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Shear resistance of endothelial cells in a pathological environment

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Abstract

Background: Endothelial cells (EC) *in vivo* are strongly influenced by changes of the milieu exterieur. Under pathological conditions EC can become activated e.g. in hypoxic areas or during sepsis. In general, the endothelialization of implant materials is evaluated *in vitro* under physiological conditions. Though, in patients who receive implant materials pathological conditions are often present. An open question is therefore, how ECs seeded on a body foreign substrate behave in a pathologic microenvironment. In this *in vitro* study a microenvironment was created mimicking the conditions present in septic patients. To simulate this situation *in vitro*, serum of patients with septic shock was added to the culture medium of EC cultured on glass. The samples were sheared in a cone-plate rheometer (shear rate of 6 dyn/cm^2) with subsequent analysis of the morphology, the microfilament organization and the shear resistance and compared to control cultures of EC without shock serum supplementation. Aim of the study was to investigate whether this *in vitro* model provides information about the functionality of an EC monolayer on a body foreign surface under pathological conditions.

Results: Septic conditions induced severe changes of the morphology of the adherent cells: there was a strong induction of stress fibers. In addition, lots of cells or cell groups were detached visible as denuded areas in the EC monolayer. After shear stress exposure only 28.7% of EC seeded in cell medium supplemented with serum of septic patients remained adherent (control cells: 96.8%).

Conclusion: The study demonstrates that the microenvironment is of extreme importance for the behavior of EC and that *in vivo* pathologies can be simulated *in vitro*. This opens the possibility to evaluate new implant materials under physiological but more important also under certain pathological conditions - simulating the implant size and the disease of the host.

Key words: Endothelial cells, *in vitro*, sepsis, shear rate

1. Introduction

Atherosclerotic diseases are still the main cause of death worldwide [29]. Based on a longer lifespan and improved medical care the number of patients receiving biomedical implants is raising in the last decades. Particularly the progress in medical technology has led to an increase of interventional procedures like angioplasty and/or stenting, valve replacement as well as of vascular bypass surgery [25]. One of the causes of dysfunction or even failure of such implants is the thrombogenicity of most of the implant materials [32,33]. This is particularly the case with small vascular prostheses (< 6 mm). Up to now no synthetic prosthesis for e.g. coronary bypasses are available [35].

One strategy to overcome this problem is to endothelialize the inner surface of bypass prostheses [23], modeling *in vivo* conditions and rendering them haemocompatible. A dense endothelial cell (EC) monolayer covers the inner surface of blood vessels. After the first description in 1865 up to the early 1970s, this monolayer was viewed to be a mere barrier separating the blood cells from the tissue. The discoveries of prostacyclin [27] and its synthesis in EC [40] as well as the groundbreaking report from Furchgott & Zawadski about the active role of the endothelium on the vasodilation of the vasculature [14] have provided the basis that the endothelium is a key player in the homeostasis of different pathophysiological processes [16]. Among other processes EC are involved in the control of platelet adherence as well as aggregation and prevent – under physiological conditions – thrombus growth and vascular occlusions.

However, under pathological conditions EC can be activated e.g. in hypoxic areas or during sepsis [21,31,36]. *In vitro* studies using EC monolayers have shown that numerous factors – occurring in the vasculature under pathological conditions – can activate EC which then may lose their antithrombotic capacity [42]; like tumor necrosis factor (TNF, [30]), endotoxin [6], Interleukin-1 [4], or thrombin [15].

An open question is how EC seeded on a body foreign substrate behave in a pathologic microenvironment. It is well known, that in septic patients noxa are circulating in the blood, which lead to severe changes of EC morphology and function inducing hyperpermeability, edema and hypotension [13,20,24]. To mimick this situation *in vitro*, serum of patients with septic shock was added to the culture medium of EC cultured on glass substrates. The samples with a confluent monolayer of EC were sheared in a cone-plate rheometer (a shear rate of 6 dyn/cm^2 was used to mimick venous shear rates) and thereafter the morphology, the microfilament organization and the shear resistance of EC was analyzed and compared to control cultures of EC without shock serum supplementation.
Our hypothesis is that this *in vitro* model might provide information about the functionality of an endothelial monolayer on a body foreign surface under pathological conditions predicting the *in vivo* situation. Further studies with polymer-based biomaterials - intended to be used as implant material - are in preparation as are also other pathological conditions.

2. Material and Methods

2.1 Study design

Human venous endothelial cells (HUVEC) of the third passage derived from human umbilical cord veins were grown *in vitro* on an extracellular matrix (ECM). HUVEC were maintained until functional confluence (stress fiber reduction and formation of a marginal filament band) under static standard culture conditions (5% CO_2, 95% humidity, 37◦C) [12]. HUVEC were experimentally exposed to shear stress 11 days after onset of culture. Shear experiments were performed with six samples (n=6). The manuscript was written in accordance with the ethical guidelines of Clinical Hemorheology and Microcirculation [3].

2.2 Endothelial cells (HUVEC)

HUVEC were harvested from the human umbilical cord vein by enzymatic digest using a mixture of 0.1% collagenase and 0.05% trypsin according to the method of Jaffé [18]. The cells were cultivated in standard culture medium supplemented with human serum pool. HUVEC of the third passage (5 x 10^4 cells / cm^2) were seeded on glass cover slips (2.7 cm diameter) coated with a tissue-type ECM secreted by bovine corneal ECs which is typical of medium and large vessel intimae. Every second day the culture medium was exchanged.

2.3 Test Procedure

After reaching functional confluence cell culture medium was exchanged and supplemented with either septic or autologous serum 20% v/v (see para 2.4). Thereafter HUVEC were inserted into the rheometer. For the exposure of adherent HUVEC to uniform shear stress a cone-and-plate shearing device (Smard-CAD Deutschland GmbH, Neu-Ulm, Germany) was used, which accommodate three samples per run at 37°C. After insertion of a sample into one of the three probe heads, the whole setup was positioned directly under a sterile truncated glass-cone (25 mm diameter and 2° angle) as described by Schulz et al. [39]. The glass cone was connected to a direct current servo motor of the shearing device and lifted under observation by a real time camera system until the correct spacing between the cell seeded surface of the investigated material and the cone tip was achieved. Thereafter the shearing of the probes was started (6 dyn/cm^2 for 30, 60, 90 or 120 minutes). Directly before and after the shear experiments images were taken to analyze cell density and morphology.

The actin content of the microfilaments was stained by Phalloidin Rhodamin. The microfilaments were documented by use of an Olympus microscope (IMT-2) equipped with a UV lamp and online connected to a TV chain (Sony XC 50 ST/monochrome) implying an OPTIMAS – Image analysis system.

2.4 Serum preparation

Freeze dried sera (from patients with septic shock) were fractionated using a Sephacryl S-200 column. 100 mg protein were solved in 2 ml of 10 mM ammonium acetate (pH-7.4), which was also used as balancing buffer, and applied to the column. The column was perfused at a linear flow rate of 7.4 ml/h and a chromatogram was recorded. Fractions of 6.5 ml were collected. One ml of each fraction was added to 24 ml Medium 199 supplemented with 20% human serum pool of apparently healthy donators. The whole solution was sterilized by filtration, filled in sterile vials and stored at 4° C in a cold storage room during the culture period.

2.5 Staining

Actin and the associated proteins myosin and α-actinin were visualized by fluorescence microscopy using rhodamin-labelled phalloidin and antibodies to calf thymus myosin and gizzard α-actinin, as described in detail in [10].

2.6 Statistics

Samples are described with mean and standard deviation. Differences between the groups over time were evaluated using ANOVA for repeated measures. The null hypothesis was rejected with a probability of error α of less than 0.05.

3. Results

Serum of patients added to the culture media (20% v/v) led to significant changes of the EC. While 96.8% of the EC remained adherent on the ECM using the pure culture medium, culture medium supplemented with serum of septic patients (20% v/v) induced a pronounced detachment of EC; in this microenvironment only 28.7% of the EC still adhered on the ECM after shearing with 6 dyn/cm^2 for 2 hours ($p<0.01$, see Fig.1).

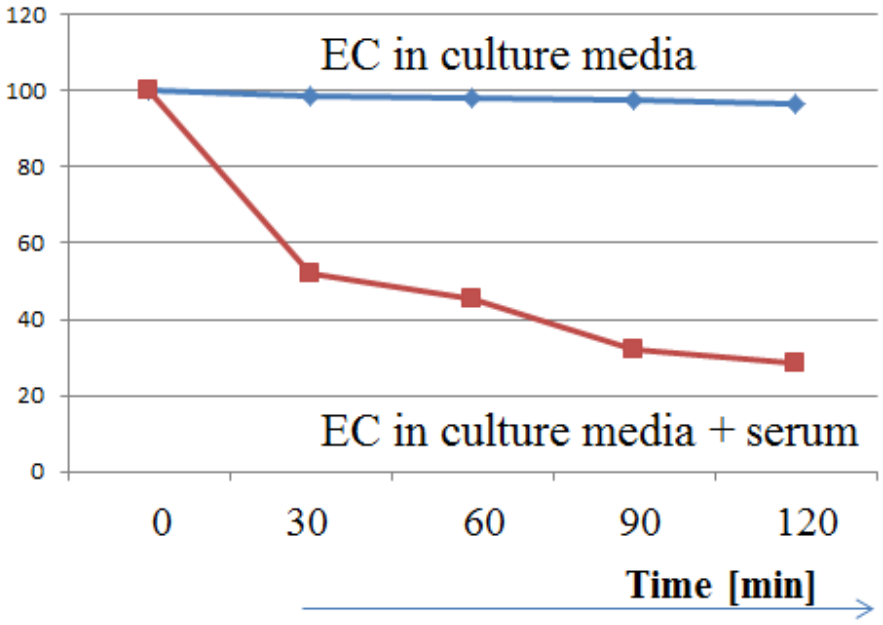

Figure 1: Percentage of adherent endothelial cells (EC) on extracellular matrix cultured in medium alone or in culture medium supplemented with serum of septic patients (20% v/v) exposed to 6 dyn/cm^2 shear stress for 2 hours.

Fig. 2a reveals that there was an increase in size of the EC, mostly by cell elongation. Also holes appeared in the cell layer demonstrating the detachment of groups of EC subjected to fluid shear stress. This was accompanied by great amounts of cell debris. Fig. 2b displays the actin part of microfilaments in EC. Below the center of the image a substantial hole in the EC layer is visible resulting from the detachment of approximately 3 EC. The majority of neighboring cells showed the marginal filament band indicative of sufficient cell-cell binding. Cells bordering on the denuded area displayed a strong induction of stress fibers indicative of an increasing reduction of cell-substrate binding.

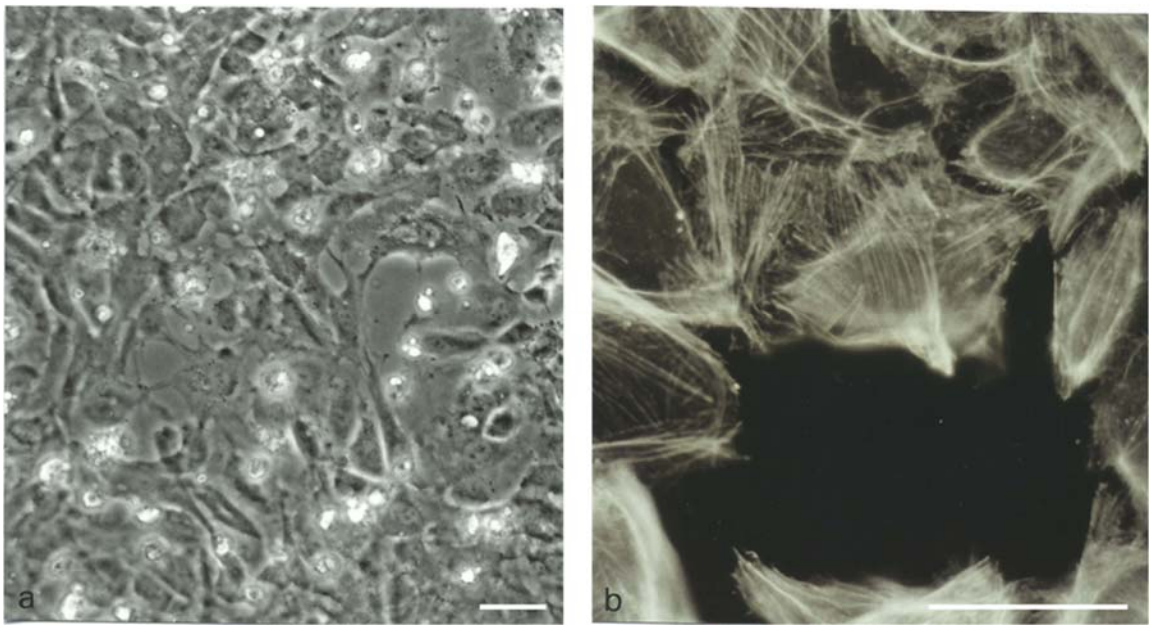

Figure 2: a: Phase-contrast visualization of HUVEC cultured on a tissue-typical extra-cellular matrix in culture medium supplemented with sera from septic shock patients and exposed to shear stress of 6 dyn/cm^2 for 30 minutes (Primary magnification 1 : 200). Scale bar: 30 µm
b: Display of the actin component of endothelial microfilaments after cytochemical preparation (Staining of F–actin using Phalloidin-Rhodamin; primary magnification 1 : 630). Scale bar: 30 µm

4. Discussion

Sepsis-associated mortality has been shown to increase with the severity of sepsis [2]. In its severe form, sepsis is accompanied by evidence of organ dysfunction, with mortality reported to be 28 to 50% [1,41]. In sepsis, infection elicits a systemic inflammatory response and endothelial dysfunction which is known to become pathologic in form of increased endothelial barrier permeability, and the resultant microvascular leakage associated with end-organ failure [13,22].
Cultures of functionally confluent venous EC on ECM can represent the endothelial state of venous blood vessels because the microfilament organization in both is characterized by a dense marginal filament band and scarce or absent stress fibers in central parts of the cells [9]. It is well known, that in septic shock patients activation of the endothelium occurs together with a desquamation of EC from the basement membrane. Here the intracellular actomyosin cytoskeleton of EC plays an important part in regulating cell shape, cell–cell and cell–matrix interactions, and cell mobility [5,7,17,28,34].

In the study presented, the morphology and shear resistance of functionally confluent EC cultured on a typical ECM was examined. It was reported that the proliferation rate of EC, as assessed by incorporation of ^{3}H-Thymidine [38], decreased only marginally when fractions of sera of shock patients were added to the culture medium. A dose response relationship, however, existed when increasing amounts of shock sera were added [37]. At a shear stress of 6 dyn/ cm^2, as applied in this study, considerable changes appeared in cell-substrate binding of EC after the addition of shock serum fractions to the culture medium. The reduction of the cell-substrate binding led to a serious reduction of endothelial shear resistivity. In most cases groups of EC detached from the substrate, as clearly demonstrated in Fig. 2. These results showed a good correlation to clinical findings [26,31]. The time course of the sequence of events reported here corresponds well to results of a recently published study [11], where the endothelial permeability, assessed by measurement of the electrical resistance, increased continuously over time for more than 50 %.

It is important to note that in this study the platelets were suspended in serum and not in plasma, because platelets in plasma would lead with increasing thrombokrit to increasing detachment of EC, even under resting conditions [19].

The presentation of the microfilament bands indicated that with the early induction of stress fibers a change of the cell-substrate binding was associated while the cell-cell binding and the marginal filament bands still were relatively intact.

Concluding, the study clearly revealed that the *in vitro* model used provided valuable findings about EC under pathological conditions exposed to shear load. Under severe pathological conditions groups of EC detached and the stress fiber formation was much more pronounced than under physiological conditions (without septic serum). The *in vitro* results were in line with changes observed in patients: EC detached in small groups which is in accordance with the loss of barrier integrity in septic patients [8]. In addition, stress fiber formation enhancing the shear resistance of EC seemed to precede the conversion of marginal filament bands.

The results demonstrate that the microenvironment is of extreme importance for the behavior of EC and that *in vivo* pathologies can be simulated *in vitro*. This opens up the possibility to evaluate new implant materials under physiological but more important also under pathological conditions mimicking conditions present at the implant size and the disease of the host.

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