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Adherence and shear-resistance of primary human endothelial cells on smooth poly(ether imide) films

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Abstract

Background: Occlusions of artificial small-diameter cardiovascular grafts are frequent events after implantation, often caused by clot formations. A main factor is the insufficient hemocompatibility of the inner artificial graft surface, which could be improved by endothelialization. Therefore, one challenge in cardiovascular graft engineering is the establishment of a shear-resistant endothelial cell layer to prevent cell detachment by shear forces after implantation.

Materials and Methods: Recently, very smooth (R_q = 2.37±1.40 nm) poly(ether imide) (PEI) films were introduced as a biocompatible candidate material for cardiovascular devices. In this study the stability of primary human umbilical vein endothelial cell (HUVEC) monolayer was investigated after long-term seeding (nine days) on PEI-films and subsequent exposure to a venous shear stress of 3 dyn/cm² for up to six hours, using the cone-and-plate shearing technique. Cell density, growth pattern and morphology of HUVEC were determined prior and after shearing compared to glass as control substrate. HUVEC adhering to the substrate after shear stress were counted and analyzed by fluorescent staining. Supernatants were collected and secretion profile analysis of vasoactive and inflammatory mediators was performed.

Results: The cell density on PEI-films compared to the controls was slightly higher after long-term seeding and exposure to shear stress (glass: 71,656±8,830 cells/cm² and 42,239±5,607 cells/cm²; PEI-film: 64,056±2,829 cells/cm² and 45,422±2,507 cells/cm² before and after shear stress, respectively). Actin- and vinculin-staining revealed a scattered re-organization of the cytoskeleton as well as a formation of stress fibers and focal adhesion points. Secretion of prostacyclin and thromboxane A2 was increased after application of shear stress, but no significant differences were detectable between cells growing on PEI-films or glass. Amounts of secreted inflammatory cytokines IL-6 and IL-8 in the supernatant were significantly lower for HUVEC seeded on PEI-films compared to glass before as well as after stress.

Conclusion: The study demonstrated that HUVEC were able to resist exposure to venous shear stress when seeded on smooth PEI-films with typical morphology and adhesion behavior. However, HUVEC adherence on PEI was not yet sufficient to retain a complete cell monolayer after shear stress exposure. Occasionally, single cells or cell plaques were disrupted resulting in cell free areas in the confluent HUVEC layer. Apart from this our data suggest that PEI is a suitable substrate for HUVEC under static and dynamic conditions and therefore a promising candidate material for cardiovascular applications. The next objective is a surface functionalization of the PEI-films in a cell specific manner to reach a functionally confluent, shear resistant HUVEC monolayer.

Keywords: Endothelial cells, shear resistance, polymer substrate, shear stress

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1. Introduction

Cardiovascular diseases (CVDs) are still the number one cause of death worldwide. In 2008 about 17.3 million people died because of CVDs representing nearly one third of all global death [27]. However not only the number of deaths increases, also the number of patients is raising, based on a longer lifespan and improved medical care. For that reason invasive therapies like angioplasty and stenting for stenosed or occluded vessels as well as vascular bypass surgery has become increasingly important during recent decades [25]. Currently more than 500,000 bypass surgeries are carried out per year [17].

For these invasive interventions the use of autologous surrogates is preferred, because of lower failure rates. Nevertheless, nearly one third of patients do not have suitable vessels because of advanced angiopathy or previous bypass surgery making the use of synthetic vascular grafts unavoidable [24, 43]. Clinically used prosthetic materials are poly(ethylene terephthalate) (PET) or extended poly(tetrafluoroethylene) (ePTFE) [18]. With regard to cardiovascular applications there is no clinical relevant difference between these materials but it had been shown in several *in vivo* trials that these synthetic grafts are inferior to autologous substitutes [41]. Especially for long term applications the patency rates are much lower with nearly 70% (PET (71%) and ePTFE (74%)) after one year and 58% (PET (59%) and ePTFE (56%)) after three years compared to 90% and 81% for autologous prosthesis, respectively [8, 19, 36, 37]. This particularly counts for small-caliber synthetic bypasses below 6 mm [16, 24]. The smaller the volume of the synthetic substitute the higher is the risk of complications or complete implant failure resulting from early thrombosis or late hyperplasia in consequence of endothelial nudation after implantation and thrombogenicity of the artificial surface [45, 47].

To improve the long-term patency and functionality of small-diameter grafts the luminal surface must be non-thrombogenic, either by surface passivation [1, 26, 32] and bioactive

coating [7, 12, 20] or endothelialization [25]. Endothelialization in form of prior cell seeding or *in vivo* recruitment is a completely different encouraging technique for overcoming the limitations of thrombogenicity by creating a natural anti-thrombotic surface on artificial grafts [4]. The endothelium plays a crucial role in regulation of the coagulation cascade, smooth muscle cell proliferation and migration as well as immunologic responses [28, 31]. However endothelial cell seeding is often limited by poor adherence of EC on the biomaterial surface once they are exposed to shear stresses [15]. Therefore, the graft prosthesis is often modified by coating with adhesion supporting RGD-peptides, matrix proteins (collagen, fibronectin, laminin), growth factors (VEGF, FGF, EGF) or a combination of all to enhance retention [38, 45].

Recently, very smooth poly(ether imide) films (R_q = 2.37±1.40 nm) were introduced as candidate material with proved cyto-, immuno- and hemocompatibility *in vitro* [33, 35]. The study revealed that primary human umbilical vein endothelial cells (HUVEC) were able to generate a confluent cell layer on PEI-films in a static test system [39]. In this study the behavior of HUVEC under shear stress in a dynamic test system was analyzed. A confluent HUVEC monolayer on PEI-films was stressed in a cone-and-plate shearing device with intermediate venous shear forces of 3 dyn/cm² for up to six hours. Subsequently, cell density, cell morphology, cell matrix interaction as well as the secretion of vasoactive and inflammatory mediators prior and after shear stress exposure in comparison to glass as control was investigated.

2. Material and methods

2.1 Preparation and characterization of poly(ether imide) films (PEI-films)

PEI-films were prepared from the commercially available polymer poly(ether imide) (ULTEM® 1000, General Electric, New York, NY, USA), sterilized and characterized as described previously [39].

2.2 Sample preparation

PEI-films were cut under sterile conditions into 30 x 30 mm squares and mounted between the two parts of the poly(ether ether ketone) (PEEK) based rheological sample holders (SmardCAD Deutschland GmbH, Neu-Ulm, Germany). Glass discs (25 mm, Gerhard Menzel GmbH, Braunschweig, Germany) were used as reference material. The growing area for the cells on the upper side of the sample holders was 4.5 cm². Before use, sample holders containing PEI-films or glass discs were pre-incubated for 30 min at 37 °C with 1 ml of endothelial cell media EGM-2 (Lonza, Cologne, Germany) to exclude leakage.

2.3 Cell culture and seeding protocol

Commercially available primary human umbilical vein endothelial cells (HUVEC, Lonza, Cologne, Germany) were used for no longer than four passages cultivating them under static cell culture conditions (37 °C, 5 vol% CO_2) in polystyrene-based cell culture flasks (TPP, Techno Plastic Products AG, Switzerland) with endothelial basal medium EBM-2 supplemented with EGM-2 Single Quots® kit and 2 vol% FCS (Lonza, Cologne, Germany). For rheological experiments confluent grown HUVEC were isolated by trypsin/EDTA treatment (0.25% v/v Trypsin and 0.53 mM EDTA in PBS(-/-), PAN-Biotech GmbH, Aidenbach, Germany), seeded on PEI-films or glass ($1.6 \cdot 10^4$ cells/cm², comparable to the static test system described previously [39]) and cultured under static cell culture conditions in the sample holders until shear stress exposure. The cell culture medium was replaced every two days and eight hours before starting the rheological experiments.

2.4 Rheological measurement

For the exposure of adherent HUVEC to uniform shear stress a cone-and-plate shearing device (SmardCAD 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), which was connected to a DC servo motor of the shearing device. Subsequently, the whole sample filled with 1.5 ml EGM-2 medium was carefully 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. With this setup, rotation of the cone at an angular speed of 11.6 rad/s generates a shear stress of 0.3 Pa (3 dyn/cm^2), which is typical for intermediate venous shear stress [22, 30]. Each sample was exposed to shear forces for six hours. Directly before and after the shear experiment images for cell density determination and morphology assessment were taken by using a QImagingRetiga™ 4000R digital camera (3 fields of view per sample with n = 6 samples; Retiga™ 4000R, QImaging, Surrey, British Columbia, Canada). Subsequently the sample holder was removed from the rotation chamber, supernatants for secretion profile analysis were isolated and material discs with remaining cells were fixed for fluorescent staining.

2.5 Cytoskeleton and focal adhesions evaluation

Actin cytoskeleton and the focal adhesion complex protein vinculin were stained by specific fluorescence labeled antibodies before and after shear stress exposure. Initially the cells were fixed with paraformadehyde (4%, v/v in 0.9% NaCl, 30 min, 4 °C) and pre-treated with Triton X-100 (0.5% v/v). F-actin was fluorescently stained with Phalloidin-Alexa555 (1:40, Molecular Probes®, Invitrogen, Germany) whereas for vinculin staining the polyclonal mouse anti-human vinculin IgG (Sigma, Taufkirchen, Germany) 1:50 and the Cy2 conjugated polyclonal goat anti-mouse IgG (Jackson ImmunoResearch, Hamburg, Germany) 1:200 was used. The genomic DNA/nuclei was stained by using 4',6-diamidino-2-phenylindole (DAPI,

1:5000, Roth, Germany). Afterwards samples were documented by taking images in various primary magnifications (20x, 40x and 100x) with the cLSM (LSM 510 META, Zeiss, Oberkochen, Germany).

2.6 Secretion profile analysis

The concentrations of the vasoactive mediators prostacyclin (PGI2) and thromboxane A2 (TXA2) as well as of prominent pro- and anti-inflammatory mediators (IL-1ra, IL-6, IL-8, IL-10, IL-12 and TNFα) and growth factors (PDGF-BB, FGF-b, GM-CSF and VEGF) were quantified from the supernatant of HUVEC seeded on PEI-films or glass prior to and after shear stress exposure as described previously [39]. As positive control recombinant human IL-1β (10 ng/ml, R&D Systems Inc., MN, USA) was added to seeded cells eight hours prior to starting the rheological measurement. The positive control was not exposed to shear stress and served only as a confirmation of the functionality of the HUVEC on the polymeric material.

2.7 Statistics

All data are reported as mean values ± standard deviation (SD) for continuous variables and were analyzed by a two-sided Student's t-test for paired samples. A p value of less than 0.05 was considered significant.

3. Results

3.1 Resistance and behavior of HUVEC monolayer on PEI-films under shear force

Microscopic images demonstrated that under static conditions HUVEC were able to form a confluent cell monolayer on the PEI-films within nine days after seeding. Generation of shear forces on the HUVEC monolayer caused a partial damage and the appearance of small cell free areas within the HUVEC monolayer (Fig. 1). The HUVEC density on the PEI-films did not differ from the HUVEC density on glass eight hours after seeding (Fig. 2; PEI-films:

11,939±2,548 cells/cm²; glass: 20,283±7,844 cells/cm²). After 48 hours, the cell density on PEI of 26,206±2,737 cells/cm² was significantly lower than on glass with 42,933±4,051 cells/cm² ($p = 9.98 \cdot 10^{-6}$). Nine days after seeding, the cell densities between both materials were comparable (PEI-films: 64,056±2,829 cells/cm²; glass: 71,656±8,830 cells/cm²).

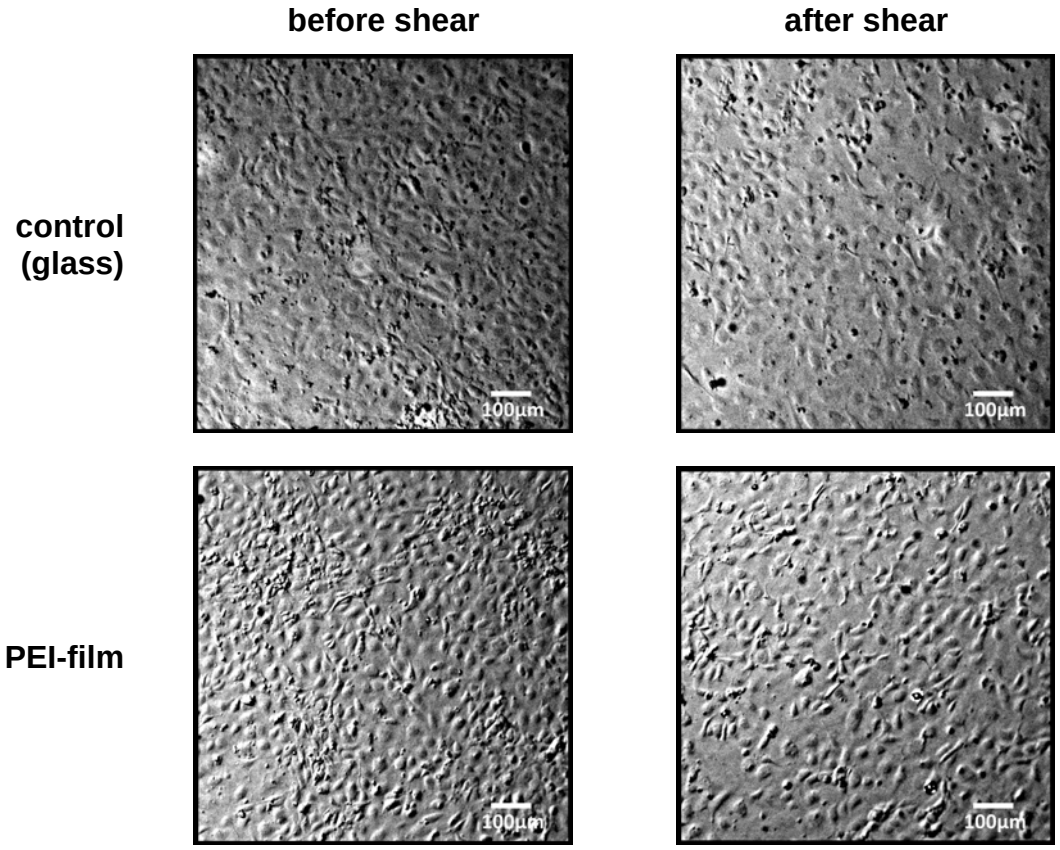

Fig. 1. Cell layer formation of primary HUVEC nine days after seeding prior and after shear stress exposure. Cells were seeded out on PEI-films (25 mm discs) and glass as a control and afterwards exposed to venous shear stress. (scale bar: 100 µm)

During and after shearing no significant differences in cell densities between both materials occurred (8 h: PEI-films: 7,706±1,377 cells/cm²; glass: 9,667±1,461 cells/cm²; after 48 h: PEI-films: 17,856±3,609 cells/cm²; glass: 20,739±2,579 cells/cm² and after nine days: PEI-films: 45,422±2,507 cells/cm²; glass: 42,239±5,607 cells/cm² after shear force exposure, respectively). The application of shear forces led to a cell loss (initial minus cell density after shearing) on PEI-films as well as on glass. The cell loss on the PEI-films was significantly lower than on glass (cell loss after 8 h: PEI-films: 35.5%; glass: 52.3% (p = n.s.); after 48 h:

PEI-films: 31.9%; glass: 51.7% (p = 0.010) and after nine days: PEI-films: 29.1%; glass: 41.1% (p = 0.0014) after shear force exposure, respectively).

3.2 Rearrangement of actin-cytoskeleton and reinforcement of focal adhesions

After exposure to shear stress no apparent differences between PEI and the reference were visible (Fig. 3). The laminar flow caused a loss of adherent cells on the material surface and the emergence of cell free areas in the layer, but had no visible effect on the cell morphology in cases of the actin cytoskeleton arrangement or a reinforcement of the focal adhesion complexes.

3.3 Secretion profile analysis

Prominent vasoactive mediators like the vasodilator prostacyclin (PGI2) and his physiological antagonist thromboxane A2 (TXA2) as well as pro-inflammatory cytokines (IL-6 and IL-8) were quantified

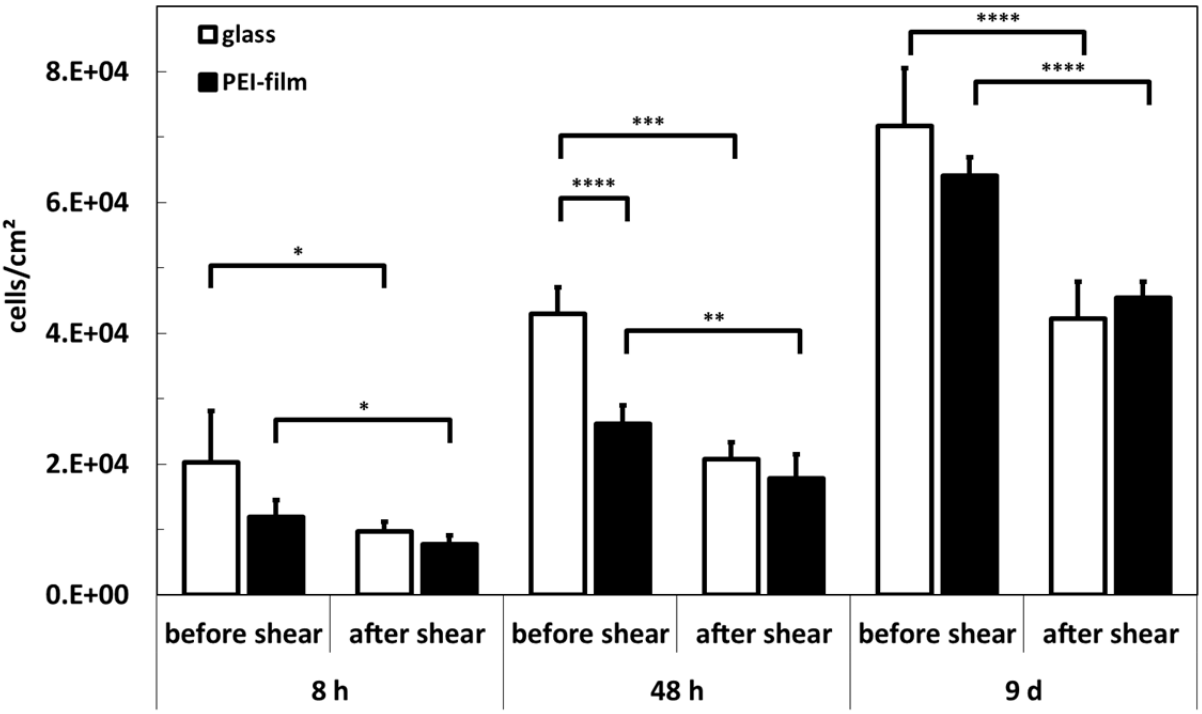

Fig. 2. Cell density of HUVEC seeded on PEI-films and glass as control for up to nine days before and after shear stress exposure, respectively. (Data analyzed by student's t-test; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$; $n = 6$.)

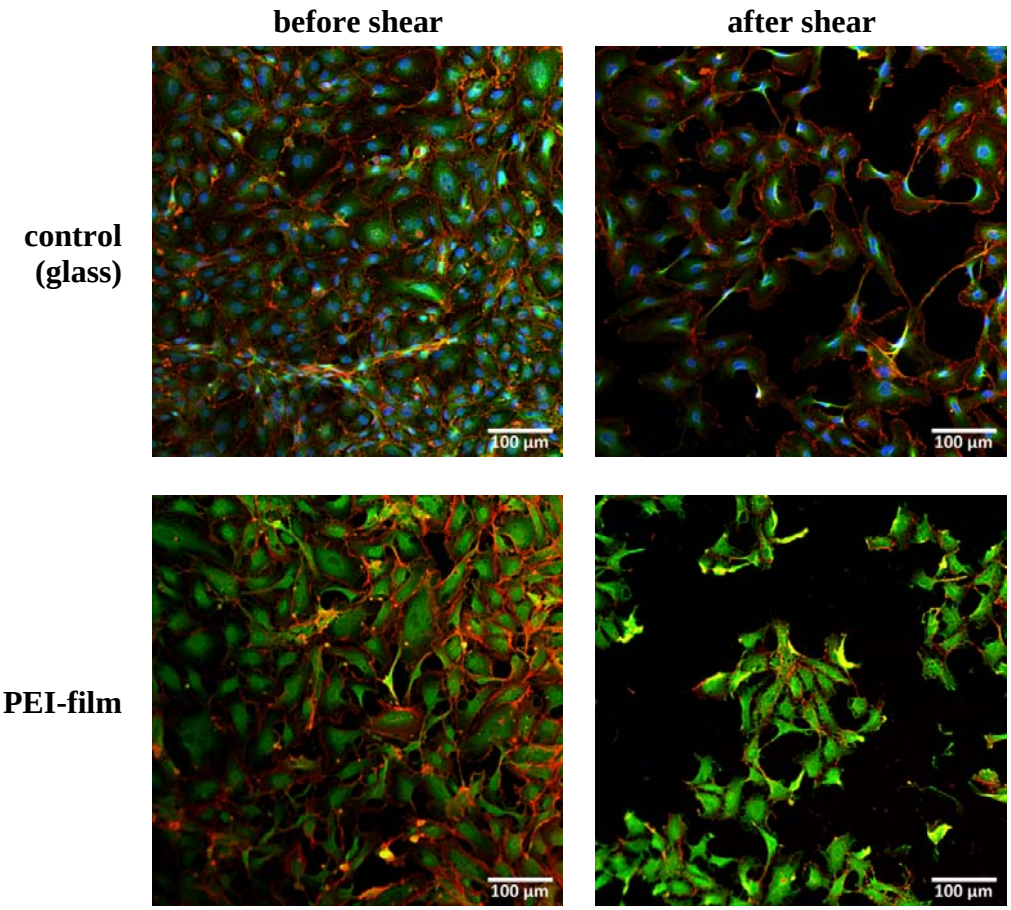

Fig. 3. HUVEC cytoskeleton and focal adhesions nine days after cell seeding on PEI-films (25 mm discs) and glass as control before and after shear stress exposure, respectively. Actin cytoskeleton (red), vinculin (green) and genomic DNA (blue) were fluorescently stained and images taken by using the cLSM with a 20x primary magnification.

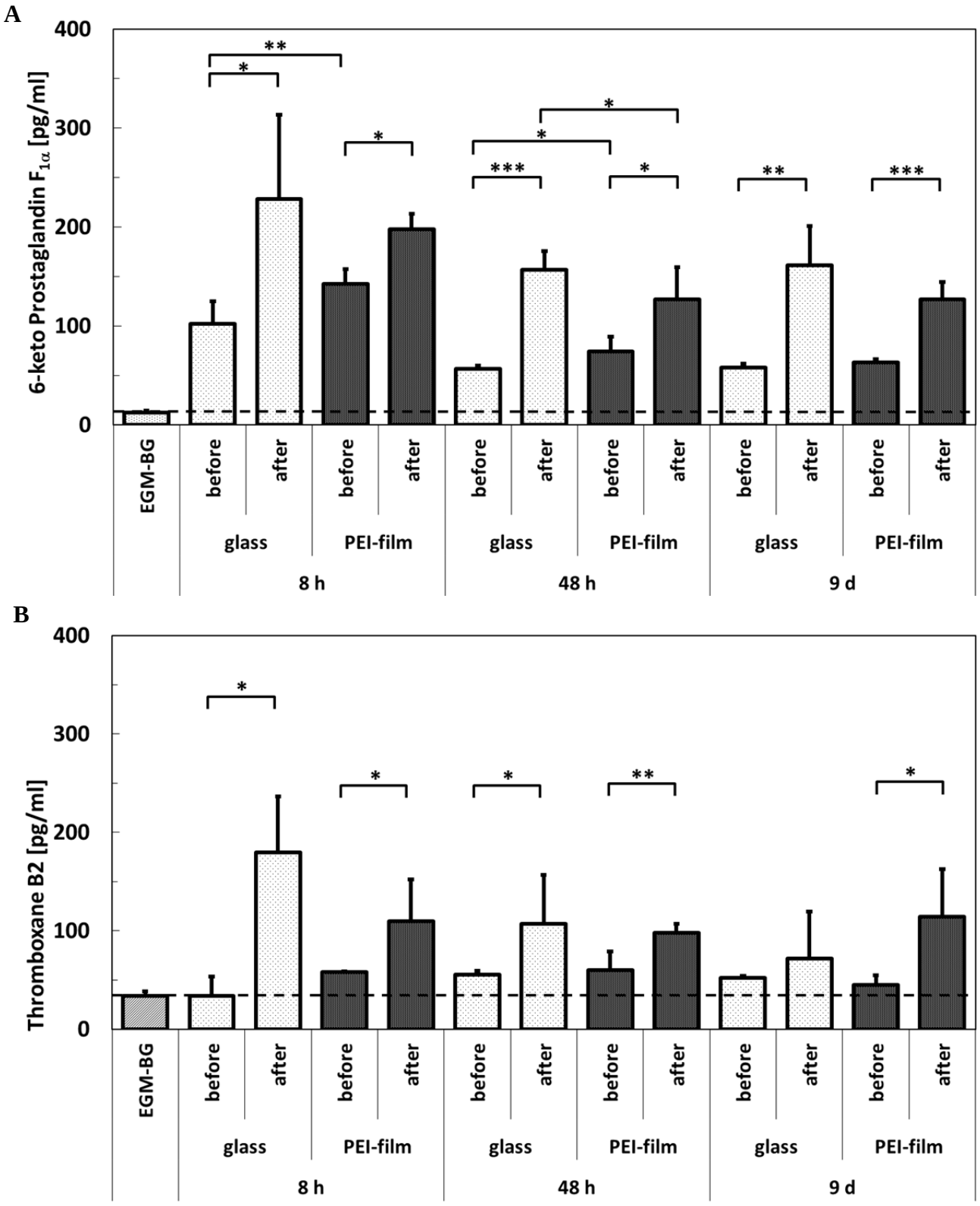

Fig. 4. Vasoactive prostacyclin (PGI2) (A) and thromboxane A2 (TXA2) (B) were quantified from the supernatant prior and after shear stress exposure by ELISA after culturing HUVEC up to nine days on PEI-film with glass as the control. (Data analyzed by student's t-test; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; EGM-BG: background control (EGM-2); $n = 6$.)

from the supernatant before and after shear stress exposure to assess the activation state and possible shear induced changes of the HUVEC secretion profile on the polymeric material in comparison to glass (Fig. 4 and 5).

No significant differences for PGI2 and TXA2 secretion between HUVEC seeded on PEI-films and on glass were detected neither before nor after shear stress exposure. Initially higher levels of PGI2 on PEI-films were detected under both static and dynamic conditions, but after 48 hours the amount of this mediator remained constant until the end of the experiment. For the un-sheared cells the secretion of PGI2 was always below 100 pg/ml, with nearly no secretion of TXA2. By introducing physiological shear forces to the cells the secretion of both mediators were significantly induced up to 3-fold for PGI2 and 5-fold for TXA2.

Additionally the amount of prominent pro- and anti-inflammatory mediators as well as several growth factors from the supernatant of PEI and glass seeded HUVEC was quantified before and after shearing.

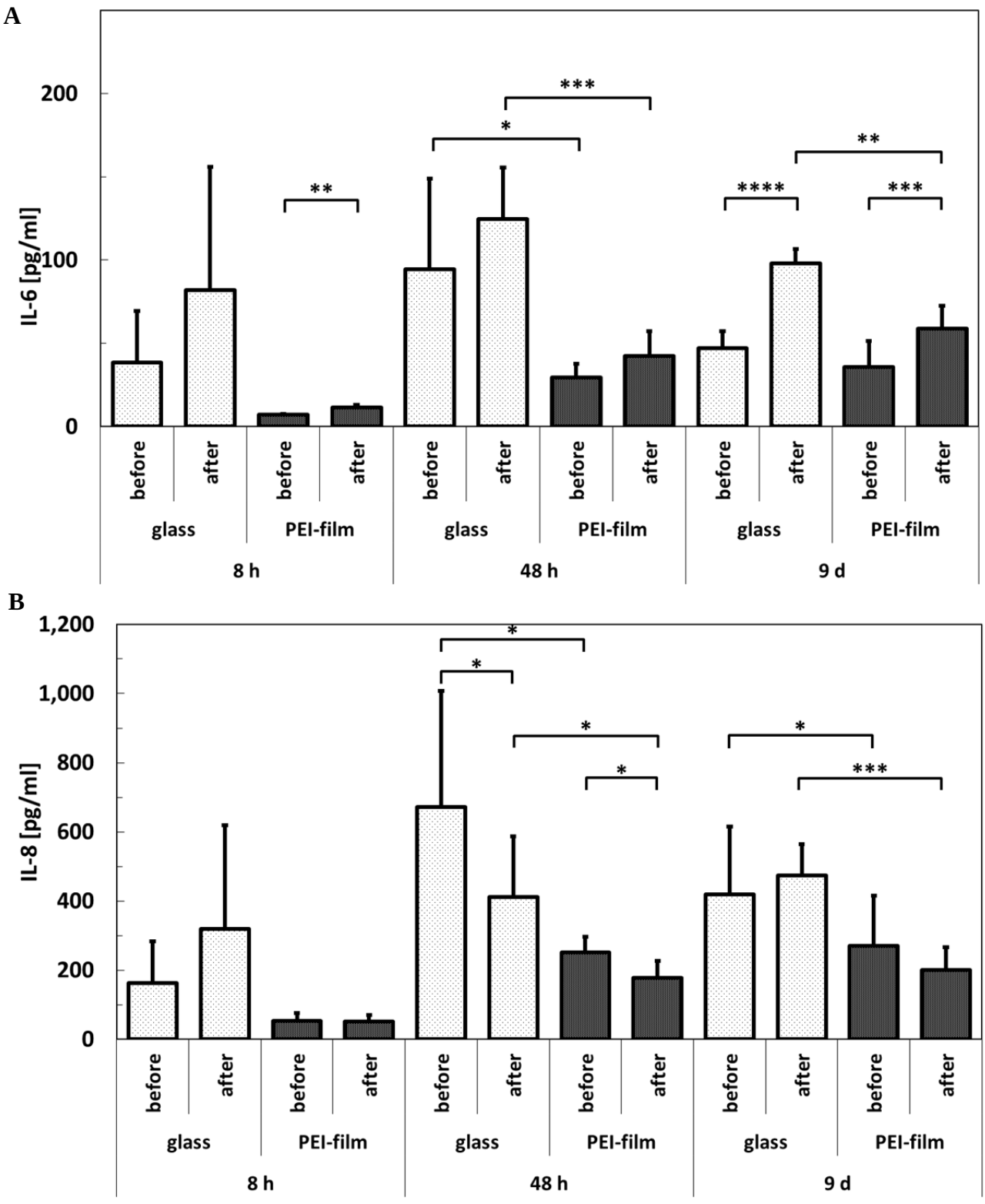

Fig. 5. Secretion profile of pro-inflammatory mediators IL-6 **(A)** and IL-8 **(B)** quantified from the supernatant prior and after shear stress exposure by ELISA after culturing HUVEC up to nine days on PEI-film with glass as the control. (Data analyzed by student's t-test; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$; $n = 6$.)

Only for the pro-inflammatory cytokines IL-6 and IL-8 shear- and material dependent differences were observed (Fig. 5).

The concentrations of both secreted mediators were always in the pg-range whereby the IL-1β activated positive controls were located in the ng-region (IL-6 between 3 – 60 ng/ml and IL-8 between 5 – 30 ng/ml for PEI-films and glass, respectively). Under static conditions the secretion of IL-6 on PEI-films was in tendency lower than on the reference material, but only significant 48 h after seeding (PEI-film: 29.58±8.19 pg/ml; glass: 94.63±54.40 pg/ml; $p = 0.03957$). The exposure of HUVEC to vein shear stress induced an increase of IL-6 secretion, which was significant for both materials after nine days of culturing (PEI-films before: 35.86±15.50 pg/ml and after shear: 59.07±13.64 pg/ml ($p = 0.00034$); glass before: 47.18±9.94 pg/ml and after shear: 97.97±8.75 pg/ml ($p = 0.00002$)). The amount of detected IL-6 on PEI was always lower than on glass and significant 48 h and nine days after seeding (after 48 h and shear forces: PEI 42.45±14.84 pg/ml and glass 124.49±31.06 pg/ml ($p = 0.00021$); after nine days and shear forces: PEI 59.07±13.64 pg/ml and glass 97.97±8.75 pg/ml ($p = 0.00098$)).

For the pro-inflammatory cytokine IL-8 there was no such clear shear force related secretion inducing effect visible. The detectable amount of IL-8 remained nearly unchanged between static and dynamic conditions. Independent of shear stress there was a marginal lower secretion of IL-8 on PEI-films detectable, which was significant after 48 h and nine days. This was observed before and after shear stress exposure (after 48 h before shear: PEI 251.06±45.36 pg/ml and glass 672.70±335.76 pg/ml ($p = 0.031$) and after shear: PEI 178.64±48.99 pg/ml and glass 411.91±174.64 pg/ml ($p = 0.012$) after nine days before shear: PEI 269.57±145.04 pg/ml / glass 418.54±196.56 pg/ml ($p = 0.0052$) and after shear: PEI 200.70±66.58 pg/ml and glass 473.23±90.38 pg/ml ($p = 0.00011$)).

4. Discussion

In this study the shear resistance of HUVEC seeded on very smooth poly(ether imide) films in comparison to glass was investigated under dynamic conditions by treating the HUVEC monolayer with venous shear stresses.

While observing the development of the HUVEC layer under static conditions there was a delayed growing of HUVEC on PEI-films visible, confirming the findings from an earlier study [39]. In comparison to the glass an initially lower adherence of the HUVEC on the PEI-films eight hours after seeding was visible which resulted in a lower cell density 40 hours later. This might be due to the very hydrophobic and smooth surface of PEI, which is known to be associated with a decreased attachment and proliferation of various cell types [44]. The passively adsorbed amorphous protein layer on the polymeric surface might have aggravated the interaction between endothelial cell receptors with the underlying matrix, which are liable for cell adhesion and extracellular matrix (ECM) formation [14, 21]. However, the formation of similar cell densities on PEI-films compared to glass after nine days showed obviously the capability of HUVEC to reach confluence on PEI-films by creating their own extracellular matrix [34, 44].

The exposure to vein shear stress over a short period of time revealed a substantial better adherence of the HUVEC monolayer on the PEI-films which became significant 48 hours and nine days after cell seeding. This is surprising because of the initially lower cell density on PEI compared to glass. This shows that the loss of HUVEC on PEI-films was much lower than on glass, particularly within the first day. On both materials cell free areas were observed after shear stress exposure. *In vivo* these areas would – because of the thrombogenic polymer surface – be accessible for thrombocytes from the blood stream, possibly resulting in thrombocyte activation and subsequent clot formation.

The investigation focused on the actin-cytoskeleton and the focal adhesion formation. Under static conditions our recent findings in terms of a strong cell-cell and cell-matrix interaction could be confirmed [39]. In contrast to former studies [3, 11] we could neither observe a re-arrangement of HUVEC with stress fiber formation or circular ruffles nor a re-enforcement of the focal adhesions as a consequence of shear stress exposure. Under long-term physiological laminar shear forces at higher shear rates endothelial cells normally change their morphology towards a spindle shape aligned in the direction of flow *in vitro* and *in vivo*, what is attended with the further mentioned intracellular changes [10]. Here the cytoskeleton acts together with the focal adhesion complexes via integrins as mechanosensors, transmitting extracellular information into the cell [46]. As an adaption to shear forces several intracellular cascades are activated resulting in changes of the gene expression, proliferation, morphology and migration [3]. In our study only single cells showed the described re-enforcement of both the cytoskeleton and the focal adhesions associated with a reduction of the spreading area. These effects were observed in the same manner for PEI-films and the reference material. It is discussed that such cells might be in another period of the cell cycle like cell division, what would likewise explain the reduced cell spreading, which is typical for human endothelial cells in this phase. Another reason for the adaption of the cells to shear forces may be the conditions during our experiments. The selection of intermediate venous shear stresses of 3 dyn/cm² over the relative short period of time might not be long enough and the shear force high enough to induce these cell reactions [10].

The quantification of the antagonistic acting vasoactive mediators PGI2 and TXA2 from the supernatant indicated no functional changes of the HUVEC seeded on PEI-films compared to the reference material for the static and the dynamic part of the study. The detected amounts were in the physiological effective range for both mediators [2, 29], whereby an appreciable TXA2 secretion was only ascertained under shear. The secretion profile of the vasodilating

PGI2 was initially elevated under both conditions but reached a steady state 48 hours after seeding. This behavior of the HUVEC monolayer under shear is already known and the amplified production of PGI2 serves to counteract an adhesion and activation of thrombocytes [9, 13, 23]. In the same manner we observed a shear induced up to 5-fold increase of the TXA2 amount in the supernatant, when the HUVEC were treated with shear forces. This observation may result from the occasional rupture of the HUVEC out of the monolayer during shear stress exposure. As mentioned above, cell free areas had appeared in the initially confluent HUVEC monolayer.

In relation to the pro-inflammatory cytokines IL-6 and -8 the results from a former study were confirmed [39]. The quantified amounts of IL-6 and -8 from the corresponding IL-1β (10 ng/ml) activated positive controls were, depending on the time point after seeding, in the range between 3 and 60 ng/ml so that the differences between both materials in the pg-range were only marginally.

Additionally we detected a significant shear induced increase of the IL-6 secretion for the HUVEC on both materials after nine days. These findings are in line with previous *in vitro* studies quantifying the IL-6 secretion of resting human endothelial cells in dynamic test systems [6, 42]. Thereby the treatment of HUVEC with low shear forces may support *in vivo* the progression of arteriosclerosis and *in vitro* to pro-inflammatory cell reactions, which go hand in hand with the detected IL-6 increase [5, 40].

5. Conclusion

The study revealed that HUVEC can generate a confluent and functional monolayer on smooth PEI films and are able to resist exposure to venous shear stress. Changes in morphology and adhesion behavior, e.g. reinforcement of stress fibres and focal adhesions, showed the typical pattern of shear stress exposed endothelial cells. However, cell adherence on PEI was not yet sufficient to retain a confluent HUVEC monolayer after shear stress

exposure. Occasionally, cells or parts of the cell layer were disrupted resulting in cell free areas in the confluent HUVEC layer. Apart from this our data suggest that PEI is a suitable substrate for HUVEC under static and dynamic conditions and therefore a promising candidate material for cardiovascular applications.The next objective is to create a cell specific, endothelialization supporting functionalization on the PEI-film surface to reach a functional and shear resistant confluent HUVEC monolayer.

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