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The rotating disc as a device to study the adhesive properties of endothelial cells under differential shear stresses

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Hemocompatibility can be conferred on a biomaterial by covering this material with a monolayer of endothelial cells. The endothelial cell is an epithelial cell of mesenchymal origin, that features a specific phenotype with homotypic intercellular interactions and with specialized cell–matrix interactions. These interactions are mandatory to the normal barrier function and the non-thrombogenicity of the endothelial monolayer and are maintained *in vivo* at shear stresses ranging from 10^{-5} to 10^{-3} N cm^{-2}. The endothelial monolayer grafted on a biomaterial should meet similar requirements. We have constructed a rotating disc device to investigate the effects of differential shear stresses on cell–cell and cell–matrix interactions in a monolayer of endothelial cells grafted on a disc-shaped biomaterial. The range of shear stresses that are being applied by the device vary from 0–10^{-4} N cm^{-2} to 0–2×10^{-3} N cm^{-2}. In a series of experiments with discs of plasma discharge treated polycarbonate (PC) that are coated with fibronectin, it has been shown that a monolayer of endothelial cells grafted on these discs starts to lose intercellular contacts and cell–fibronectin interactions at shear stresses of 10^{-4} N cm^{-2}. Coating of the PC discs with a complex extracellular matrix, synthesized by arterial smooth muscle cells in culture, prior to endothelial cell seeding results in the formation of a monolayer, which retains its integrity at shear stresses up to 2×10^{-3} N cm^{-2}.

1. Introduction

To confer hemocompatibility on biomaterials which must interface with blood on a long-term basis, by coating them with the natural non-thrombogenic lining of the blood vessel, the endothelium [1], is a concept currently under investigation in many research laboratories and in clinics. Endothelial cells are anchorage-dependent mesenchymal cells that line the lumen of blood vessels. The cells assemble through homotypic intercellular contacts into a monolayer that separates physically blood constituents from structures of the subendothelium. Specific interactions anchor the cells to the extracellular matrix, which is a subendothelial meshwork of predominantly glycoproteins and proteoglycans [2]. The matrix glycoproteins fibronectin, vitronectin, laminin, and von Willebrand Factor are well studied for their capacity to mediate endothelial cell attachment. They all contain linear amino acid sequences, that are recognized by members of a family of transmembrane receptors, the integrins (for reviews see [3, 4]).

In the cardiovascular system the blood flow exercises hemodynamic forces on the endothelial cells. Under physiologic conditions the cells resist these forces and the sheet of endothelium remains intact by virtue of the specialized homotypic intercellular and cell–matrix interactions.

Cell attachment to a biomaterial can be improved by precoating the material with the glycoprotein fibronectin or by the coupling of an integrin specific peptide to the surface [5]. The fibronectin and the peptide bridge between the material surface and the cell by invoking the integrin receptors. Spreading, growth and monolayer formation occur at such coated surfaces, at least *in vitro* under static conditions. *In vivo* the cells are submitted to hemodynamic forces. The monolayer covering a biomaterial should remain intact under these stringent conditions in order to satisfy its intended purpose.

Here we report the results of studies with cultured human umbilical vein endothelial cells and a rotating disc device to introduce differential shear stresses to a monolayer of cells grafted on a coated disc-shaped biomaterial. A monolayer of cells attached to a simple substratum of fibronectin on the support has little capacity to maintain its integrity during the application of shear stresses. A monolayer of cells on a complex matrix that was synthesized by smooth muscle cells shows, however, a well-developed feature of resisting high shear stresses.

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2. Materials and methods

2.1. Cell culture

Endothelial cells (HUVEC) were isolated from veins of freshly obtained human umbilical cords and cultured essentially according to the method of Jaffe *et al.* [6]. Briefly, endothelial cells were grown to confluence at 37 °C and 5% CO_2 in culture medium (RPMI 1640, Flow laboratories), supplemented with 20% Newborn Calf Serum (Gibco), heparin (50 $\mu g\,ml^{-1}$, Sigma H.8514), bovine brain ECGF (20 $\mu g\,ml^{-1}$, Boehringer Mannheim), L-glutamine (2 mM, Flow Lab.), streptomycine (100 $\mu g\,ml^{-1}$, Flow Lab.) and penicillin (100 IU ml^{-1}, Flow Lab.) in tissue culture flasks (25 cm^2, Costar) coated with human plasma fibronectin. At confluency ($4–6 \times 10^4$ cells cm^{-2}) cells were passaged using trypsin (Flow Lab.) as follows. The cells were washed with phosphate buffered saline (PBS). After aspiration of the PBS 1 ml of a 0.25% (w/v) trypsin solution in PBS was added. When the trypsin solution covered fully the monolayer surface it was removed immediately to limit trypsin action. The remaining trypsin detached the cells within 5 min. To inhibit trypsin, 4 ml of culture medium was then added. The suspended cells were further diluted with culture medium to a certain count per ml and replated. All experiments were performed with cells of the first passage.

Arterial smooth muscle cells (SMC) were derived from tissue explants of human umbilical cord arteries as described by Ross [7]. The SMC were further cultured on fibronectin-coated tissue culture polystryrene in RPMI 1640, supplemented with 20% Newborn Calf Serum, L-glutamine (2 mM), streptomycine (100 $\mu g\,ml^{-1}$) and penicillin (100 IU ml^{-1}. SMC were passaged by trypsin as described above.

2.2. Preparation of polycarbonate discs for cell seeding

Transparent polycarbonate (PC) discs of 16 mm diameter and 2 mm height were cut out of PC-plates of LEXAN PA-III 2.2347. The PC-discs were washed with 2-propanol, air-dried and then submitted to three cycles of plasma discharge treatment in air at an output energy of 0.6 kW per cycle with an electrode distance of 3 mm (Sherman Treaters, Tame Oxon UK). The treated PC-discs were sterilized by autoclaving for 20 min at 120 °C prior to their use in the experiments.

2.3. HUVEC monolayer establishment on PC-discs coated with fibronectin or with the extracellular matrix of SMC

HUVEC were detached from the culture flask as described above and plated with 5×10^4 cells cm^{-2} on a fibronectin or extracellular matrix coated PC-disc in wells of a 12-well plate (Costar).

The fibronectin coating was achieved by physically adsorbing fibronectin to the PC-discs from a solution of 10 $\mu g\,ml^{-1}$ fibronectin in PBS for 1 h at room temperature. In order to coat the PC disc with extracellular matrix, SMC were plated on a fibronectin coated PC-disc with a density of 5×10^4 cells cm^{-2}. The cells were allowed to attach, spread and grow in culture medium for 48 h. Then the medium was replaced by a solution of 0.1 M NH_4OH and the plate was incubated in this solution for 15 min at room temperature. This procedure removed the cells while leaving behind the extracellular matrix on the PC-disc. The matrix was washed with PBS and immediately seeded with HUVEC as described for the fibronectin-coated PC-discs. The plated HUVEC were kept in culture medium at 37 °C and 5% CO_2 and, if relevant, the culture medium was changed every 48 h. At predetermined time points, the PC-discs with the HUVEC were carefully washed with Hepes buffered RPMI 1640 (Flow Lab.) and either subjected immediately to the analysis procedures or were first submittted to shear stresses using a rotating disc device as described below.

2.4. Rotating disc device and rotation procedure

The shear stress was applied to the endothelial cells on the PC-discs using a rotating disc device. that was modified from a version of the apparatus previously described [8] (Fig. 1). The essential modification is the addition of a collar around the disc holder and four triangular planes mounted perpendicularly on the bottom of the rotation chamber in order to minimize circumferential movement of the bulk liquid around the imaginary rotation axis during the experiment. PC-discs covered with endothelial cells were mounted on the nylon holder. The holder with disc was then

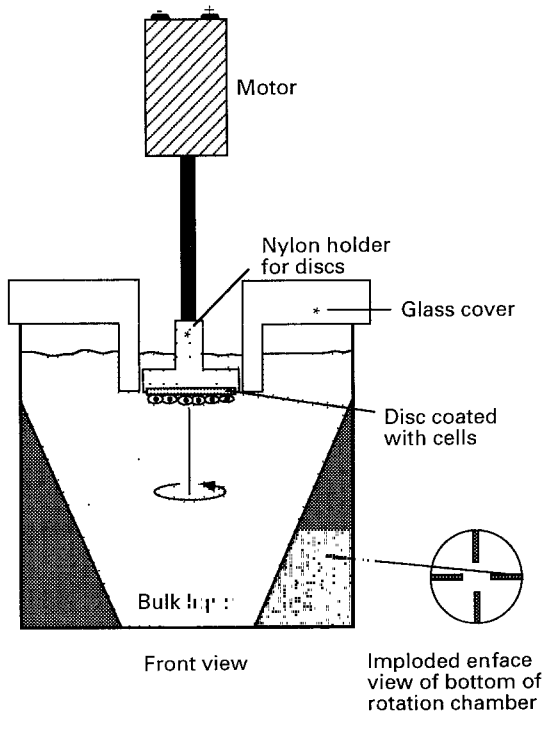

Figure 1 Schematic presentation of the rotating disc apparatus. The device consists of a round glass rotation chamber ($h = Ø = 40$ mm). Four triangular planes are mounted at the bottom of the chamber to break any rotational movement of the bulk liquid in the plane of the disc. The disc ($Ø = 16$ mm) is connected via a nylon holder to the shaft of the motor. The nylon holder is inserted into a glass collar to minimize forces acting on the bulk liquid during rotation.

inserted via the collar into the rotation chamber containing prewarmed rotation buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, 4 mM glutamine and 1 mg ml^{-1} BSA). Much care was taken to avoid exposing the cells to air during the transfer. The motor was connected to a tunable voltage output power supply to allow the setting of the angular velocity. Rotation was performed for 30 min at 37 °C.

2.5. Cell–cell contact analysis

The intercellular contacts of the cells on the PC-disc were evaluated by two staining procedures. (i) Silver staining: the HUVEC were stained with silver nitrate by a modification of the procedure of Poole *et al.* [9]. The cell layer on a PC-disc was washed with RPMI 1640, supplemented with 10% Newborn Calf Serum (NCS). The PC-disc was then immersed sequentially in the following solutions. 0.25% (w/v) $AgNO_3$ in water for 30–40 s, 5% (w/v) glucose in water, three times for 10 s, and finally in 4% (w/v) formaldehyde in Hepes-buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl). In this latter solution the PC-disc is exposed to u.v. light (254 nm) for 1 min. The disc was then dehydrated in methanol for 10 min and air-dried. At this point the disc entered the second procedure. (ii) Immunofluorescent staining of PECAM-1: the PC-disc treated as described above was rehydrated in RPMI 1640, 10% NCS for 15 min and then incubated sequentially with CLB-HEC 75 and anti-mouse IgG-fluorescein (Dako-Patts), all diluted in RPMI 1640, 10% NCS. Washing in between the incubations with antibodies was carried out with PBS, containing 0.05% Tween 20. The stained PC-disc was photographed using an Olympus BH-2, equipped with phase contrast and fluorescence optics.

2.6. Analysis of extracellular matrix exposure

Exposure of extracellular matrix was evaluated using antibodies directed against fibronectin and von Willebrand Factor (vWF). The PC-discs with HUVEC, either rotated or not, were fixed in 4% (w/v) formaldehyde in Hepes-buffer for 1 h in ice. Fibronectin and vWF were stained by indirect immunofluorescence using anti-fibronectin (CLB-HEC 140) or anti-vWF (Dako-Patts), respectively. In some experiments extracellular matrix was exposed fully by removing the cells from the PC-discs using 0.1 M NH_4OH as described above.

2.7. Transmission electron microscopy (TEM)

The PC-discs with HUVEC, either rotated or not, were fixed in 2.5% (w/v) glutytraldehyde in PBS at 4 °C for 1 h. After fixation the discs were rinsed twice in PBS and then post-fixed in 1% (w/v) OsO_4 in PBS at 4 °C for 1 h. Subsequently the discs were rinsed in PBS and dehydrated in a graded series of ethanol and embedded in Epon 812. The embedding in Epon 812 of a PC-disc occurred in physically separated circular compartments (diameter 1.5 mm) with known positions on the surface of the disc. Hence, the sections of a PC-disc prepared from these embedded compartments for visualization by TEM could be directly related to the shear stresses they were subjected to in the experiment. Ultra-thin sections were cut and contrasted with a solution of 7% (w/v) uranyl-acetate in distilled water (5 min), followed by immersion in lead citrate for 2 min according to Reynolds [10]. The contrasted sections were examined with an electron microscope (Philips CM10) at 80 kV.

3. Results and discussion

3.1. Rotating disc properties

The hydrodynamic properties of a system in which round disc rotates around an imaginary central axis in a round reaction chamber containing fluid at rest, was described by Levich [11]. The mathematical descriptions apply only under the assumption that the liquid column does not rotate around the same imaginary axis. In order to prevent this as much as possible the apparatus as described by Pratt *et al.* [4] was modified by placing a collar around the rotating disc holder and four triangular planes on the bottom of the rotation chamber (Fig. 1).

The shear stress at the surface of the rotating disc is described as follows:

$$\tau = 0.7996\, r\, \rho\, \nu^{1/2}\, \omega^{3/2}$$

where r is the distance to the centre [cm], ρ is the density of the rotation buffer [1.008 g cm^{-3}], ν is the kinematic viscosity of this buffer [0.0069 $cm^2\, s^{-1}$] and ω is the angular velocity [rad s^{-1}]. At given ρ, ν and ω the shear stress at the surface of the disc depends linearly on the distance to the centre of the disc. Hence, a range of shear stresses can be applied to a single monolayer of cells and the broadness of the range can be set by the speed of rotation.

Before performing the experiments the voltage settings of the power supply were calibrated in terms of angular velocity using a stroboscope with tunable frequency.

3.2. Establishment of a monolayer of HUVEC on a coated PC-disc

When HUVEC are seeded on a fibronectin-coated PC-disc with a density of 5×10^4 cells cm^{-2}, a typical time-dependent formation of a monolayer is observed. This monolayer formation is monitored by using two different staining procedures for cell–cell contacts. (i) Polyanionic structures of unknown chemistry accumulate at the intercellular contact regions and can be visualized by silver precipitates; (ii) PECAM-1, an endothelial protein of the immunoglobulin gene superfamily, accumulates at the cell–cell borders of confluent monolayers [12] and is involved in cell–cell adhesion [13].

Freshly seeded cells attach to the surface as rounded structures and start to spread immediately. Within 40 min attached cells are spread and cell–cell contacts start to develop, as indicated by occasionally occurring silver lines. PECAM-1 present in granular

structures during adhesion and spreading of the cells becomes redistributed to the cell borders around 40 min post-seeding. At 24 h after seeding a monolayer of endothelial cells is established with pronounced cell–cell contacts as illustrated by the continuous silver lines and the PECAM-1 distribution (Fig. 2).

Examination of the extracellular matrix of a 24-h-old monolayer shows a fibrillar network of fibronectin with vWF deposited in small patches (Fig. 3). The fibronectin probably originates from the first coating procedure. Endothelial and serum derived fibronectin, however, may also be incorporated in this network. Seeding of the cells on uncoated PC-plates does result in adhesion and spreading of the cells but never in the formation of an intact monolayer with cobblestone morphology (not shown). The vWF in the matrix is of endothelial origin.

Electron microscopic analysis (TEM) of cross-sections shows a continuous layer of endothelial cells on the fibronectin-coated PC-discs. The cells are arranged in monolayer side-by-side with regions of overlap similar to those seen *in vivo*. The cross-sections that we have examined show very few junctions between adjacent cells. It is known that passaged endothelial cells on a fibronectin coating establish junctions less frequently than primary cultures [14]. In contrast, electron dense regions could be observed at the albuminal plasma membrane, indicating adhesion plaque formation (Fig. 4). When endothelial cells are seeded on a preformed matrix of smooth muscle cells no appreciable differences could be observed in attachment, spreading and monolayer formation, compared to a coating of fibronectin. TEM reveals the presence of adhesion plaques and, in comparison to a monolayer on fibronectin, frequently occurring electron dense regions on the borders between cells, indicating junction formation between adjacent cells (Fig. 4). This suggests that the extracellular matrix plays a role in the establishment and organization of intercellular contacts. Whereas silver lines and PECAM-1-mediated cell–cell adhesion are similar on a monocomponent and a complex matrix, the latter induces a more frequent appearance of electron dense junctions between the cells.

3.3. Effects of shear stresses on the integrity of the monolayer

Except for junctions, no differences appear to exist between monolayers of HUVEC formed on a simple substratum of fibronectin and a complex extracellular matrix of SMC. This holds for the static condition. Application of shear stresses to these monolayers reveals a substantial difference. A 24-h-old HUVEC monolayer that is formed on a fibronectin substratum is damaged by shear stresses of 10^{-4} N cm^{-2}. Intercellular contacts are disrupted and subendothelial structures become exposed (Figs 5 and 6). At 7 $\times 10^{-4}$ N cm^{-2} the endothelial layer is increasingly damaged and cells are dislodged from the surface. Cell–cell contacts are disrupted and PECAM-1 is redistributed in the remaining attached cells from the cell borders to granular structures. Similar phenomena are observed for 96-h-old monolayers.

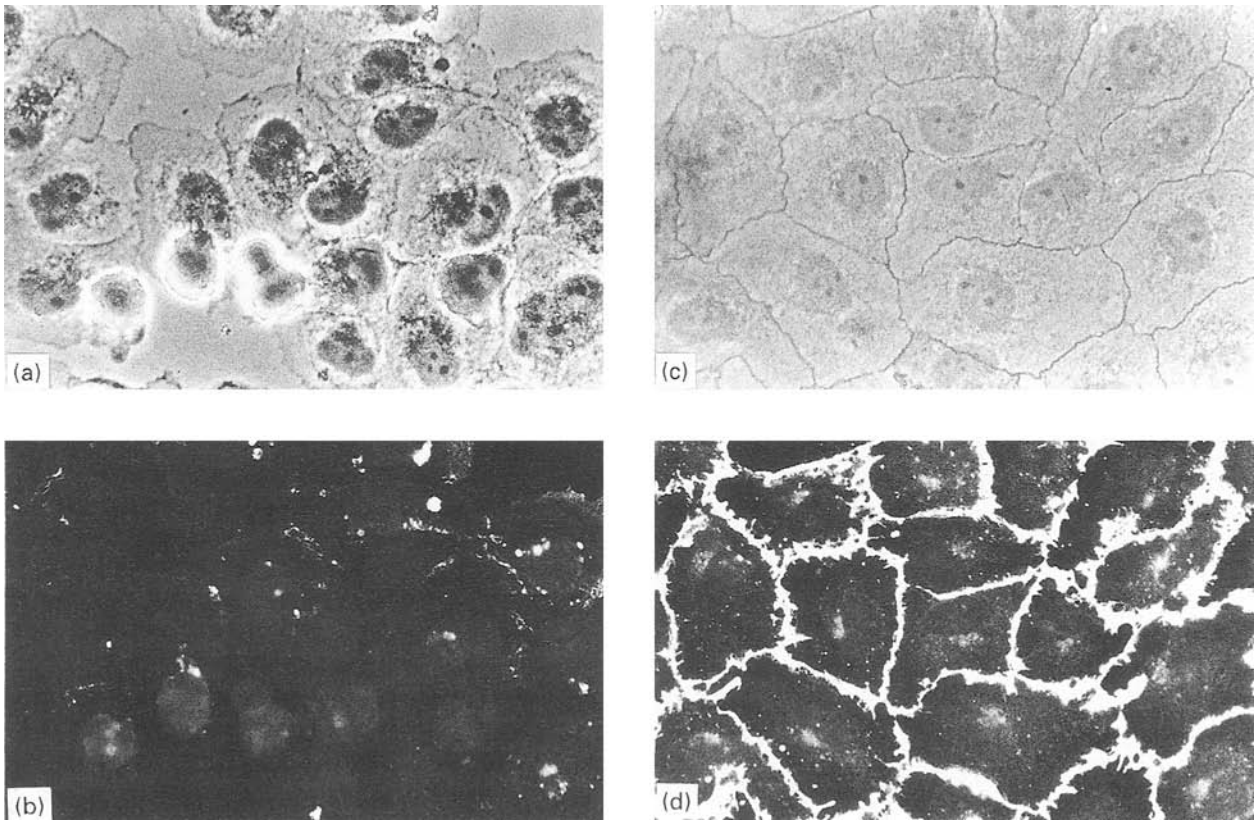

Figure 2 Establishment of a HUVEC monolayer on a fibronectin-coated PC-disc. The cells are seeded on the disc at a density of 50 000 cells cm^{-2}. After 40 min (a,b) and 24 h (c,d) the discs are processed, first for silver staining (a,c) and second for indirect immunofluorescent staining of PECAM-1 (c,d). (a) and (b) cover the same area on a single disc as (c) and (d).

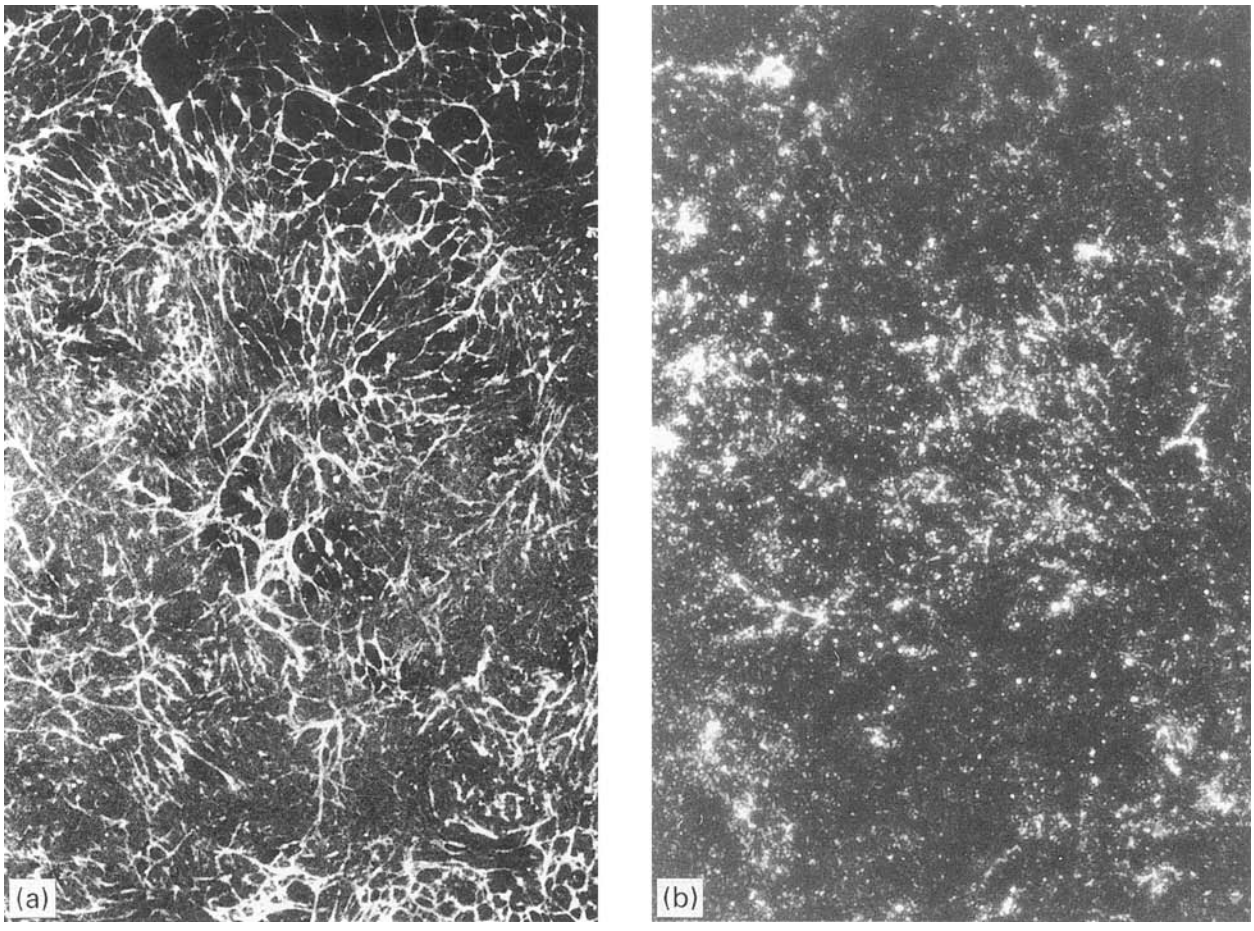

Figure 3 Indirect immunofluorescent staining of fibronectin (a) and von Willebrand Factor (b) of the extracellular matrix formed by a 24-h-old HUVEC monolayer on a fibronectin-coated PC-disc.

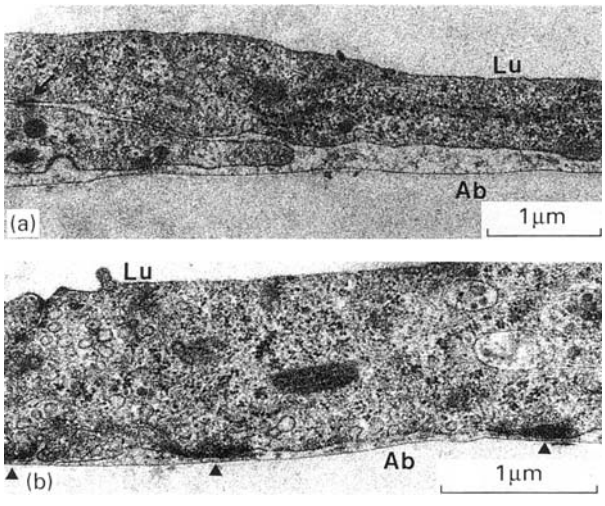

Figure 4 Transmission electron microscopy of cross-sections of a HUVEC monolayer on a PC-disc covered with the extracellular matrix of smooth muscle cells. A 24-h-old HUVEC monolayer was processed for TEM. (a) Close contacts with junctions (arrow) between adjacent cells. (b) Adhesion plaques (arrowheads) formed on the abluminal side of the cells. Panel (b) is also representative for a HUVEC monolayer on a fibronectin-coated PC-disc. Lu = luminal side of the cell, Ab = abluminal side of the cell.

The shear stress exposed areas are still covered with a fibrillar network of fibronectin (Fig. 6), indicating that the shear stress detaches the cells from the fibronectin coating. Whether integrin–fibronectin interactions are disconnected, or cells detach while leaving behind integrin clusters (a phenomenon observed for motile fibroblasts [15]), is a subject for further investigation.

As a result of the stress, vWF no longer exhibits an evenly patched distribution but accumulates around the remaining attached cells, from which also long filament-like vWF-containing structures depart parallel to the vector of the shear stress (Fig. 6). The intensity of the vWF staining is increased suggesting that the shear stress induced vWF release. In contrast to the above-mentioned events, a 24-h-old HUVEC monolayer on a substratum of SMC extracellular matrix resists shear stresses up to 2×10^{-3} N cm^{-2} and retains its integrity with intact intercellular contacts under these gravitating conditions (Fig. 7). This suggests that the endothelial sheet interacts more strongly on a complex matrix than on a simple substratum of fibronectin, although the latter likely becomes increasingly complex in time due to deposition of extracellular matrix proteins by the attached endothelial cells. It is postulated that endothelial cells strengthen their adhesion to a monocomponent matrix by deposition of adhesive proteins like fibronectin, laminin and collagens [16]. This deposition occurs within 4 h of seeding. Our results show that such mechanisms do not necessarily imply strengthened shear stress resistance.

In the light of these results it is tempting to speculate that intercellular junctions are more important for

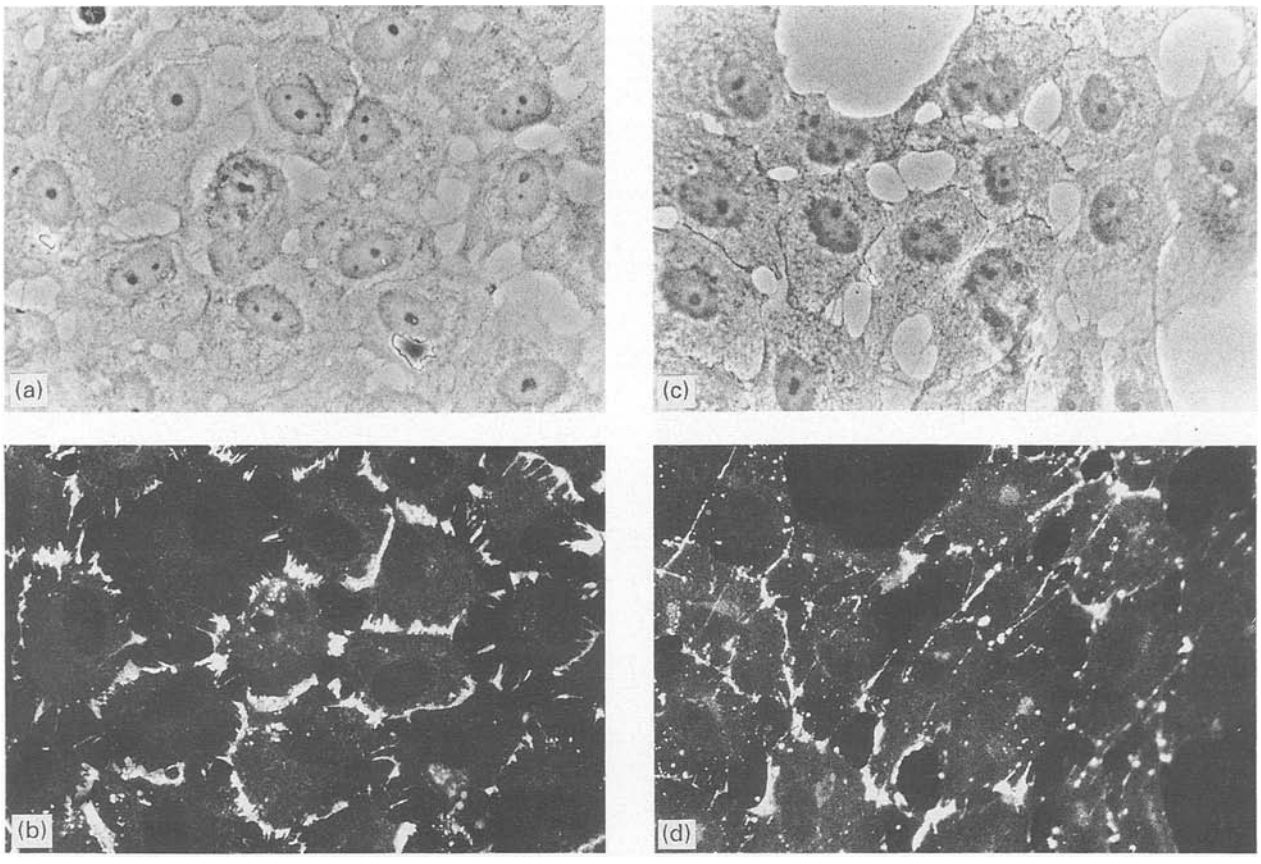

Figure 5 Shear stress induced loss of integrity of a HUVEC monolayer on a fibronectin-coated PC-disc. A 24-h-old HUVEC monolayer is submitted to shear stresses in the rotating disc device. Following rotation the disc is processed for silver staining (a,c) and indirect immunofluorescent staining of PECAM-1 (b,d). Photographs are taken at positions on the disc that correspond to 10^{-4} N cm^{-2} (a,b) and 7×10^{-4} N cm^{-2} (c,d). Pairs (a) and (b) and (c) and (d) cover identical areas on a single disc.

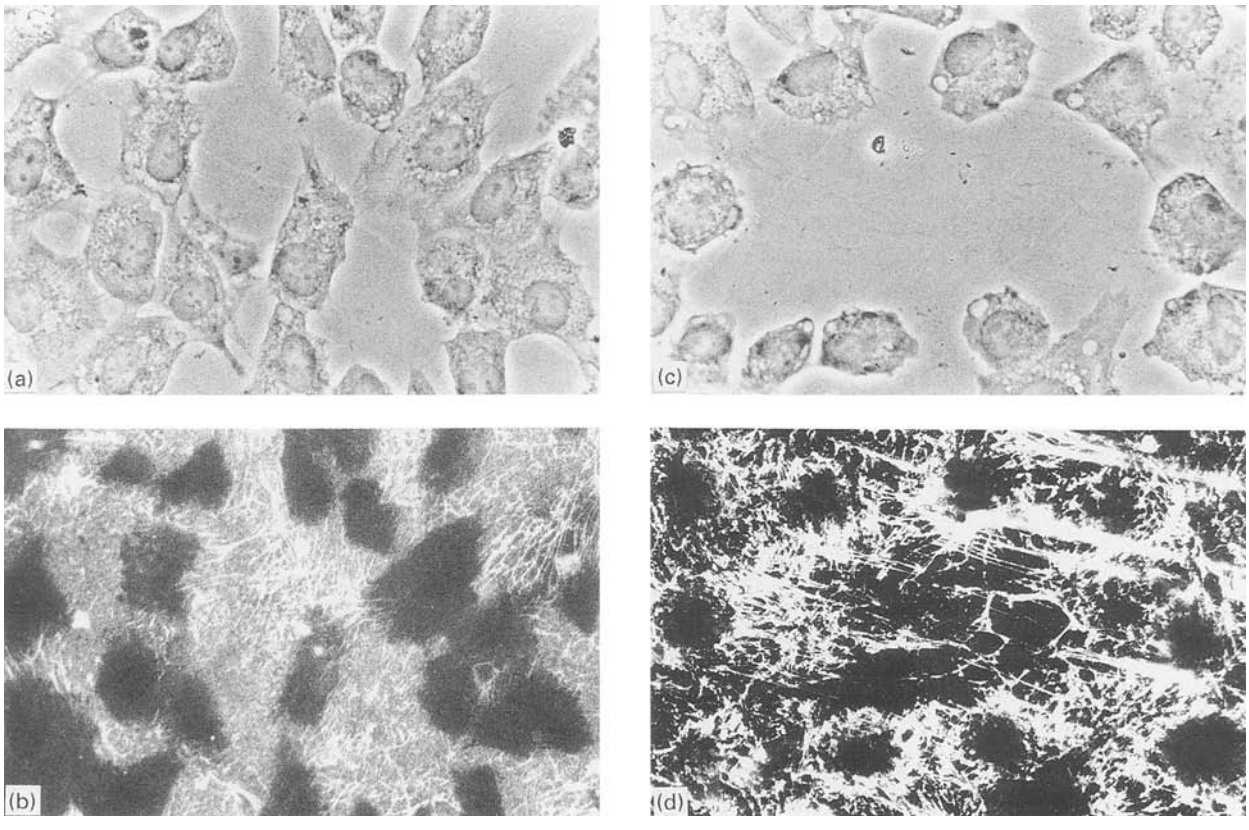

Figure 6 Shear stress induced exposure of extracellular matrix in a HUVEC monolayer on a fibronectin-coated PC-disc. The experiment is identical to that described in Fig. 5, except that now fibronectin (b) and von Willebrand factor (d) are targeted by indirect immunofluorescent staining. Photographs are taken at positions on the disc, that correspond to 7×10^{-4} N cm^{-2}.

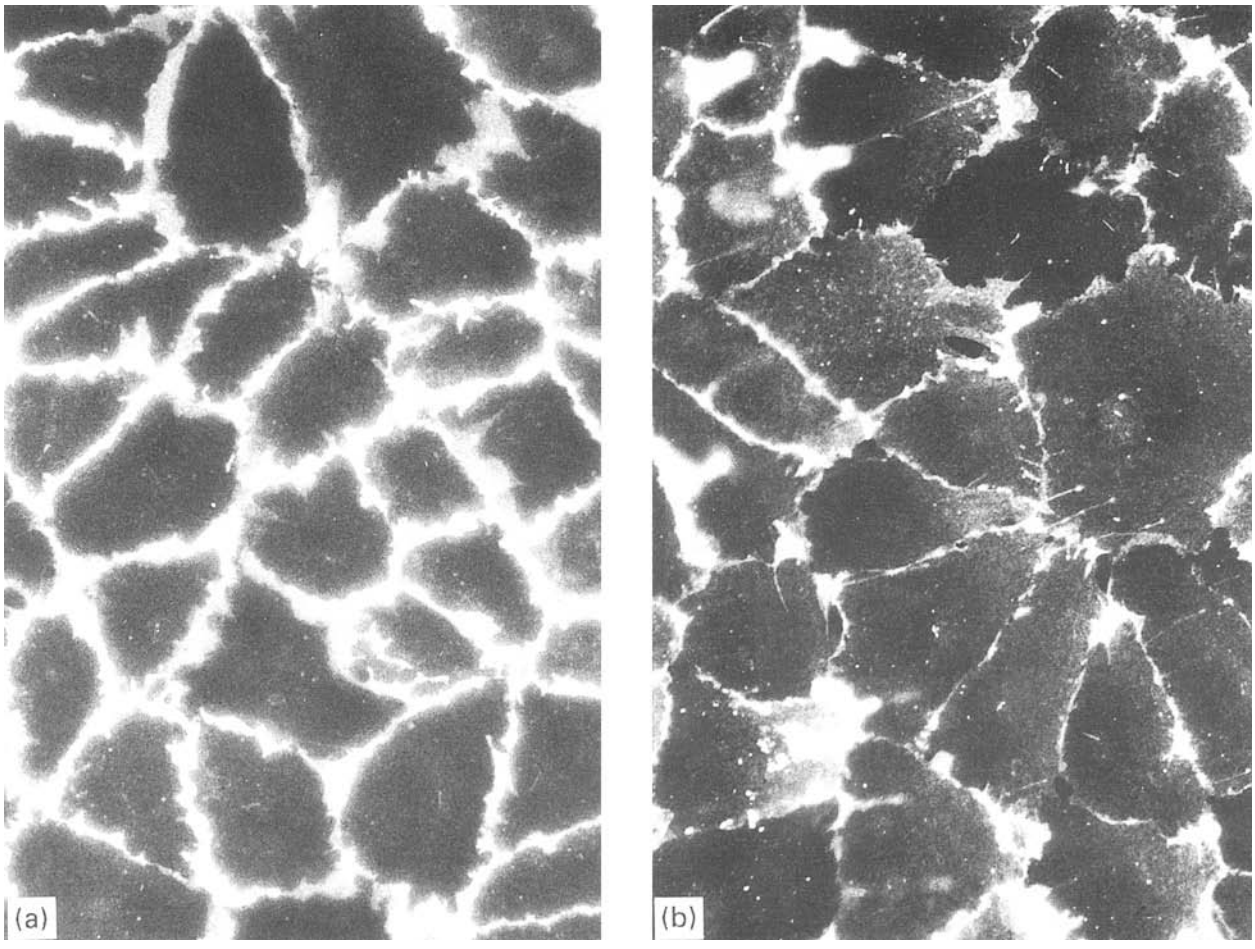

Figure 7 Shear stress resistence of a HUVEC monolayer on a PC-disc covered with the extracellular matrix of smooth muscle cells. A 24-h-old HUVEC monolayer is submitted to shear stresses in the rotating disc device. Following rotation the disc is processed for indirect immunofluorescent staining of PECAM-1. Photographs are taken at positions on the disc that correspond to 0 (a) and 2×10^{-3} N cm^{-2} (b).

the shear stress resistance of a sheet of endothelial cells than cell–cell adhesion through PECAM-1 and adhesion plaque mediated cell–matrix interactions. The formation and frequency of occurrence of intercellular junctions might be regulated by the composition of the extracellular matrix.

4. Conclusions

The rotating disc is a versatile device to study the adhesive properties of cells seeded on a biomaterial under various shear stress conditions. It helps to depict the aptness of materials and coatings in cell seeding applications, and it rapidly exposes differences between materials and coatings which appear similar in respect of cell attachment under static conditions.

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