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Effects of different components of the extracellular matrix on endothelialization

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Abstract
The endothelialization of cardiovascular prostheses is known to improve their haemocompatibility. As such body-foreign materials often do not endothelialize spontaneously. A lot of *in vitro* studies are ongoing how endothelialization of biomaterials can be improved. In this study the influence of different components of a tissue-typical extracellular matrix (ECM) like laminin, fibronectin or gelatin on the formation of an endothelial cell monolayer and on the shear resistance of adherent cells on these substrates was studied.
The study revealed that the density of human venous endothelial cells (HUVEC) monolayers differed markedly between cells grown on a natural ECM and cells grown on singularized components of an ECM ($p<0.001$). Only HUVEC grown on laminin showed similar densities and a stress fiber pattern comparable to HUVEC grown on the ECM. HUVEC grown on gelatin- or fibronectin-coated coverslips were less firmly attached to the substrate; frequently individual HUVEC and even groups of cells detached.
Concluding it seems that coating of implants with laminin supports the formation of shear resistant endothelial cell (EC) monolayer - superior to other ECM components.

1. Introduction
Vascular grafts are widely used for the treatment of cardiovascular disease, which is still the leading cause of mortality in industrialized countries [1, 25]. Beside re-stenoses, formation of thrombi - caused by the thrombogenicity of the graft surface or by an incomplete endothelialization - are discussed as main causes of graft failure [12, 18, 19, 23, 27].
Bio-functionalization of vascular prostheses and subsequent *in vitro* lining with endothelial cells (EC) was previously shown to reduce the risk of thrombosis and graft failure and to improve the clinical performance of vascular prostheses [5, 32].

Unfortunately, cardiovascular prosthetic bypass grafts do not endothelialize spontaneously in humans. Therefore, different strategies are applied to accelerate the endothelialization of biomaterials. Biomimetic surface modifications are regarded as promising approach to stimulate cellular adherence and proliferation at the interface of implant materials [2, 4, 8, 11, 13, 24, 26, 28, 31]. It was shown that e.g. components of the extracellular matrix (ECM) could influence the endothelialization of materials. Components of the ECM include fibrous collagen, hyaluronic acid, proteoglycans, laminin, and fibronectin. In the study presented here the influence of singularized components of the ECM like collagen type I (constituent of the macro-vasculature), laminin, or fibronectin on the endothelialization was examined in comparison to the effects of a tissue-typical ECM.

2. Material and Methods

Endothelial cells were harvested from the human umbilical cord vein (HUVEC) by enzymatic digest using a mixture of 0.1% collagenase and 0.05% trypsin according to the method of Jaffé [17] and incubated in standard culture medium supplemented with human serum pool. HUVEC were seeded on differently pre-coated coverslips. The coverslips (2.7 cm diameter) were pre-coated either with gelatin (0.2 %, Sigma, Deisenhofen, Germany), or with laminin (3.5 µg/cm^2, Sigma, Deisenhofen, Germany), or with fibronectin (5 µg/cm^2, Sigma, Deisenhofen, Germany) or with a tissue-typical ECM. The ECM was secreted by bovine corneal ECs on glass coverslips and maintained until functional confluence (stress fibre reduction and formation of a marginal filament band) under static standard culture conditions (5% CO_2, 95% humidity, 37°C). Thereafter the corneal ECs were mechanically removed.

First passage HUVEC were seeded in equal densities (5 x 10^4 cells / cm²) on the four differently prepared substrates, $n = 6$ for every time point (for a total of 13 time points 4×13 = 52 samples had to be prepared). At every time point the HUVEC were enzymatically detached and the cell number quantified using a cell counter. Every second day the culture medium was exchanged.

F-actin was fluorescently stained with Phalloidin-Alexa555 (1 : 40, Molecular Probes®, Invitrogen, Germany), VE-cadherin with a monoclonal mouse anti-human VE-cadherin IgG (1 : 500, Abcam, Cambridge, Great Britain) and a Cy2 conjugated polyclonal goat anti-mouse IgG (1 : 200, Jackson ImmunoResearch, Hamburg, Germany) as described in detail in [6, 21]. The genomic DNA/nuclei was tagged by using intercalating 4',6-diamidino-2-phenylindole (DAPI, 1 : 5000, Roth, Germany).

Confluent monolayers of HUVEC of the first passage (8 – 13 days after seeding) were exposed to a venous level (2 dyn/cm^2) of shear stress for 3 hours. The fluid shear stress was produced by a rotating cone placed with its tip on the centre of the coverslips [10].

All samples are described with mean and standard deviation. Differences between the four groups at day 13 were evaluated using pairwise multiple

comparison procedures (Holm-Sidak method). The null hypothesis was rejected with a probability of error α of less than 0.05.

3. Results

3.1 Density of HUVEC of the first passage on ECM in comparison to HUVEC-density on single components of the ECM

The growth of a HUVEC monolayer was nearly identical on ECM- and fibronectin-coated coverslips up to day 9 in culture (see figure 1). Actual cell numbers were related to the numbers of seeded cells. The relative number of HUVEC on fibronectin increased from 1 to 2.75, on ECM from 1 to 2.65. The relative number of HUVEC on fibronectin did not increase further after day 9, oscillating instead around a value of 2.7. The relative number of HUVEC on ECM, however, increased further arriving at a value of 2.97 after day 12 in culture. HUVEC on laminin or gelatin showed similar growth tendencies up to day 6 in culture. The relative number of HUVEC on gelatin increased linearly after day 6 and amounted to 2.7 on day 11 in culture. The HUVEC layer was optically confluent, now, but did not show a further increase in cell density.

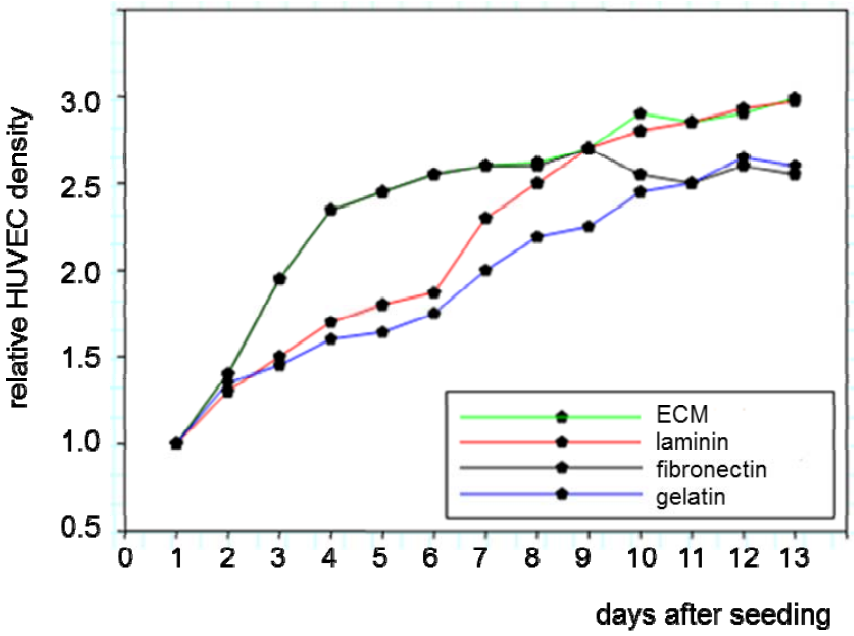

Figure 1: Relative HUVEC density on the four substrates over 13 days after seeding (ECM; laminin-coated or fibronectin-coated or gelatin-coated coverslips; mean ratios of $n = 6$ samples each).

HUVEC seeded on laminin revealed a remarkable proliferation gain after 6 days in culture. Their relative cell number amounted to 2.93 on day 11 in culture.
The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.001$). Results of the pairwise multiple comparison procedures (Holm-Sidak

method) are shown in table 1.

Table 1: Holm Sidak Analysis of Pairwise multiple comparison procedure

Group comparison	Difference of mean	*t*	Unadjusted p	Critical level	Significance
ECM vs. gelatin	0.302	5.175	<0.001	0.009	yes
ECM vs. fibronectin	0.285	4.889	<0.001	0.010	yes
laminin vs. gelatin	0.267	4.575	<0.001	0.013	yes
laminin vs. fibronectin	0.250	4.289	<0.001	0.017	yes
ECM vs. laminin	0.0350	0.600	0.555	0.025	no
Fibronectin vs. gelatin	0.0167	0.286	0.778	0.050	no

The numbers of adherent HUVEC at day 13 after seeding were higher ($p<0.05$ each) on ECM and laminin (which did not differ ($p>0.05$)) than on gelatin or fibronectin (which did also not differ between each other ($p>0.05$)).

3.2 Stress fibre pattern during growth on the four substrates

The HUVEC had settled on the ECM already two hours after seeding. The cells had changed their morphology from a more rounded to a flattened shape and contained several rather short and thin stress fibres continuously labelled with rhodamin-phalloidin.

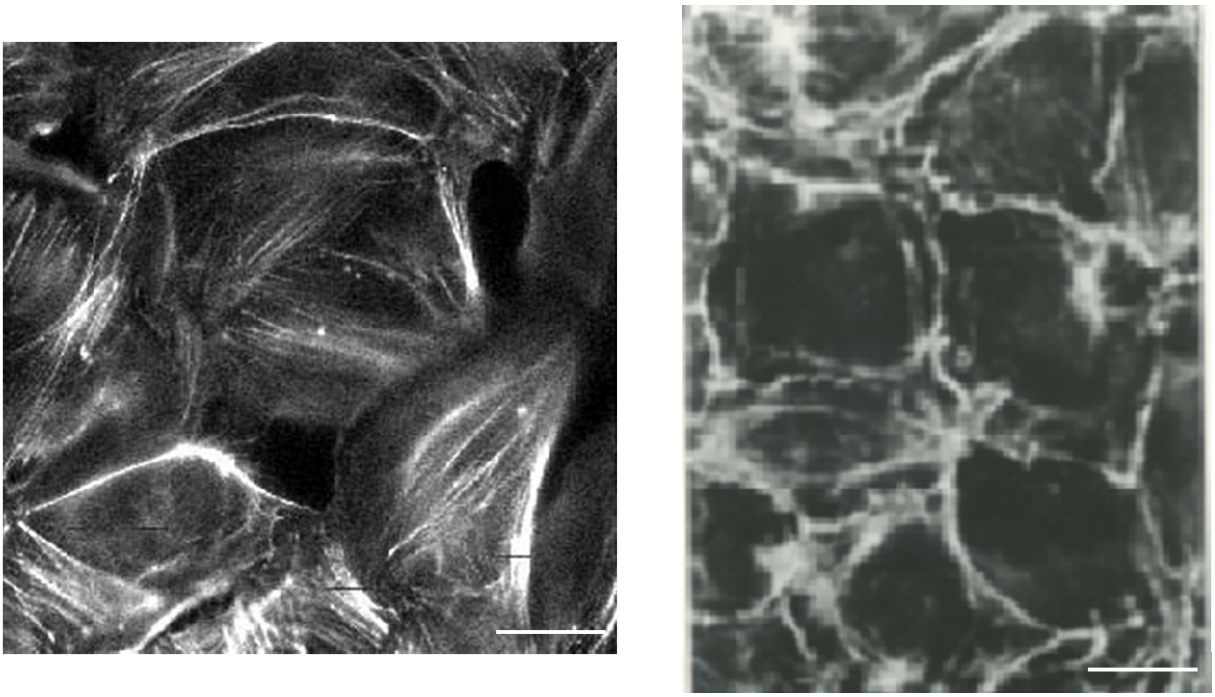

Figure 2: Pattern of the microfilament network in primary HUVEC of the first passage on ECM produced by bovine corneal endothelial cells at day 1 (left) and day 12 of cultivation (right). (F-actin was stained using rhodamin labelled phalloidin; scale bar corresponds to 10 μm).

The stress fibres were confined to the basal cell surface and did not show any preferential orientation. During days 5 to 7 HUVEC reached optical confluence and turned to a uniform polygonal shape and size still containing numerous stress fibres. During days 8 to 12 stress fibres gradually disappeared from the central parts of the HUVEC and were finally incorporated into a brightly stained continuous band of actin filaments along the entire cell rim (Fig. 2, right image). HUVEC on gelatin exhibited a similar behaviour. In the early state they were less well spread in a non-uniform pattern on the substrate and showed several micro-spikes. The stress fibres were more prominent than in cells adhering to ECM. Importantly, at day 12 HUVEC had acquired a more or less uniform cell shape with presenting marginal filament bands along the cell rim, however, not so dense as in HUVEC on ECM (see Fig. 3). Individual cells still showed tiny stress fibres and some began to detach, leaving small cell-free areas in the monolayer.

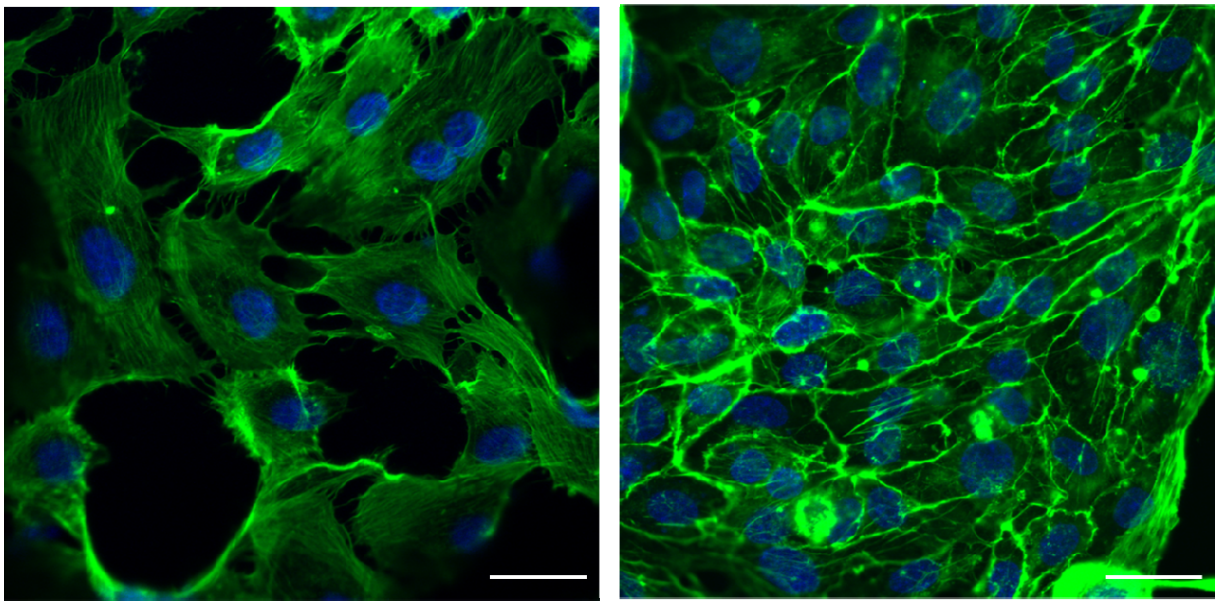

Figure 3: Pattern of the microfilament network in primary HUVEC of the first passage on gelatin at day 1 (left) and day 12 of cultivation (right). (F-actin was stained using rhodamin labelled phalloidin (green) and genomic DNA using DAPI (blue); Scale bar corresponds to 20 µm)

HUVEC seeded on fibronectin-coated coverslips showed a similar stress fiber pattern as described for cells seeded on ECM up to day 9 but then cells started to detach.
Laminin-coated coverslips provided a substrate which resembled the ECM as judged by the stress fibre pattern.

3.3 Shear stress exposure

Early confluent cultures (8 – 10 days of cultivation) grown on ECM and gelatin were exposed to controlled levels of fluid shear stress (2 dyn/cm^2 for 3 hours). HUVEC grown on gelatin-coated coverslips (Fig. 4) were less firmly attached to

the substrate as revealed by frequent detachment of individual HUVEC and even groups of cells. Some of these cells did not show regular stress fibre pattern. The central parts of the cells were more or less weakly stained with phalloidine. Intensively stained structures (possibly remnants of stress fibres) were regularly found at the site of intercellular contacts.

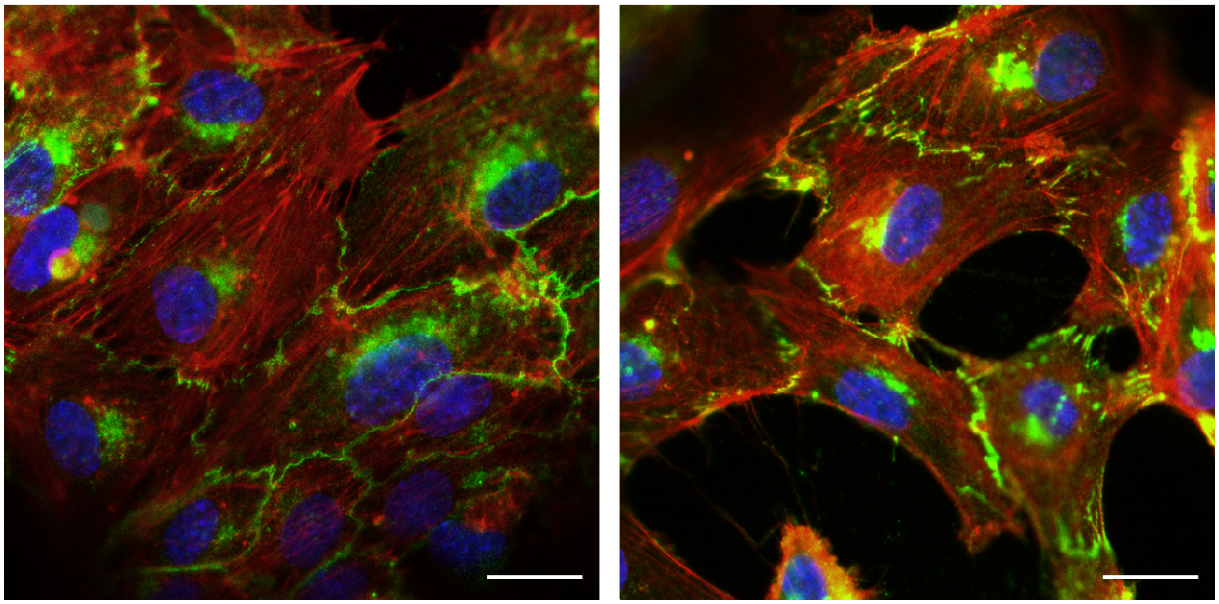

Figure 4: HUVEC of the first passage seeded on coverslips coated with gelatin after 10 days cultivation (left) and after 3 hours of shear stress exposure with 2 dyn/cm^2 (right). (Actin cytoskeleton (red), VE-cadherin (green) and genomic DNA (blue) were fluorescently labelled and images taken by using the cLSM with a 63-fold primary magnification; scale bar corresponds to 20 µm).

Studies on monolayers grown on fibronectin revealed similarity to the cells grown on gelatin. Cells grown on laminin resembled cells grown on ECM.

4. Discussion

Cardiovascular implant surface engineering is focused on rendering surfaces as non-thrombogenic as the endothelium, but despite decades of attempts to solve the problem the burden of coagulation has not been overcome. Since it was shown that the endothelialization of vascular prostheses can help to ameliorate clinical results [5, 32], the number of trials increased to engineer vascular implant surfaces in a way that endothelial cells would couple quickly to the surface and exhibit high shear resistivity. This was not possible, however, for a variety of standardly used polymers [16]. Many of the published studies cannot be compared because differing ECM components were used for the pre-coating, or different cells or culture conditions were used. Most seriously, these studies in general do not examine the behavior of cultured cells on a tissue-typical ECM as control system.

Therefore, we investigated in the present study the influence of substrates coated with different biomolecules on the endothelialization of surfaces up to 13 days of cultivation under static conditions and after 3 hours of low arterial shear stress exposure.

4.1 Static conditions

HUVEC grown on the basal lamina-like ECM formed a monolayer that resembled – after 12 days of cultivation - the endothelium of the vena cava inferior *in situ* by both the uniform polygonal shape of the cells and by the intracellular organization of the actin filament system (see Fig. 2); a behaviour in line with Schnittler et al. [28]. HUVEC grown on substrates precoated with singularized components of the ECM revealed under static conditions a nearly similar colonization pattern as HUVEC on a tissue-typical ECM [10]. This was unexpectedly true even in the case of denatured collagen type I (gelatin). On day 13 in culture only HUVEC seeded on laminin arrived at relative cell numbers (2.93) comparable to HUVEC seeded on ECM (2.97), although the proliferation of HUVEC on laminin was clearly retarded during the first 6 days in culture. Not only proliferation was retarded in the initial culture period. Another short term cultivation experiment lasting for three hours revealed that much less HUVEC adhered to laminin than to fibronectin [3]. Seeding human EC derived from the V. saphena magna of patients with cardiac disease on substrates covered either with collagen, or fibronectin, or laminin resulted in similar growth curves [2]. Something similar was reported by Shi; substrates coated with fibronectin and laminin significantly promoted the adherence of HUVEC cultured in a steady environment [30]. Heller et al. reported contrasting results. They found higher numbers of adherent EC on substrates coated with fibronectin or laminin compared to substrates coated with collagen [15]. According to Palotie, bovine aortic EC cells became attached most readily to surfaces coated with fibronectin or type III or type IV collagen. Laminin and collagen types I and V served as less effective substrates [26]. Form et al. reported that during a 5 day period the proliferation of microvascular EC as well as of bovine aortic endothelial cells was significantly greater on laminin than on either plasma fibronectin; the interstitial collagen types I and III, or on the basement membrane collagen type IV [8].
A further study demonstrated that the coating of ePTFE with laminin resulted in significantly increased adhesion of HUVEC [20]. This comparative study corroborated earlier results published by Form et al. and by Schnittler et al. [8, 28], showing that under static conditions laminin evidently offered the best conditions for adherence and proliferation of HUVEC, accompanied by a cell morphology and a structure of the cell layer coming near to the performance of HUVEC seeded on a tissue-typical ECM [10].

4.2 Dynamic Conditions

Exposure of the HUVEC monolayers to arterial levels of fluid shear stress induced the formation of numerous actin filament stress fibres which have been shown to be the most prominent feature of the cytoskeleton in endothelial cells of human arteries in situ. In view of observations that stress fibres can contract [6, 9] it seems reasonable to assume that the shear-stress-induced stress fibres can exert tension that may allow the cells to withstand the rheological shear forces. The shear resistance of adherent cells was reported to be associated with an increased assembly of vinculin in focal adhesion plaques densely and diffusively spread over the whole basal cell surface [29, 30], what also was described, too, by Filova and coworkers [7]. Filova et al. described higher EC densities on collagen, laminin, and fibronectin coated 2D polystyrene than on the pristine polystyrene surface after 3 and 7 days of culture.
In line with our study, Schnittler et al. reported that the ECM protein laminin strongly increased EC adhesion under fluid shear stress [28]. This could be confirmed by adding doxorubicin (which inhibits the stress fibre formation) resulting in significant detachment of EC exposed to medium levels of fluid shear stress (5 dyn/cm^2).
Results can differ not only due to substrate characteristics (in the present study biomolecules derived from the ECM were coupled to the substrates) but also due to the use of different cell types and differing culture conditions [14]. Krüger et al. could show that the proliferation rate of HUVEC significantly decreased with increasing numbers of cell passages [22], coinciding with remarkable changes in the secretory efficiency of endothelial cells.

5. Conclusion

All of this reveals that a valid comparison of published results concerning the endothelialization of polymer surfaces is only possible, when the number of cell passages, the provenance of EC used, the secretory efficiency of cells, and the culture conditions, e.g. culture medium exchanges, supplements of fetal calf serum or human serum pool etc. are carefully described, since these are besides the substrate very critical factors considerably influencing the endothelial proliferation and performance. In addition, the study revealed that EC grown on gelatin and fibronectin contained higher amounts of stress fibers and F-actin than cells grown on ECM or laminin. In contrast to fibronectin and gelatin, laminin turned out to provide a much better substrate for cell adhesion and growth as judged by a rather low background level of stress fibers in confluent cultures, however, still more stress fibres were visible than in HUVEC grown on ECM, leading to an ability of the HUVEC to withstand fluid shear stress.

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