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Effect of lipopolysaccharide on the adherence of human umbilical vein endothelial cells (HUVEC) on a natural substrate

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Abstract

Polymers are often contaminated with lipopolysaccharides also known as endotoxins. Even small amounts of endotoxins can have strong effects on endothelial cell function so that the endothelialisation of cardiovascular implants might be hampered. An open question is how endothelial cells seeded on a body foreign substrate respond to shear load after adding Lipid A (LPA), the domain, which is responsible for much of the toxicity of gram-negative bacteria, and whether morphological changes of endothelial cells occur.

LPA supplementation to the culture medium in increasing concentrations (5, 25 and 50 µg/ml) resulted in progressive reductions of the density of adherent human umbilical vein endothelial cells (HUVEC) after shear load (p<0.001). 48% of the HUVEC in control cultures (0 µg/ml LPA) were still adherent after 2 hours of

shearing at 6 dyne/cm^2, while 80 minutes after addition of 50 µg/ml LPA, 88% of the HUVEC had already detached from the substrate and after 100 minutes no more HUVEC were attached.

The results demonstrate that endotoxins are of extreme importance for the behavior of HUVEC and that *in vivo* pathologies can be increasingly simulated *in vitro*.

1. Introduction

Implant failure is discussed to be often associated with bacterial contamination of the material surface [1, 2]. One important class of contaminants are lipopolysaccharides (LPS), also known as lipoglycans or endotoxins, which were found to be constituents of the outer membrane of Gram-negative bacteria [3]. Endotoxins are shed upon cell death but also during bacterial growth and division [4, 5]. They can be present, circulating or adherent to surfaces, even in the absence of viable bacteria. Most critically, even small amounts of bacterial endotoxins can have very strong biological effects. Besides influencing erythrocyte aggregability and deformability [6, 7] in particular, LPS may be responsible for endothelial dysfunction and microcirculatory disorders [8-12] and endothelial cell (EC) injury associated with Gram-negative sepsis and septic shock [13, 14]. LPS were shown to comprise three parts: The O antigen (O polysaccharide), the core oligosaccharide and the Lipid A (LPA). LPA is, in normal circumstances, a phosphorylated glucosamine disaccharide decorated with multiple fatty acids, but it is responsible for much of the toxicity of Gram-negative bacteria.

It has been demonstrated often that polymer-based biomaterials can be contaminated e.g. with pyrogenic substances including endotoxins and other microbial products [15]. Therefore, the load of implant materials with soluble and adherent endotoxins has to be tested and should be below 0.5 EU/ml according to FDA limits [15].

Several studies revealed that endotoxins can activate macrophages and neutrophils, followed by secretion of different interleukins (IL-1, IL-6, IL-8) and tumor necrosis factor. Endotoxins can also activate the classic and alternative complement pathways and the arachidonic acid cycle [16-21].

In addition, a direct effect *in vitro* - in the absence of immune cells - on the morphology of EC was reported [18]. It was described that EC exposed to LPS could undergo cell death by means of a NAD(P)H oxidase–dependent ROS generation mechanism [22]. While moderate ROS generation facilitated normal cellular function, overproduction resulted in deleterious changes in gene expression and protein malfunction, thus promoting endothelial damage in a dose-dependent way

[22, 23]. However, even in the presence of a high concentration of LPS, a death of all EC was never complete, with a significant portion of cells remaining alive and fully attached to the substrate [23]. A previous study revealed that after addition of 20% v/v of either septic or autologous serum, 28.7% of human umbilical vein endothelial cells (HUVEC) remained attached on the substrate in comparison to 96.8% of the HUVEC in the controls [24].

Endotoxin-induced shock in pigs was followed by endothelial damage within 30 minutes after onset of shock and by endocarditis thrombotica at the heart valves. Endotoxins can apparently affect EC from macro- and micro-vessels of different organs (e.g. kidneys, lungs and heart) to a variable extent with respect to cell morphology, cell damage, cell proliferation and endothelial resistivity to hemodynamic shear load [25].

Our hypothesis is that this *in vitro* model can be used to study how macro-vascular EC seeded on a body foreign substrate respond to shear load after adding LPA and whether the morphological changes described [26] coincide with changes of the marginal filament net and the formation of stress fibers and finally in a detachment of singularized HUVEC or of the complete HUVEC monolayer.

This *in vitro* model might provide information on the shear resistance of an endothelial monolayer in the presence of such endotoxin levels adherent on implant materials of cardiovascular devices.

2. Material and Methods

2.1. Endothelial cells (HUVEC)

Human venous endothelial cells (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 Jaffe [27]. The cells were cultivated in standard culture medium supplemented with human serum (pool). HUVEC of the third passage (5×10^4 cells/cm^2) were seeded on glass cover slips (2.7 cm diameter) pre-coated with a tissue-type extracellular matrix (ECM), secreted by bovine corneal EC [28], which is typical of medium and large vessel intimae [29]. Every other day the culture

medium was exchanged.

2.2. Study design

HUVEC were maintained until functional confluence with stress fiber reduction and formation of a marginal filament band under static standard culture conditions (5% CO_2, 95% humidity, 37 °C) [29]. Six samples each were supplemented with 0, 5, 25 or 50 µg/ml LPA. HUVEC were experimentally exposed to shear stress of 6 dyne/cm^2 which is typically found in large veins or aortae [30] 11 days after the onset of cell cultures. For the application of uniform shear stress to adherent HUVEC, a cone-and-plate shearing device (Smard-CAD Deutschland GmbH, Neu-Ulm, Germany) was used, which accommodates three samples per run at 37 °C. After insertion of cells onto one of the three probe heads the culture in the probe head was positioned directly under a sterile truncated glass-cone (25mm diameter and 2° angle) as described by Krüger-Genge *et al.* [24]. The glass cone, which was connected to a direct current servo motor of the shearing device, was slightly elevated under observation by a real time camera system until the correct spacing between the EC seeded surface of the investigated material and the cone tip was achieved. Thereafter the shearing of the probes was started (6 dyne/cm^2 for 30, 60, 90 or 120 minutes). Directly before and after the shearing experiments images were taken to analyze the cell density.

The actin content of the microfilaments was stained by Phalloidin Rhodamin [31]. 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.

2.3. Statistics

Samples are described with mean values and standard deviations. 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

LPA supplemented to the culture medium in increasing concentrations (5, 25 and 50 µg/ml) resulted in significant changes of the density of adherent HUVEC (p<0.001). 50% of the HUVEC in control cultures (0 µg/ml LPA) were still adherent after 2 hours of shearing at 6 dyne/cm^2. The density of adherent HUVEC decreased significantly with increasing LPA concentration and duration of shearing (see Figure 1). Student-Newman-Keuls tests showed that increasing supplementation of LPA led to a significant reduction of adherent HUVEC (p<0.05 each). 80 minutes after addition of 50 µg/ml LPA, 88% of the HUVEC had already detached from the substrate and after 100 minutes no more HUVEC were attached.
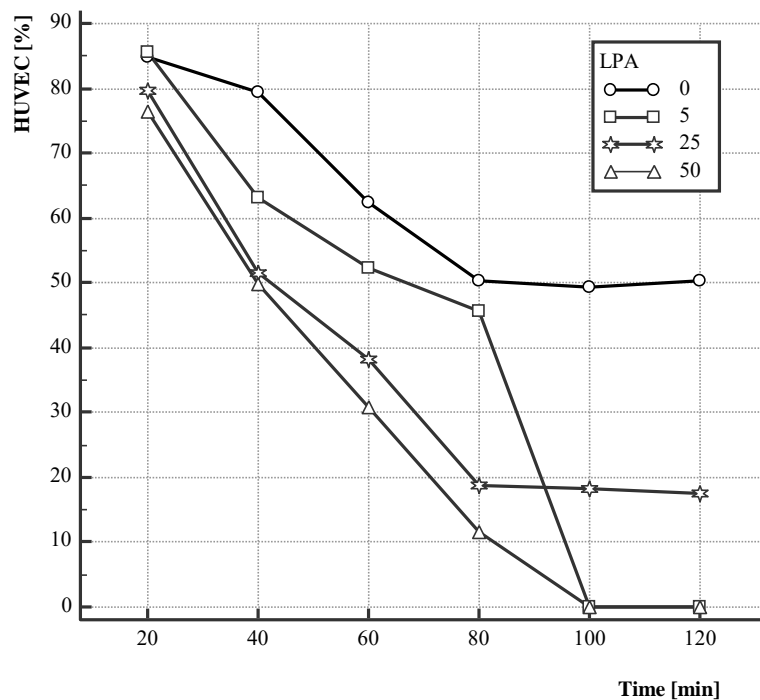

Figure 1: Percentage of adherent HUVEC [%] after supplementation of the culture medium with increasing concentrations of LPA (0, 5, 25, 50 [µg/ml]) and after application of shear load (6 dyne/cm^2) over 120 minutes. Cell numbers are given in percentage of the initial numbers of cells in control HUVEC cultures (without LPA) under static conditions.

For all the cases where the culture media had been supplemented with doses of LPA,

the highest relative amounts of so far adherent EC detached directly after onset of shear application between the 20th and the 40th minute. 26% of EC detached after addition of 50 µg/ml LPA, 27% of EC after addition of 25 µg/ml LPA and 23% of EC after addition of 5 µg/ml LPA. This was clearly different from the control cultures. Here the relative numbers of detached EC per period of shearing did not differ so much: in the shearing period between 20 and 40 minutes 15% of EC detached, between 40 and 60 minutes 8% of EC detached and between 60 and 80 minutes 12% of EC detached. No more EC detached thereafter.

4. Discussion

Cultures of functionally confluent HUVEC 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 [29, 32]. It is well known that in Gram-negative sepsis, activation of the endothelium occurs together with a desquamation of EC from the basement membrane. In this process, the intracellular actomyosin cytoskeleton of EC plays an important part in regulating cell shape, cell-to-cell and cell–to-matrix interactions, and cell mobility [33 - 35].

Supplementation of variable amounts of LPA to the culture medium of HUVEC under static culture conditions reduced only marginally the numbers of adherent HUVEC and also revealed only a weak dose-response effect. Schöffel attributed the slight reduction of adherent HUVEC to a marginal decrease of HUVEC proliferation [36]. Supplementation of culture media with sera (20% v/v) from patients with either septic or hypovolemic shock gave similar results under static culture conditions. When HUVEC cultures were exposed to shear stress, however, there was a dose dependent and clear reduction of adherent cells (Fig. 1). Similar results were published by Krüger-Genge *et al.* where, after addition of shock sera to culture medium [24], more and more HUVEC detached from the substrate with increasing time of exposure to shear rates typically occurring in the human vena cava or the aorta. The LPA concentrations applied here were markedly higher than those

evaluated in the circulating blood of shock patients [37], while local concentrations of LPA can be much higher. In experimental studies, LPA concentrations up to 10 mg/ml, which is a 1000-fold concentration, were applied [38, 39]. These results showed that the applied endotoxins evoked mechanisms, which finally reduced the cell-substrate binding *in vitro*. Changes in the arrangement of HUVEC microfilaments resembled those appearing in HUVEC after the supplementation of culture media with sera from patients with septical shock (see Figure 2). Important to note were the well-marked marginal filament bands, which were also observed after addition of shock sera to the culture medium of HUVEC [24], and which are thought to coincide with a well-developed cell-cell binding, as well as the clear induction of stress fibers, even when their induction was not as strong as in experiments when shock sera were applied.
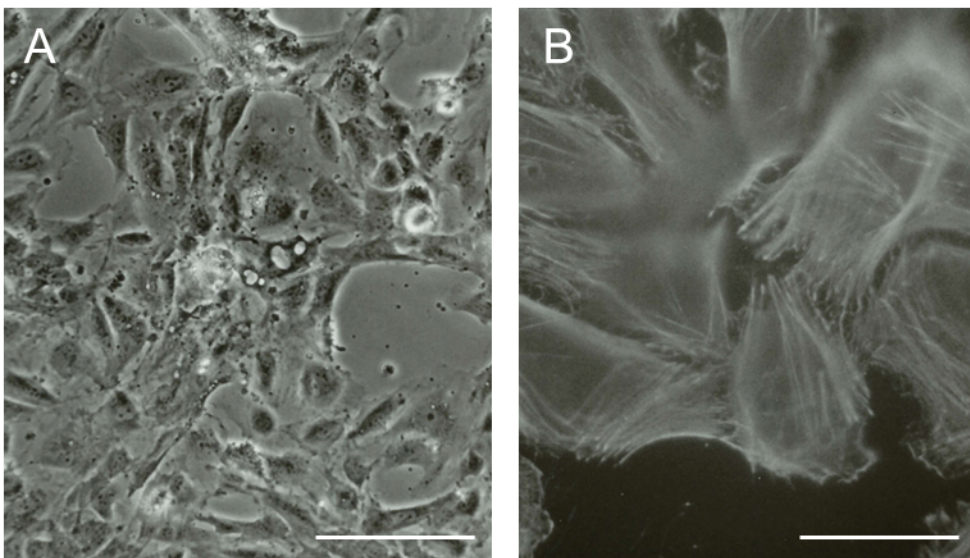

Figure 2: A) Phase contrast image of functionally confluent HUVEC, cultured for 11 days on extra cellular matrix (ECM), then incubated with Lipid A and exposed to hydrodynamic shear stress ($\tau=6$ dyne/cm^2) in a cone-plate rheometer (magnification 1 : 200). Well to observe are the detachment of cells and some cell debris. Bar: 100 µm. B) Fluorescent microscopy of actin filaments (F-actin) in HUVEC after cytochemical staining with Phalloidin-Rhodamine. Beside denuded areas, where HUVEC were detached already, were neighboring cells whose marginal filament band was under decomposition, but still visible. In some cells strong stress fibers developed (magnification 1: 630). Bar: 10 µm.

The results of this *in vitro* study coincide well with clinical findings. In the initial phase of a Gram-negative sepsis the activation of the endothelium is a key event, accompanied and followed by endothelial dysfunction, shedding of EC, an increase in vascular permeability, a loss of the barrier function of the vascular walls, death of EC and, finally, the destruction of tissues [40, 41]. The results demonstrate that the microenvironment is of extreme importance for the behavior of EC and that *in vivo* pathologies can be increasingly simulated *in vitro*.

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