-albumin and RITC-dextran. Bars represent the albumin concentration in µg/ml of permeated albumin (hatched bars) and dextran (filled bars) after 1 (left panel), 6 (middle panel) and 24 (right panel) h of equilibration. Data represent at least four independent measurements originating from two or more experiments and are expressed as mean ± SD. Culture inserts: COL = collagen coated, FN = fibronectin coated, GEL = gelatin coated.

Permeability of HUVEC monolayers

Culturing of HUVEC for six days resulted in the growth of confluent monolayers that had formed tight junctions throughout the culture. As shown in Fig. 2, these junctions were present between cells grown on all three different matrix proteins. On all membranes the permeability to albumin and dextran was significantly restricted by HUVEC monolayers (Fig. 3). Although monolayers cultured on collagen-coated PTFE membranes allowed more albumin and dextran passage than monolayers cultured on gelatin- or fibronectin-coated PE membranes, comparing the permeability to the corresponding unseeded membranes showed that the relative restriction to albumin and dextran by HUVEC monolayers was similar for the three coatings. For instance, after 1 h, monolayers cultured on collagen, fibronectin or gelatin reduced passage of albumin by $87.0 \pm 4.8\%$ ($n = 12$), $79.8 \pm 5.6\%$ ($n = 4$) or $82.2 \pm 4.7\%$ ($n = 4$), respectively, when compared to the permeability of unseeded membranes. After 24 h, these percentages were 55.5 ± 7.2 , 61.7 ± 1.9 and 59.3 ± 8.2 , respectively. Similar data could be calculated for the dextran permeability, but the relative restriction was not significantly different between the three coatings.

In order to investigate whether the extracellular matrix, produced by the cells during culture, differently contributed to the permeability after initial seeding on either protein, HUVEC were grown for six days and the permeability was measured. Thereafter, the cells were lysed, leaving membranes with matrix (coating plus the extracellular matrix laid down by the cells) only and the permeability was measured again. After cell lysis, albumin permeability measured after 1 h of diffusion was increased by a factor 4.4 ± 0.8 ($n = 4$) on collagen-coated membranes, 4.9 ± 0.1 on fibronectin-coated membranes and 4.5 ± 0.1 on gelatin-coated membranes. Dextran permeability changed accordingly, but as with albumin, the permeability increase after cell lysis did not significantly differ between the collagen-, gelatin- and fibronectin-coated membranes.

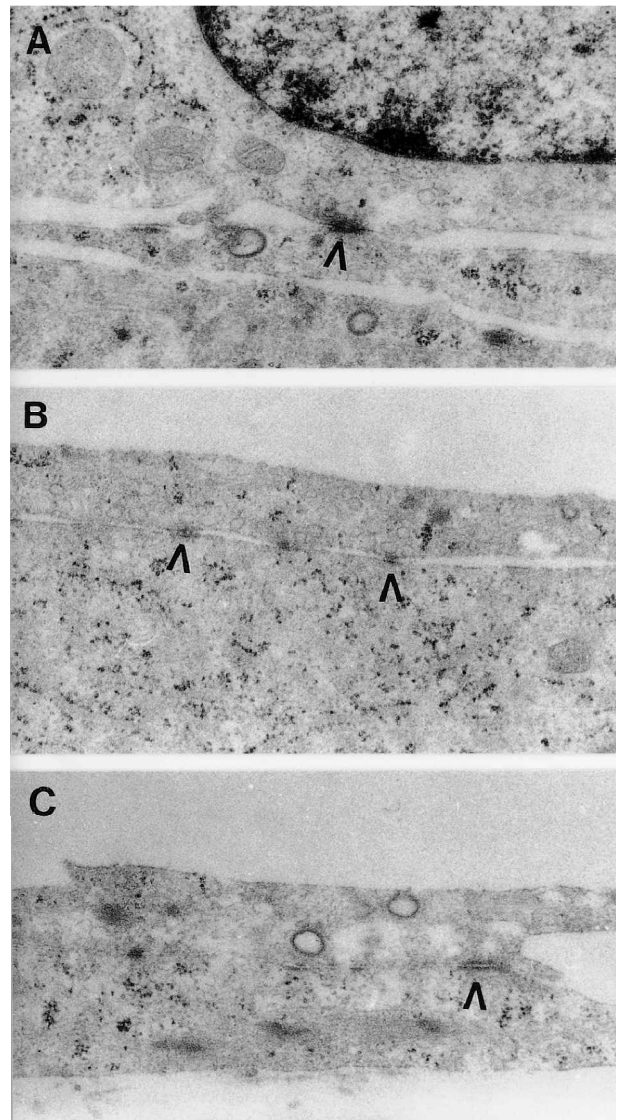


FIG. 2. Ultrastructural characteristics of HUVEC monolayers cultured on different matrices. HUVEC cultured on (A) collagen- (B) fibronectin- and (C) gelatin-coated membranes. Note junctional complexes between adjacent cells (arrowheads). Magnification 30,000x.

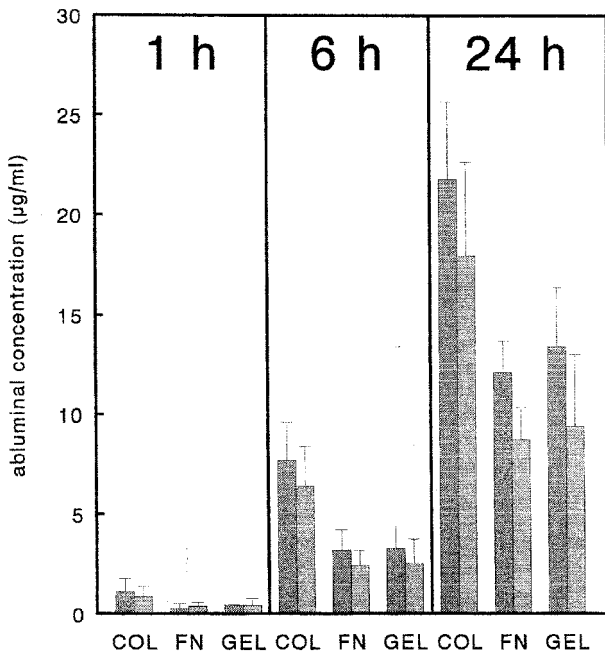


FIG. 3. Permeability of HUVEC monolayers cultured on collagen, fibronectin and gelatin. Bars represent the albuminal concentration in $\mu\text{g/ml}$ of permeated albumin (hatched bars) and dextran (filled bars) in time (cf. legend to Fig. 1). Data represent at least four independent measurements originating from two or more experiments and are expressed as mean \pm SD.

Permeability of TNF- α or LPS-pretreated HUVEC monolayers

Pre-incubation of the monolayers with increasing doses of TNF- α or LPS resulted in a dose-dependent increase in monolayer permeability. In order to allow a quantitative comparison of the effects induced by these inflammatory mediators on the monolayers grown on the different coatings, permeability was expressed as the ratio between treated and untreated monolayers. While a dose of 50 $\mu\text{g/ml}$ of TNF- α was insufficient to enhance the permeability for albumin, a dose of 500 $\mu\text{g/ml}$ of TNF- α induced a moderate permeability increase of the monolayers cultured on collagen, fibronectin or gelatin (Fig. 4A). A further stimulation was observed at a concentration of 10 ng/ml TNF- α . After 1 h of diffusion, the relative increase ranged from 2.0 ± 0.4 (on gelatin) to 2.3 ± 0.2 (on collagen). The TNF- α effect could be observed on all the coatings and no significant differences were observed between monolayers cultured on either collagen, gelatin or fibronectin. After 6 and 24 h of diffusion, the permeability difference between TNF- α treated monolayers and control monolayers gradually declined, resulting in a ratio close to 1. Still, at the highest dose the effect seemed persistent for at least 24 h: at this time the ratio between treated and non-treated monolayers ranged between 1.2 ± 0.2 (on collagen) to 1.3 ± 0.04 (on gelatin). A similar picture emerged for the permeability to dextran (Fig. 4B).

Pre-incubation with bacterial LPS also induced a dose-dependent increase in permeability which, like in the TNF- α experiments, seemed qualitatively and quantitatively independent of the protein on which the monolayers were initially cultured. However, the enhanced permeability seemed less persistent after LPS pre-incubation than after pre-incubation with TNF- α . After 1 h of diffusion, 500 ng/ml LPS had induced an albumin permeability which was a factor 1.2 ± 0.2 (on fibronectin) to 1.5 ± 0.3 (on collagen) higher than the permeability in untreated monolayers (Fig. 5A). At 10 $\mu\text{g/ml}$ LPS a further increase was observed ranging from a factor 1.9 ± 0.7 (on gelatin) to 2.3 ± 0.2 (on collagen). Similar values were obtained from dextran measurements (Fig. 5B). However, during further incubation of albumin and dextran on LPS-treated monolayers a rapid decline towards control monolayer permeability was observed, reaching a ratio after 6 and 24 h that was not significantly different from 1.

Discussion

We have investigated the influence of different, but commonly used, matrix protein substrates on the permeability of resting, and TNF- α - or LPS-treated HUVEC monolayers and found no essential differences between cells seeded on collagen, fibronectin or gelatin.

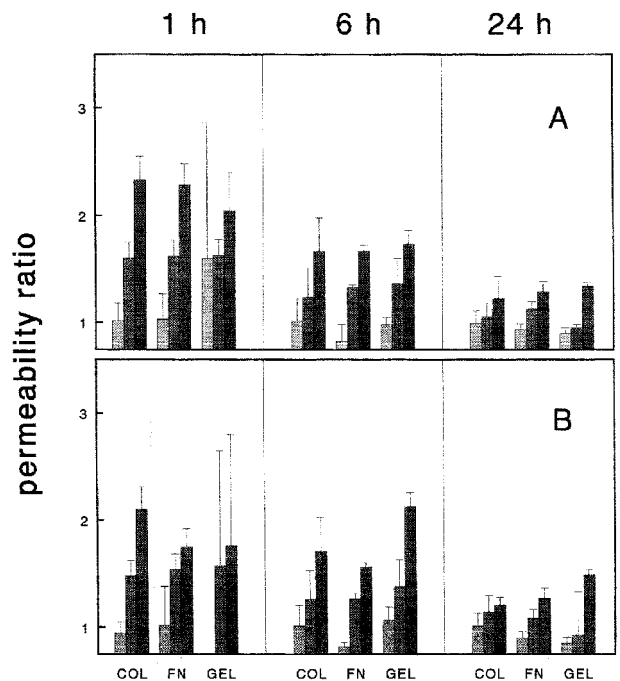


FIG. 4. Permeability of TNF- α -treated HUVEC monolayers. The increase in permeability to albumin (panel A) and dextran (panel B) is expressed as the permeability ratio (\pm SD, $n = 4$) between treated and untreated monolayers. In panels, from left to right, the permeability ratio of monolayers cultured on collagen (COL), fibronectin (FN) and gelatin (GEL), after 1, 6 and 24 h of diffusion is shown. Open bars: 50 $\mu\text{g/ml}$ TNF- α ; grey bars: 500 $\mu\text{g/ml}$ TNF- α ; black bars: 10 ng/ml TNF- α .



FIG. 5. Permeability of LPS-treated HUVEC monolayers. The increase in permeability to albumin (panel A) and dextran (panel B) is expressed as the permeability ratio (\pm SD, $n = 4$) between treated and untreated monolayers. In panels, from left to right, the permeability ratio of monolayers cultured on collagen (COL), fibronectin (FN) and gelatin (GEL), after 1, 6 and 24 h of diffusion is shown. Open bars: 50 ng/ml LPS; grey bars: 500 ng/ml LPS; black bars: 10 μ g/ml LPS.

One could think of several reasons for such a difference to exist. The attachment and growth of endothelial cells *in vitro* depends at least in part on specific binding sites provided by the ECM. Also, the ECM is involved in mechanisms by which inflammatory mediators cause increased endothelial permeability and loss of monolayer integrity. These receptor-mediated mechanisms include: disruption of cell-cell contacts by cytoskeletal and/or junctional reorganization and alteration of endothelial adhesion molecule expression,²⁴ disruption of cell-matrix contacts by altering endothelial matrix receptor expression and loss of cell attachments sites in the ECM;²⁵ disruption of matrix-matrix contacts by proteolytic degradation and remodelling of the ECM.¹⁶ Despite the apparent contribution of ECM proteins on endothelial permeability, different ECM proteins, as well as mixtures of ECM proteins, have been used for endothelial cell culturing.

For the evaluation of endothelial monolayer permeability, multi-well culture inserts suited with microporous membranes were used in this study. A major advantage of these culture inserts is that they allow an easy and multiple analysis of monolayer permeability, with only minor modifications of the standard culture conditions. Moreover, these inserts all allowed visual examination of the monolayer before and after the permeability assay. Across unseeded membranes, the

accumulation of albumin appeared always slightly faster than that of dextran. Physical properties, like shape, diameter and charge, of these molecules could be responsible for this difference. Untreated cultured HUVEC monolayers, and TNF- α - or LPS-treated monolayers, did not show a clear preference for the passage of either albumin or dextran. Since albumin passage occurs by both active vesicular and paracellular transport, while dextran only passively diffuses through the intercellular clefts,^{9,26} we conclude that in our experiments diffusion via the transcellular route contributes less to the permeability of resting and activated monolayers and that the majority of albumin and dextran pass the endothelial monolayer by means of the paracellular route.

HUVEC monolayers cultured on collagen-, fibronectin- or gelatin-coated membranes did not display different morphologic characteristics, as was revealed by light and transmission electron microscopy. All monolayers displayed typical cobblestone morphology and tight junctions were abundantly present between neighbouring cells. Confluence was usually achieved within six days and was independent of the type of coating used. In the permeability experiments all differences observed between the monolayers cultured on the three proteins could be attributed to the original differences observed in the unseeded filters. Thus, collagen, fibronectin and gelatin are all suitable as a substrate for the attachment and growth of endothelial cells *in vitro*, and in our model, these substrates do not induce different responses to inflammatory mediators. We assume that additional ECM proteins, produced by the endothelial cells and laid down into the ECM, mediate most of the endothelial-ECM interactions involved in monolayer permeability. The production of ECM proteins, including fibronectin, laminin and type IV collagen, by endothelial cells *in vitro* has been previously shown by others.²⁷ In our experiments we did not see a difference in the membrane permeability changes induced by a procedure which removed the cells while leaving the ECM on the membrane. Although the eventual composition of the ECM may differ between monolayers cultured on collagen, fibronectin and gelatin, its contribution to the permeability of untreated and TNF- α or LPS-HUVEC monolayers apparently is of minor significance. When the monolayers were exposed to TNF- α , a dose-dependent increase of permeability was observed, which is in accordance with previous observations.²⁰ Although loss of fibronectin has been associated with a TNF- α -mediated increase of permeability,¹² we did not observe a difference between monolayers cultured on collagen-, fibronectin- or gelatin-coated membranes. Possibly, in our experiments TNF- α treatment did not induce significant matrix degradation or matrix degradation equally affected the three ECM proteins investigated.

When compared to TNF- α , LPS pre-treatment resulted in a moderate and more transient increase of permeability, suggesting that continuous exposure of LPS may be required for a prolonged permeability enhancement. Others have shown that the effect of LPS on endothelial permeability depends on the availability of soluble CD14 and lipopolysaccharide binding protein in serum.²⁸ Further studies are needed to reveal eventual differences between TNF- α - and LPS-induced endothelial permeability and the reversibility of these changes.

In conclusion, we have demonstrated that cultured HUVEC monolayers seeded on various proteins are equally suitable as a model for vascular permeability, showing physiologically relevant responses to inflammatory stimuli. Apparently, the initial differences in cellular environment are unimportant with respect to the eventual permeability characteristics of the confluent monolayer, possibly as a result of additional ECM laid down during culture.

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