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Comparison of two substrate materials used as negative control in endothelialization studies: glass versus polymeric tissue culture plate

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Abstract

The endothelialization of synthetic surfaces applied as cardiovascular implant materials is an important issue to ensure the anti-thrombotic quality of a biomaterial. However, the rapid and constant development of a functionally-confluent endothelial cell monolayer is challenging. In order to investigate the compatibility of potential implant materials with endothelial cells several *in vitro* studies are performed. Here, glass and tissue culture plates (TCP) are often used as reference materials for *in vitro* pre-testing. However, a direct comparison of both substrates is lacking.

Therefore, a comparison of study results is difficult, since results are often related to various reference materials. In this study, the endothelialization of glass and TCP was investigated in terms of adherence, morphology, integrity, viability and function using human umbilical vein endothelial cells (HUVEC).

On both substrates an almost functionally confluent HUVEC monolayer was developed after nine days of cell seeding with clearly visible cell rims, decreased stress fiber formation and a pronounced marginal filament band. The viability of HUVEC was comparable for both substrates nine days after cell seeding with only a few dead cells. According to that, the cell membrane integrity as well as the metabolic activity showed no differences between TCP and glass. However, a significant difference was observed for the secretion of IL-6 and IL-8. The concentration of both cytokines, which are associated with migratory activity, was increased in the supernatant of HUVEC seeded on TCP. This result matches well with the slightly increased number of adherent HUVEC on TCP.

In conclusion, these findings indicate that both reference materials are almost comparable and can be used equivalently as control materials in *in vitro* endothelialization studies.

Key words: Negative control, endothelial cells, glass, TCP, reference

1. Introduction

The lack of a blood compatible synthetic interface is one of the largest challenges in the field of biomaterials science. This technological shortcoming hinders e.g. the successful clinical application of small diameter vascular grafts but also of other cardiovascular devices such as stents or artificial heart valves [1-3]. Materials, which are considered for use as cardiovascular implants, should provide a broad range of properties or functionalities, which are necessary for a long-term function of the device [4]. This implies i) short term an appropriate hemocompatibility, in order to reduce the thrombus formation at the luminal site of the implant, ii) a region-specific elasticity to avoid a mechanic mismatch, iii) long term a degradation behavior, which is adapted to the function, and iv) a fast and shear-resistant endothelialization of the luminal site of the implant, in order to prevent long term the adherence of blood and blood components thereby hindering the dysfunction of the implant.

Usually, the endothelialization of implant materials is evaluated in i*n vitro* studies [1, 5 - 13]. For this purpose, endothelial cells are seeded and subsequently the adherence of the endothelial cells until monolayer formation is investigated in terms of endothelial cell density, morphology, viability and cell function. The assessment of the endothelial cells is performed in comparison to cells seeded on a control material (negative control). In most studies glass or tissue culture plates (TCP) are applied as control materials. However, a study comparing both materials is not existing until yet. Therefore, the direct comparison of different study results in the literature is difficult. Thus, the seeding of endothelial cells was investigated on glass and TCP until monolayer formation in the frame of the present study.

2. Material and Methods

The formation of an endothelial cell monolayer on glass or tissue culture plates (TCP) was evaluated using human umbilical venous endothelial cells (HUVEC). A time period of nine days was considered to be sufficient to form functional confluence [14]. The study was performed in accordance with the ethical guidelines of Clinical Hemorheology and Microcirculation [15].

2.1 Materials

As control or reference materials i.) uncoated glass microscope coverslips made of colorless borosilicate glass (Gerhard Menzel GmbH, Braunschweig, Germany) or ii.) corona discharged treated polystyrene tissue culture plates (TCP; 24 well plate, Trasadingen, Switzerland) were used. The surfaces of both materials were very smooth, the roughness of the coverslips was R_q = 12.9 ± 2.0 nm and comparable to the roughness of TCP with R_q=6.1±0.7 nm. Both materials were hydrophilic; the glass coverslips had a wettability of 43±9 °, TCP of 56±7 °.

2.2 Contact angle measurements

Dynamic contact angle (DCA) measurements were conducted in ultra-pure deionized water with a conductivity of 0.055 µS/cm (Ultra Clear UV clean water system, SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany) at ambient temperature on a drop shape analyzer (DSA 100, Krüss GmbH, Hamburg, Germany) using the captive bubble method. Advancing and receding contact angle measurements were performed

by stepwise withdrawing/adding of air from/to the captured bubble, while the bubble was increased with each measurement cycle from 2 to 5 mm in diameter. Prior to the DCA measurement, all samples were preconditioned for 24 hours in deionized water at ambient temperature for equilibration. At least five measurements for advancing and receding angles on three different locations were performed for each sample. The average of the contact angles was calculated as well as the standard deviation.

2.3 Profilometry

The surface profile was analyzed with an optical profilometer type MicoProf 200 (FRT GmbH, Bergisch Gladbach, Germany) equipped with a CWL 300 chromatic white-light sensor by scanning an area of 50 × 50 μm^2 at three different areas of each sample. The square surface roughness mean values (R_q) of the substrates were obtained using the analysis module of the software MARK III.

2.4 Endothelial cells

Primary human umbilical venous cells (HUVEC; Lonza, Cologne, Germany) of the fourth passage [14] were cultured in EGM-2 under static cell culture conditions (37 °C, 5% CO_2) with endothelial basal medium EBM-2 supplemented with EGM-2 Single Quots® kit and 2 vol% FBS (Lonza, Cologne, Germany). For cell culture experiments HUVEC were harvested by trypsin/EDTA treatment (0.25% v/v Trypsin and 0.53 mM EDTA in PBS(-/-), PAN-Biotech GmbH, Aidenbach, Germany) and 158 HUVEC/mm^2 were seeded on the two reference materials. The experiments including cell density analysis, viability analysis, analyzes of released HUVEC mediators, protein expression, and immuno-cytochemistry were performed at day 2, and 9 after cell seeding. The cell culture medium was replaced every two days and eight hours before starting the rheological experiments.

2.5 Cell viability assessment

Viability and monolayer formation of HUVEC were examined after two and nine days by fluorescence staining. Viable cells were stained with fluorescein diacetate (FDA, 25 µg/ml, Invitrogen, Carlsbad, CA, USA) whereas propidium iodide (PI, 2 µg/ml, Sigma, Taufkirchen, Germany) staining indicates dead cells. Downstream documentation was realized by taking three pictures from different areas of each sample by using a confocal laser scanning microscope (cLSM) with a 10x primary magnification (LSM 510 META, Zeiss, Oberkochen, Germany).

2.6 Cell membrane integrity and metabolic activity

After two days of cultivation HUVEC membrane integrity and metabolic activity was examined by lactate dehydrogenase (LDH) activity in the extracellular fluid (Cytotoxicity Detection Kit LDH, Roche, Grenzach, Germany) and by quantification of the mitochondrial activity using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, Mannheim, Germany).

2.7 Cytoskeleton and focal adhesions evaluation

Actin cytoskeleton and the focal adhesion complex protein vinculin were stained by specific fluorescence labeled antibodies after seeding 3 x 10^4 HUVEC out on TCP (n = 6) or 14 mm glass discs as inlays in the TCP well (n = 6; Gerhard Menzel GmbH, Braunschweig, Germany) up to nine days [16]. The cells were fixed with paraformaldehyde (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 were stained by using 4',6-diamidino-2-phenylindole (DAPI, 1:5000, Roth, Germany). Samples were documented by taking three different fields of view in various primary magnifications (20x, 40x and 100x) with the cLSM (LSM 510 META, Zeiss, Oberkochen, Germany).

2.8 Secretion profile screening

The prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) concentrations from glass and TCP seeded HUVEC were determined by using a competitive enzyme immuno assay for the stable hydrolysis products of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ and TXA_2, thromboxane B_2 (TXB_2) after two and nine days of culturing (6-keto-Prostaglandin $F_{1\alpha}$ EIA Kit and Thromboxane B_2 EIA Kit from Cayman Chemical Company, Ann Arbor; MI, USA). Similarly, the concentrations of prominent secreted inflammatory cytokines (IL-6 and IL-8) were quantified from the supernatant by a Bioplex™ Cytokine Assay (Bio-Rad Laboratories Inc., Munich, Germany) whereas cell culture medium EGM-2 was always used as a negative control.

2.9 Statistics

Data are reported as arithmetic mean ± standard deviation for continuous variables and were analyzed by a two-sided Student's t-test for paired or unpaired samples, respectively. A *p* value of less than 0.05 was considered significant.

3. Results

3.1 Morphology and density of HUVEC on glass and TCP

Seeding experiments up to nine days confirmed that HUVEC were able to adhere and proliferate to confluence on TCP as well as on glass. Two days after seeding 457 ± 8 HUVEC/mm² adhered on TCP and 429 ± 41 HUVEC/mm² on glass (p=0.46), nine days after seeding 951±148 HUVEC/mm² were adherent on TCP and 717 ± 88 HUVEC/mm² on glass (p=0.046) (Table 1). On both substrates a nearly confluent HUVEC monolayer had formed with more HUVEC adhering on TCP at day nine (p=0.0046).

Table 1: HUVEC density on TCP or glass 2 and 9 days after seeding

Substrate	**HUVEC/mm²**		
	seeded	after 2 days	after 9 days
TCP	158	457 ± 8	951±148
Glass	158	429 ± 41	717 ± 88
		p=0.46	p=0.0046

Figure 1 shows HUVEC on TCP or glass after live/dead staining. After two days of seeding, most HUVEC on both substrates exhibited a large, spindled shape and were randomly organized. On glass HUVEC exhibited an elongated shape and showed more migratory activity than HUVEC on TCP.

HUVEC on TCP HUVEC on glass

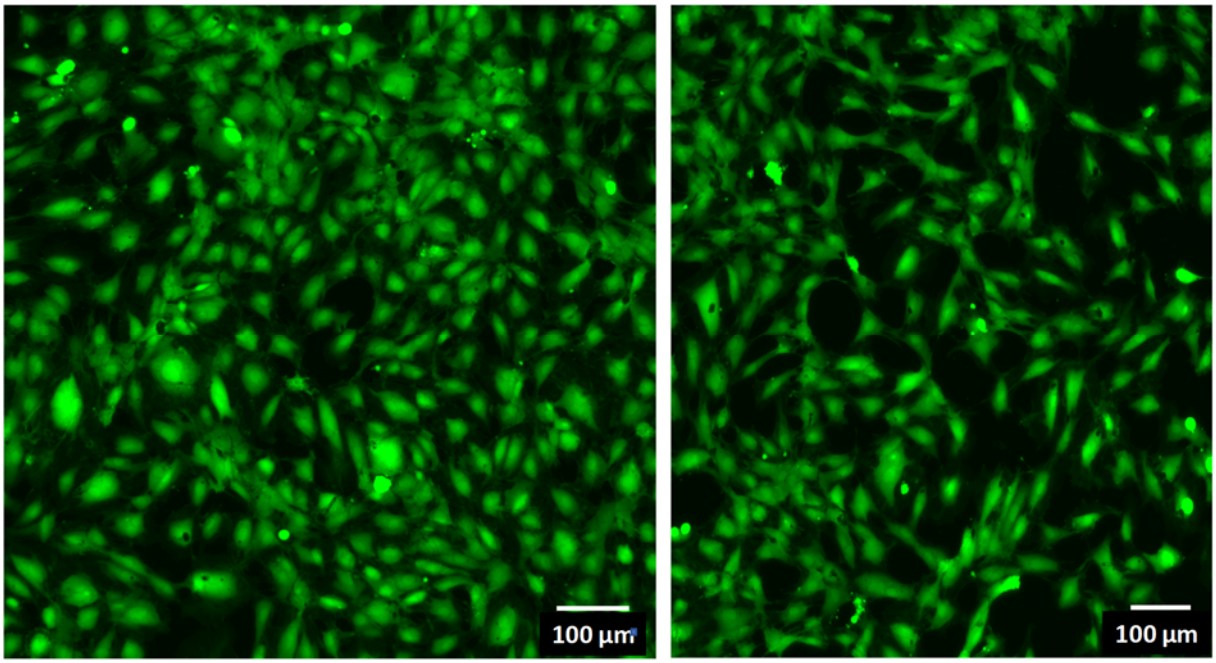

Figure 1: HUVEC on TCP or glass two days after seeding (before culture medium change)

After nine days of seeding, a confluent HUVEC monolayer had formed on both substrates. Cell rims were clearly visible and no overgrowing had occurred.

The number of adherent dead cells after two or nine days did not differ between the two substrates ($p>0.05$; TCP: after 2 days 6 ± 8 HUVEC/mm² (1.3%) after 9 days 62±22 HUVEC/mm² (6.5%); Glass: 2 days 14 ± 9 HUVEC/mm² (3.3%), after 9 days 22 ± 13 HUVEC/mm² (3.1%)).

LDH activity in the supernatant (TCP: 0.10 ± 0.02 AU; glass: 0.04 ± 0.04 AU) as well as the mitochondrial activity (TCP: 0.54 ± 0.04 AU; glass: 0.32 ± 0.01) showed 48 hours after seeding no considerable differences neither for the cell membrane integrity nor for the metabolic activity between both substrate materials.

3.2 Cytoskeleton and focal adhesion

Cytoskeleton and focal adhesion complex formation were evaluated through immuno-fluorescence staining of actin filaments and vinculin. Two days after seeding HUVEC showed the typical pattern of a sub-confluent monolayer on both substrates (Fig. 1). After nine days, they exhibited clearly less actin stress fibers in central parts of the cells, which are usually

formed during the adhesion process (see Fig. 2 left). The terminal ends of these cytoskeleton fibers were in most cases co-localized with vinculin, which is an essential structural protein of focal adhesion complexes. On both substrates the tendency to a condensation and narrowing of the marginal filament band could be observed. In general, the HUVEC approached the state of a functionally confluent monolayer [17]. This process seemed to be more advanced on glass.

Staining cell genomic DNA (nuclei) with DAPI showed the nuclei of the cells on both substrates very clearly; nearly all with good visible nucleoli. A few HUVEC showed more than 1 nucleus, without a difference depending on the substrate.

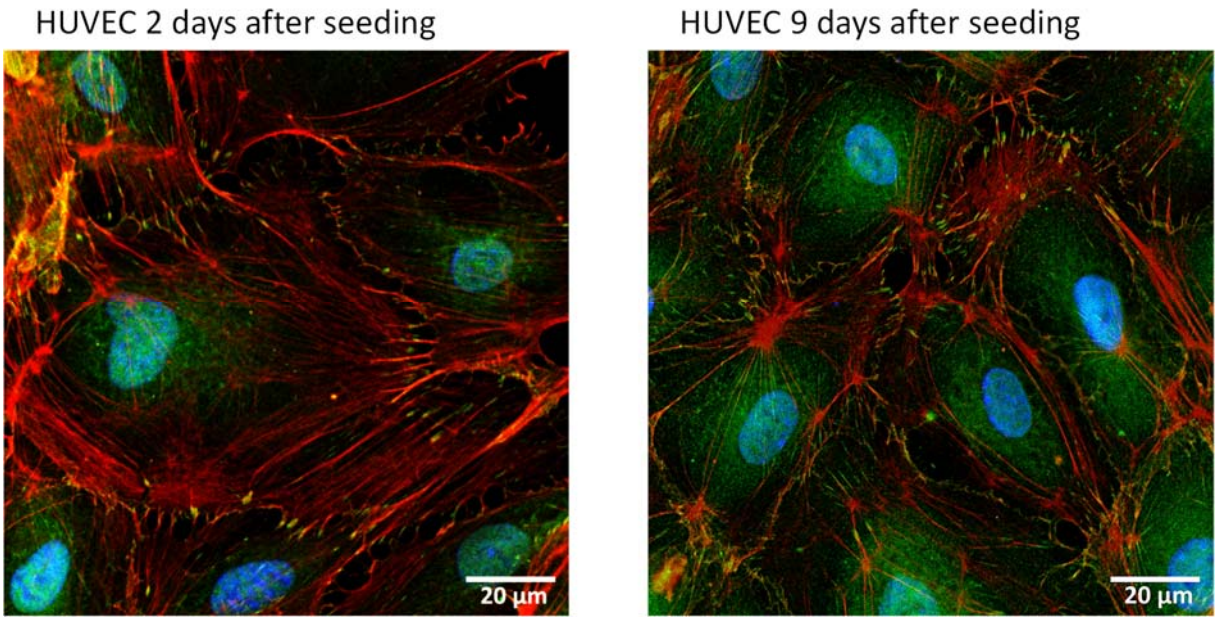

Figure 2: HUVEC cytoskeleton and focal adhesions 2 or 9 days after seeding 158 HUVEC/mm^2 on glass.
(Actin cytoskeleton (red), vinculin (green) and genomic DNA (blue) were fluorescently stained and pictures taken by using the cLSM with a 40x magnification.)

3.3 Secretion profile screening

Vasoactive mediators like prostacyclin (PGI_2) and his physiological antagonist Thromboxane A2 (TXA_2) as well as prominent inflammatory cytokines (IL-6 and IL-8) were quantified using the supernatant by competitive ELISA assay to assess the activation state of the HUVEC on both substrates.

In the supernatant of HUVEC PGI_2-values around 100 pg/ml were found two days and values around 60 pg/ml nine days after seeding for both materials ($p>0.05$ each).

Overall there was no relevant TXA_2 secretion during the whole experiment after two days (TCP: 48.6 ± 8.4 pg/ml; glass: 55.6 ± 3.8 pg/ml), and after nine days (TCP: 50.7 ± 4.7 pg/ml; glass: 52.2 ± 2.3) for HUVEC on both materials.

Secretion of the pro-inflammatory cytokine interleukin 6 (IL-6) in the pg-range raised after two days on TCP from 62.4 ± 9.2 pg/ml to 128 ± 33 pg/ml ($p<0.0001$) but decreased on glass from 94.63 ± 54.4 pg/ml to 47.2 ± 9.9 pg/ml ($p=0.0619$). The increase of IL-6 on TCP with 65.8

±12.8 pg/ml differed significantly from the decrease on glass with -47.4 ± 50.9 pg/ml (p=0.0004).
The concentration of interleukin 8 (IL-8) was always in the ng-range increasing during the experiment: IL-8 raised from 615.5 ± 286.3 pg/ml on TCP after two days up to 1,717 ± 254 pg/ml after nine days (p=0.0001) while the concentration on glass decreased in the same time period from 673 ± 336 to 418.5 ± 196.5 pg/ml (p=0,1406). The increase of IL-8 on TCP with 1,101 ± 565 pg/ml differed significantly from the decrease on glass with -254 ± 163 pg/ml (p=0.0002).

4. Discussion

In the present study the adherence, morphology, integrity, viability and function of HUVEC was evaluated on two different substrates, which are often used in *in vitro* endothelialization studies as control or reference material [18]. The morphology of adherent endothelial cells is characterized by their shape as well as by their microfilament organization; a dense marginal filament band at the rim of the cells and scarce or absent stress fibres in central parts of the cells [19]. In addition, the pattern of released vasoactive, inflammatory substances depends on the status and the microenvironment of the cells.

Proteins in blood plasma or in culture medium adhere very quickly to body foreign materials, so that a direct contact between material surfaces and surrounding cells is unlikely [20]. *In vitro*, endothelial cells adhere, when proteins are in the culture medium [21]. The adherence as well as spreading and proliferation of the cells depend clearly on the proteins adsorbed, their conformation and the integrin receptors presented on the cell surface [21, 22]. To support the adherence of the HUVEC, the culture medium was supplemented with fetal bovine serum (2% v/v). On both substrates HUVEC could adhere and a nearly confluent cell monolayer had developed after nine days. On TCP significantly more HUVEC adhered compared to glass. Dissociated peripheral filament bands and stress fibers were visible after two days on both substrates. This development was less pronounced after 9 days with the peripheral filament band getting condensed and a decrease of the stress fibers in central parts of the HUVEC (see Fig. 2). Especially on glass the tendency to a condensation and narrowing of the marginal filament bands together with the decrease of stress fibres approached the typical physiological cobblestone pattern. The morphology of the HUVEC indicated a pronounced cell-to cell and cell-to-substrate binding, which is necessary for the formation of a functionally-confluent and shear resistant HUVEC monolayer [23]. Vinculin is visible at the cell rims typically at actin-anchored cadherin adhesions demonstrating the high cell-cell binding (see Fig 2). On both substrates, up to 6% of the adhered cells were dead.
Figure 2 clearly shows a lot of nucleoli - suborganelles within the nucleus - in HUVEC. With the identification of ribosomal RNA (rRNA) and proteins within the nucleolus, the organelle was determined as the site for nascent ribosomal biogenesis and assembly [24]. The nucleolus has been associated with many diverse cellular functions. Approximately 30% of 4,500 nucleolar proteins discovered, are linked to ribosomal assembly and processing [25]. Such nucleolar proteins play vital roles in cell cycle control, signal recognition particle assembly, cell growth, microRNA biogenesis, viral replication, cell death, nuclear transport, cellular

senescence, and stress response signaling [24, 26-28]. Especially 2 days after seeding the cells are in the proliferating state, the number of nucleoli is described for actively synthesizing cells [29, 30].

Prostacyclin (PGl_2) is involved in the regulation of the vascular tone, in pro-inflammatory and anti-thrombogenic processes *in vivo* in order to limit potential thromboses. In physiological conditions, endothelial cells show an anti-thrombotic phenotype characterized by the degradation of the aggregating agent ADP with ectonucleotidases; the prevention of platelet activation, adhesion and aggregation by expressing 13-hydroxyoctadecadienoic acid (13-HODE) and releasing prostacyclin (PGI_2), and NO [31, 32]. Endothelial cells are able to respond to changes in their environment within minutes. This acute response follows receptor-mediated cell signaling accompanied by influx of calcium ions and induction of various types of phosphorylations, which result i) in activation of enzymes that generate NO, prostacyclin or endothelin-1, and ii) in the recruitment of vesicles with pre-formed proteins to the plasma membrane reinforcing endothelial cell activation [33]. Increasing amounts of released prostacyclin indicate a state described as perturbation of endothelial cells [34 - 37]. The findings of this study clearly show for both materials that the HUVEC were not in an activated or perturbated state but showed a behavior comparable to physiological conditions. Also, the concentrations of thromboxane A2 did not differ between the two materials at both time points. The anti-thrombogenic ratio of PGI_2/TXA_2, which is balanced under physiological conditions, indicated for both substrates a slight shift to an anti-thrombotic behavior and normalized after 9 days.

However, there was a marked difference according to the release of interleukins. HUVEC on TCP released significantly more interleukin-6 compared to HUVEC on glass after nine days (the concentration of interleukin-6 increased from day 2 to day 9 by 105% on TCP but decreased by 50% on glass). Since interleukin-6 release can lead to an increase of the migration and proliferation of endothelial cells, this could explain the migratory activity and the higher density of HUVEC on TCP [38, 39]. The same is true for interleukin-8 [40]; the increase from day two to day nine on TCP differed also significantly from the decrease on glass.

However, the concentrations of interleukin-6 released on TCP or glass between 47 – 127 pg/ml were clearly below concentrations reported to induce maximal responses (100 – 600 ng/ml) of endothelial cells [32], though a certain influence cannot completely be ruled out. In this regard, the situation concerning interleukin-8 was different. Here, the concentrations of IL-8 on TCP (not on glass) were with 1.7 pmol/l in the range described to induce an effect on the proliferation and migration of endothelial cells [41, 42]. Already at concentrations above 1.25 pmol/l a doubling of the HUVEC number was reported [34]. Very recently Yu et al. could show that autocrine IL-8 promoted endothelial cell migration via the Src/Vav2/Rac1/PAK1 signaling pathway [42], which supports the endothelialization process and seemed to be one of the reasons for the higher HUVEC density on TCP.

5. Conclusion

HUVEC adhered on TCP as well as on glass remaining viable and presented a typical monolayer pattern. On both substrates a nearly confluent HUVEC monolayer had formed with TCP slightly more HUVEC adhering after nine days of cultivation. On both substrates a condensation and narrowing of the marginal filament band could be observed with a decrease of stress fibres in central parts of the cells gradually approaching a functionally-confluent

HUVEC monolayer. The higher HUVEC density on TCP after nine days is most likely attributable to the higher release of interleukin-8.
These findings indicate that both materials are comparable according to their adherence and proliferation during the formation of an endothelial cell monolayer and can be used equivalently as control materials in *in vitro* endothelialization studies.

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