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Angiogenically stimulated alternative monocytes maintain their pro-angiogenic and non-inflammatory phenotype in long-term co-cultures with HUVEC

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Abstract

Angiogenically stimulated alternative monocytes (aMO2) could be established as cellular release system accelerating the endothelialization of polymers rendering their surfaces hemocompatibility in a short-term study. However, for their clinical application it is essential that aMO2 do not switch back to the MO1 state sustaining their capability as cellular release system over an extended period of time.

We explored whether aMO2 can maintain their differentiation state over 21 days in a mono- and in a co-culture with HUVEC. In comparison, the influence of recombinant VEGF-A_{165} on the endothelialization of biomaterials was assessed including endothelial cell (HUVEC) density, organisation of the endothelial cytoskeleton, cytokine secretion profile and release of prostacyclin, thromboxane A2 and matrix metalloproteinases.

In mono-culture aMO2 secreted high amounts of VEGF and other growth factors/cytokines. Co-cultured with HUVEC, aMO2 accelerated the formation of a confluent HUVEC monolayer. Furthermore, no pro-inflammatory cytokines were found, neither in aMO2-mono, nor in co-cultures with HUVEC indicating that the majority of the aMO2 remained stable in their aMO2 state during the 21 days of cultivation.

In contrast, the addition of recombinant VEGF-A_{165} instead of the co-culture with aMO2 resulted in the formation of stress fibres, dissociated marginal filament bands, and a detachment of HUVEC. In addition, the profile of bioactive agents of HUVEC (e.g. prostacyclin, thromboxane A2, matrix metalloproteinases, IFN-γ and TNF-α) was influenced by the VEGF-A_{165} treatment inducing the detachment of HUVEC.

In conclusion, in co-culture with HUVEC aMO2 remained stable in their type 2 state over 21 days confirming the suitability of aMO2 as biological release system for the

endothelialization of biomaterial surfaces with constant release of angiogenic factors but without secretion of pro-inflammatory cytokines over three weeks. Therefore, this endothelialization approach seems to be appropriate to improve the hemocompatibility of cardiovascular implant materials *in vitro*, and proved to be superior to the use of recombinant VEGF-A_{165}.

KEYWORDS: angiogenically stimulated alternative monocytes, endothelialization, biomaterials, hemocompatible surface

1. Introduction

Artificial heart valves, stents, or vascular grafts are widely used for the treatment of cardiovascular diseases, which are still the leading causes of mortality in industrialized nations [5, 16]. Besides restenoses, thrombus formations caused by an incomplete endothelialization are discussed to contribute to the failure of cardiovascular devices [13, 16, 32] Surface modifications such as heparin coating, seeding with stem cell, and/or endothelial cells (EC) are used to layer the surface of cardiovascular devices in order to provide a non-thrombogenic and hemocompatible surface [3, 14, 16] The antithrombotic properties of EC *in vivo* result from a multitude of mechanical, anti-platelet, anti-coagulant, and fibrinolytic systems including the expression of a glycocalix on the surface of EC [39, 46]. However, endothelialization of biomaterials *in vivo* after implantation ("one-stage") or *in vitro* ("two-stage") before implantation is still challenging [24].

Therefore, different strategies were applied to enhance the endothelialization of biomaterials. One current approach is the application of endothelial progenitor cells (EPC) [40]. However, this method still has some limitations, e.g. the coating of synthetic surfaces with antibodies or integrin-binding peptides for the selective capture of circulating EPCs from the blood flow [30, 42] and, in addition, the low numbers of circulating EPCs [2, 8, 25]. Different studies proved that also the addition of VEGF and/or other growth factors to HUVEC cultures could support the endothelialization of polymeric biomaterial surfaces [1, 15]. However, the optimal dosage of VEGF is not known to induce the formation of a functionally-confluent EC-monolayer. Recently, we suggested a new strategy to enhance the endothelialization of polymer surfaces *in vitro* by co-culturing of angiogenically stimulated alternative monocytes (aMO2) as biological growth factor release system with HUVEC in a short-term study [19]. Two subsets of monocytes have been described – classical monocytes (MO1) participating in the defence of microorganisms and alternative monocytes (MO2) foremost involved in

angiogenesis, tissue remodelling and repair [4, 35, 43]. These types can be distinguished by their surface molecule expression pattern, effector function and secretion of cytokines, and growth factors [17]. While MO1 produce mainly pro-inflammatory cytokines, aMO2 predominantly secrete anti-inflammatory molecules. Therefore, it is of crucial importance for polymer-based biomaterials, which are difficult to endothelialize, to know whether aMO2 can maintain their pro-angiogenic and non-inflammatory properties over an extended period of time without polarizing into a pro-inflammatory state [4]. Since the formation of a functionally confluent EC monolayer can require 10 up to 30 days - for certain materials confluence could not be reached at all [12] - it is of utmost importance that aMO2 do not switch back to MO1 during adherence on polymers . Up to now, it is unclear whether aMO2 can maintain their phenotype for such long periods of time. Therefore, the hypothesis that the aMO2 phenotype can be maintained over longer cell culture times thereby supporting the endothelialization of the substrate was proven in this study. This was verified by cytokine secretion profile, EC density, cytoskeleton organization, and functional analysis of HUVEC in mono-culture as control, HUVEC in mono-culture supplemented with VEGF-A_{165} and HUVEC in co-culture with aMO2 investigated in a long-term study over 3 weeks.

2. Material and Methods

Within this study the morphology of HUVEC, the cytokine and MMP profile, the release of prostacyclin (PGI2) and thromboxane A2 (TXA2) was measured in mono- and co-culture with angiogenically stimulated monocytes. For each group n=3 was used.

2.1 Ethical statement

The study protocol received an approval by the institutional review board of the Medical Faculty of the Charité University Medicine Berlin. Written informed consent was obtained from each subject before entry into the study.

2.2 Cultivation of primary human umbilical venous endothelial cells

Primary human umbilical venous endothelial cells (HUVEC, Lonza, Basel, Switzerland) were cultured for 3 - 5 passages in endothelial cell growth medium (EGM-2, Lonza, Basel, Switzerland). 7.6×10^4 HUVEC in monoculture (referred to as untreated control) or co-cultured with 3.8×10^4 aMO2 were seeded on glass slides (diameter 13 mm) (Th. Geyer, Renningen Germany). In addition, 500 µL of the medium in all HUVEC cultures were

exchanged daily supplemented with either 0 ng, 10 ng or 20 ng VEGF-A_{165} (Sigma-Aldrich, Missouri, United States) per mL^{-1} EGM-2.

2.3 Isolation and differentiation of pro-angiogenic alternative monocytes (aMO2)

Primary peripheral blood monocytes (MO) were isolated from buffy coats (n=3 donors) (provided by DRK, Berlin). Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation as previously described [21].

In brief, monocytes were isolated using the Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured in ultra-low-attachment dishes (Sigma-Aldrich, Missouri, USA) in D-MEM (Dulbecco`s Modified Eagle Medium, Life Technologies ™, Carlsbad, USA) with 10% fetal calf serum. The differentiation towards aMO2 was achieved by adding IL-4 (10 $ng \cdot mL^{-1}$, Miltenyi Biotec) and dexamethasone (100 µM, Sigma-Aldrich) to the culture medium for six days followed by angiogenical stimulation with recombinant VEGF-A_{165} (10 $ng \cdot mL^{-1}$, Sigma-Aldrich) for another 24 hours [21].

2.4. Morphology and cell numbers of adherent HUVEC cultivated with recombinant VEGF-A_{165} or co-cultured with aMO2 on glass

After different cultivation periods (day 1, day 3, day 6, day 10, day 14, day 21), cell morphology and numbers of adherent HUVEC per mm^2 were determined after paraformaldehyde (PFA) fixation and fluorescent staining as previously described [14]. Briefly, HUVEC were fixed in 4 wt% PFA incubated on ice for 30 minutes. After PFA fixation; cells were permeabilized with TritonX-100 (0.5 vol%) and F-actin was stained with phalloidin-AlexaFluor555® (Life Technologies,) at room temperature. In addition, nuclei were stained with DAPI (Sigma-Aldrich,) [34, 41]. Images (five fields of view per sample) were taken using InCell Analyzer 2000 (GE healthcare, Berlin, Germany).

2.5. Cytokine secretion profile of HUVEC and aMO2

Supernatants from cultured cells were collected and frozen at -80 °C after cultivation of 1, 2, 3, 6, 10, 14, and 21 days. Concentrations of VEGF, IL-1β, IL-6, IFN-γ, and TNF-α were assessed using Multiplex technique Bio-Plex200® and Bio-Plex Pro Human Cytokine 27 plex Assay, (Bio-Rad, Germany) according to manufacture´s instruction.

2.6. Analysis of HUVEC functionality in mono- or co-culture with aMO2

MMP activity was determined with pan-MMP fluorogenic peptide substrate Mca-PLGL-Dpa-AR-NH_2 from R&D Systems [45]. Recombinant human MMP-9 (R&D Systems, Minneapolis, USA) was used as positive control. The release of prostacyclin and thromboxane were measured using 6-keto Prostaglandin $F_{1\alpha}$ EIA Kit and Thromboxane B_2 EIA Kit (Cayman Chemical Company, Michigan, USA).

2.7 Statistical analysis

Data of n=3 samples are presented as arithmetic mean ± standard deviation. Statistical significance was calculated using two-way analysis of variance (ANOVA). Bonferroni-adjusted p values <0.05 were considered significant.

3. Results

3.1 Investigation of cell density and morphology

All cultures showed similar cell densities after one and three days of cell seeding. From day six until day 21, untreated HUVEC or HUVEC supplemented with VEGF-A_{165} showed significant lower cell densities compared to HUVEC co-cultivated with aMO2 (p values see Table 1).

Table 1: Cell densities of adherent cells in HUVEC monoculture and HUVEC + aMO2 co-culture on glass slides after cultivation times (t_c) of t_c = 1, 3, 6, 10, 14, and 21 days. HUVEC were treated with 10 ng·mL^{-1} or 20 ng·mL^{-1} VEGF-A_{165} or co-cultured with aMO2. Untreated HUVEC were used as control. Mean value ± standard deviation of n=3 are presented.

Numbers of adherent HUVEC per mm^2				
t_c [d]	HUVEC	HUVEC + 10 ng·mL^{-1} VEGF- A_{165}	HUVEC + 20 ng·mL^{-1} VEGF- A_{165}	HUVEC + aMO2
1	19 ± 6	35 ± 13	48 ± 14	51 ± 16
3	64 ± 15	100 ± 34	114 ± 36	112 ± 19
6	171 ± 29	188 ± 51	194 ± 65	391 ± 68$^{+++***\$\$\$}$
10	183 ± 49	279 ± 47	304 ± 86	464 ± 90$^{++++**\$}$
14	282 ± 71	330 ± 49	411 ± 40	683 ± 107$^{++++****\$\$\$\$}$
21	306 ± 39	236 ± 43	344 ± 59	596 ± 32$^{++++****\$\$\$\$}$

For statistical analysis Two-way ANOVA with a Bonferroni posttest was performed: +: compared to HUVEC, * : compared to HUVEC with 10 ng·mL^{-1} VEGF, $: compared to HUVEC with 20 ng·mL^{-1} VEGF; $<0.05, **: p<0.01; +++/***/$$$: p<0.001; ;++++/****/$$$$: p<0.0001

The morphology of HUVEC was comparable in the four groups 24 hours after cell seeding. Thereafter, in both HUVEC cultures supplemented with VEGF-A_{165} stress fibres and dissociated marginal filament bands developed. In addition, plaques of HUVEC detached during the fluorescent staining procedure. In contrast, in HUVEC co-cultured with aMO2, the marginal filament band was prominent at the cell rim and no stress fibres were visible. Representative images are shown in Fig. 1.

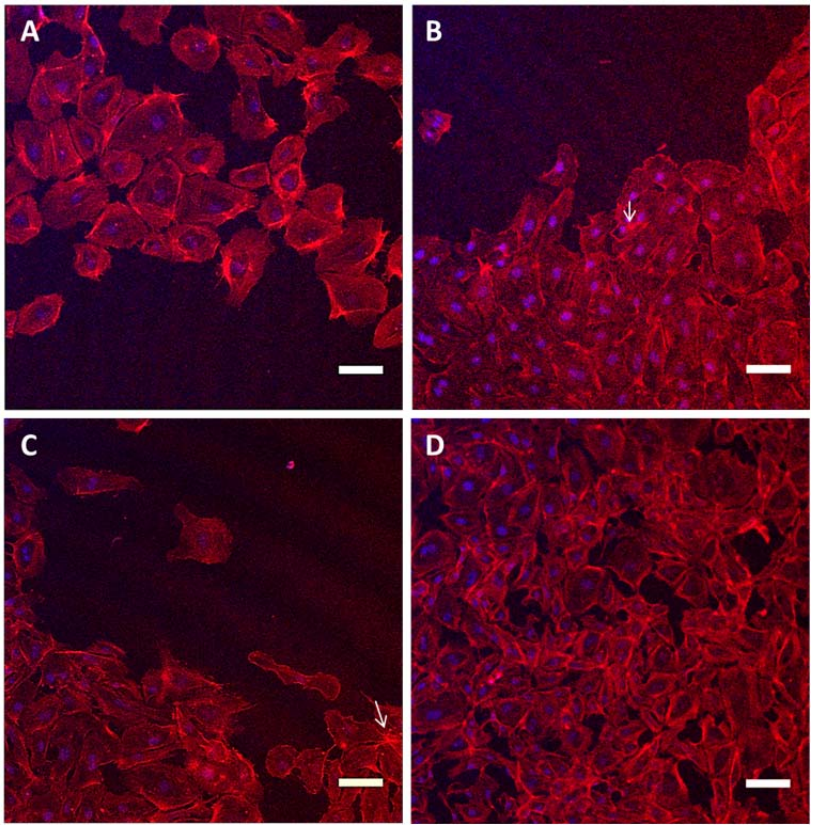

Figure 1: Representative images of adherent HUVEC after six days in mono-culture cultured in (A) regular EGM-2, (B) HUVEC supplemented with 10 ng·mL^{-1} VEGF-A_{165}, (C) HUVEC supplemented with 20 ng·mL^{-1} VEGF-A_{165}, (D) HUVEC co-cultured with aMO2. While in (A) a non-confluent HUVEC monolayer is visible due to a decreased number of adherent cells, (B) and (C) represents HUVEC cultures with a detachment of cells after the staining procedure. Fluorescent F-actin as cytoskeleton component is visualised in red, nuclei are stained with DAPI in blue. White arrows point out stress fibres. Primary magnification: 10x, InCell Analyzer 2000. Scale bar: 10 µm.

3.2. Cytokine secretion analysis of HUVEC with and without VEGF-A_{165} or co-cultured with aMO2

Pro-inflammatory cytokines such as IL-6, TNF-α (Fig. 2), and IL-1β (data not shown in Fig. 2) were not secreted by aMO2 in monoculture during the 21 days of cultivation. HUVEC cultivated either with recombinant VEGF-A_{165}, monocytes, or untreated controls secreted IL-6, TNF-α, and IFN-γ during the cultivation period, whereas a higher trend of pro-inflammatory cytokine release of IFN-γ and TNF-α was observed for HUVEC treated with

VEGF-A_{165}. The angiogenic cytokine VEGF was released in constant amounts by aMO2 starting after six days of cultivation until the end of the experiment ($p>0.05$ each).

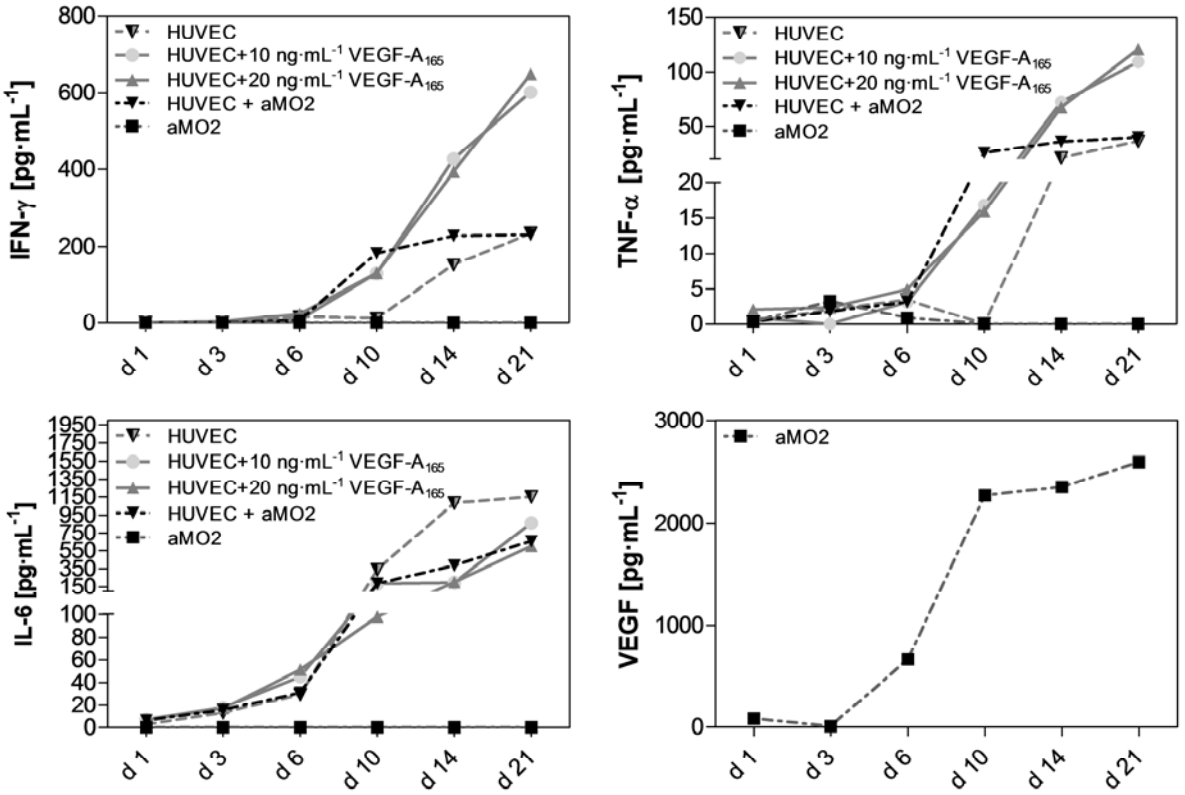

Figure 2: Cytokine release of HUVEC mono-culture without VEGF-A_{165}, HUVEC with 10 ng·mL^{-1} VEGF-$_{A165}$, HUVEC with 20 ng·mL^{-1} VEGF-A_{165}, HUVEC co-cultured with aMO2 and aMO2 in monoculture after t_c = 1, 3, 6, 10, 14, and 21 days. Cytokines were detected using a multiplex system (Bio-Plex®). The cytokine secretion of n=3 samples is shown.

3.3. PGI2 and TXA2 secretion profile analysis of HUVEC over 21 days with and without VEGF-A_{165} or co-cultured with aMO2

HUVEC supplemented with VEGF-A_{165} secreted significantly higher amounts of PGI2 compared to control cells and to HUVEC co-cultured with aMO2 24 h after cell seeding ($p<0.01$ for HUVEC + 10 ng·mL^{-1}, $p<0.0001$ for HUVEC + 20 ng·mL^{-1}) (Fig.3). In contrast, at day three, six and ten, no significant differences in the secretion of PGI2 between the four groups were found. This trend changed on day 14. Here, HUVEC controls showed a markedly higher PGI2 release compared to HUVEC supplemented with VEGF-A_{165} ($p<0.0001$ each) or co-cultured with aMO2 ($p<0.05$). However, HUVEC co-cultured with aMO2 showed a higher release than HUVEC supplemented with VEGF-A_{165} (HUVEC + 10 ng·mL^{-1}: $p<0.0001$ HUVEC + 20 ng·mL^{-1}: $p<0.01$). At day 21 after cell seeding, the release of PGI2 in the HUVEC control cultures and in the co-cultures of HUVEC and aMO2 differed from the cultures with HUVEC supplemented with VEGF-A_{165}. For both VEGF-A_{165}-

supplementations a diminished PGI2 release occurred compared to controls (HUVEC + 10 $ng \cdot mL^{-1}$: $p<0.05$, HUVEC + 20 $ng \cdot mL^{-1}$: $p<0.001$). In HUVEC cultures supplemented with the higher VEGF concentrations a lower amount of PGI2 was detected in contrast to co-cultures of HUVEC and aMO2 ($p<0.05$; Fig. 3).

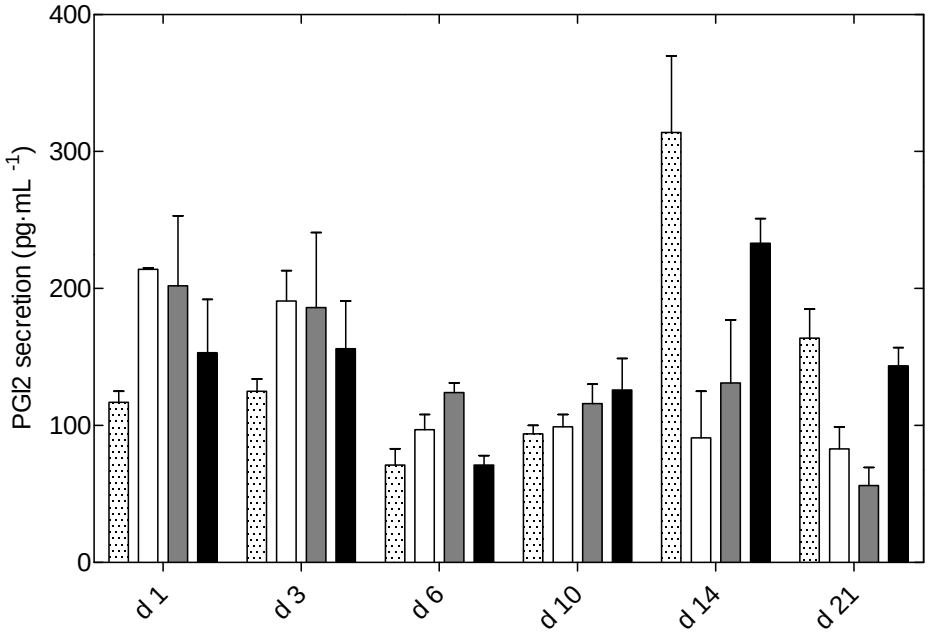

Figure 3: Secretion of vasodilator prostacyclin (PGI2) by HUVEC without VEGF-A_{165} (spotted), HUVEC with 10 $ng \cdot mL^{-1}$ VEGF-$_{A165}$ (white), HUVEC with 20 $ng \cdot mL^{-1}$ VEGF-A_{165} (grey), co-cultured with aMO2 (black) after t_c = 1, 3, 6, 10, 14, and 21 days of cultivation. Concentration of prostacyclin PGI2 is given in $pg \cdot mL^{-1}$. The PGI2 secretion and standard deviation n=3 is shown. For statistical analysis Two-way ANOVA with a Bonferroni posttest was performed. For the sake of clarity statistical results are described in the text.

The release of the vasoconstrictor TXA2 (Fig.4) was comparable for HUVEC, HUVEC supplemented with recombinant VEGF-A_{165}, or HUVEC co-cultured with aMO2 during the first six days of cell cultivation. At day 10, a significantly reduced secretion was detected for HUVEC supplemented with the lower VEGF-A_{165} concentration compared to HUVEC control cells ($p<0.01$). At day 14, a decreased TXA2 secretion was observed for HUVEC supplemented with VEGF-A_{165} ($p<0.0001$) and co-cultured HUVEC/aMO2 cells ($p<0.05$) in comparison to controls. In addition, in HUVEC/aMO2 co-cultures more TXA2 was detected in contrast to HUVEC supplemented with VEGF ($p<0.0001$ for HUVEC + 10 $ng \cdot mL^{-1}$, $p<0.01$ for HUVEC + 20 $ng \cdot mL^{-1}$). After 21 days, HUVEC/aMO2 co-cultures showed the highest TXA2 release compared to the other cell culture conditions ($p<0.05$). By trend, the secretion of TXA2 increased during the duration of the experiment.

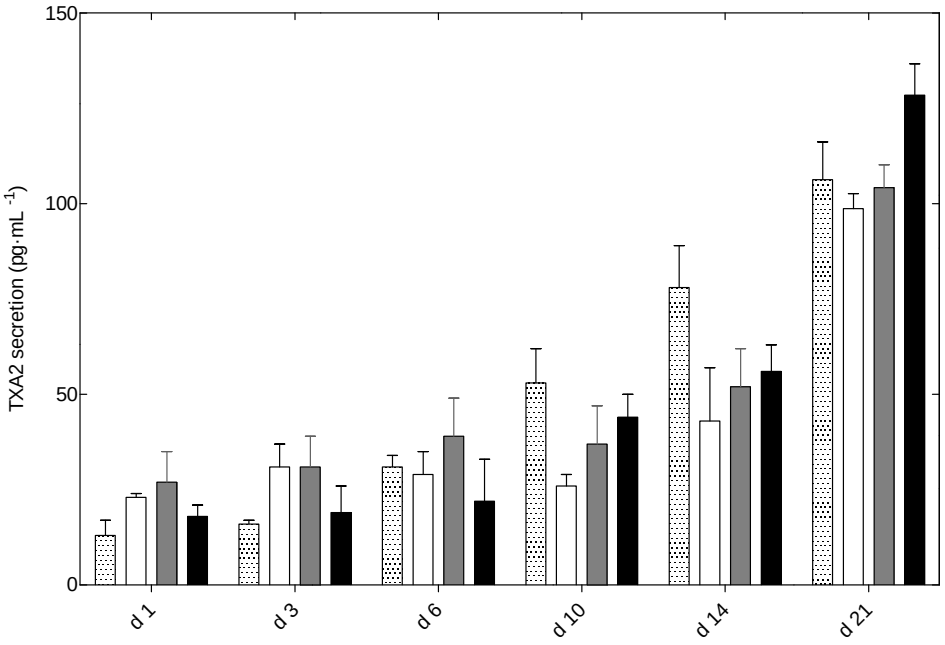

Figure 4: Secretion of the vasoconstrictor thromboxane (TXA2) by HUVEC without VEGF-A_{165} (spotted), HUVEC with 10 ng·mL^{-1} VEGF-$_{A165}$ (white), HUVEC with 20 ng·mL^{-1} VEGF-A_{165} (grey), co-cultured with aMO2(black) after t_c = 1, 3, 6, 10, 14, and 21 days of cultivation. Concentration of thromboxane TXA2 is given in pg·mL^{-1}. The TXA2 secretion and standard deviation of n=3 is shown. For statistical analysis Two-way ANOVA with a Bonferroni posttest was performed. For the sake of clarity statistical results are described in the text.

3.4. Matrix metalloproteinase activity of HUVEC

At days one, three, and six, no significant differences in matrix metalloproteinase (MMP) activity were measured in the four sets of cell cultures. Ten days after cell seeding, HUVEC supplemented with VEGF-A_{165} showed a high MMP activity ($p<0.0001$), while HUVEC/aMO2 in co-cultures showed decreased MMP activity ($p<0.0001$) compared to controls. At days 14 and 21 after cell seeding, HUVEC supplemented with VEGF-A_{165} revealed a significantly higher MMP activity compared to HUVEC and HUVEC/aMO2 co-cultures. In tendency, an increasing MMP activity was detected for all cell culture conditions within the cultivation time of 21 days (Fig. 5).

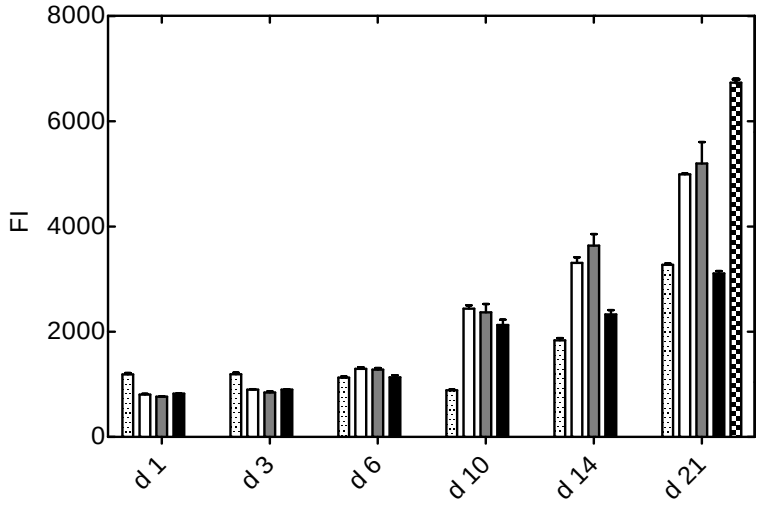

Figure 5: MMP activity of HUVEC at different time points. Fluorescence intensity (FI) of fluorogenic MMP peptide substrate after enzymatic cleavage is presented after 1, 3, 6, 10, 14, and 21 days of cultivation The fluorescence intensity and standard deviation of n=3 is shown. MMP activity of HUVEC without VEGF-A_{165} (spotted), HUVEC with 10 ng·mL^{-1} VEGF-$_{A165}$ (white), HUVEC with 20 ng·mL^{-1} VEGF-A_{165} (grey),co-cultured with aMO2 (black). As positive control 200 ng·mL^{-1} MMP 9 was used (checked). For statistical analysis Two-way ANOVA with a Bonferroni posttest was performed. For the sake of clarity statistical results are described in the text.

4. Discussion

To avoid thrombus formation on cardiovascular implants (e.g. vascular grafts, stents, left atrial appendage occluder systems, etc.), an early endothelialization of the implant or hemocompatible material surfaces are needed [13, 23]. Most polymeric or metallic biomaterials impair the adhesion, the migration or the proliferation of EC, thereby slowing or even retarding the endothelialization process [23, 32]. As mammalian cells in general prefer to adhere to hydrophilic surfaces, the endothelialization of hydrophobic surfaces is still challenging [31].

Recently, Mayer et al. reported that co-culturing of HUVEC with aMO2 led to an accelerated formation of a functionally confluent HUVEC monolayer within three days even on a hydrophobic polymeric surface [22]. In this study, it could be demonstrated that adherent aMO2 in co-culture with HUVEC remained in their pro-angiogenic and non-inflammatory phenotype up to 21 days and induced a functionally-confluent EC monolayer. This might be due to the release of growth factors (e.g. VEGF, bFGF, PDGF) [19, 20] and other cytokines from aMO2. At the same time, pro-inflammatory cytokines such as TNF-α and IL-1β - known to be involved in graft failure by impairing endothelial cell function [7, 29] - were not released by these co-cultures.

The morphology and cell densities differed between the four sets of cultures at day 21. The co-culturing of aMO2 with HUVEC led to significantly higher cell densities than the supplementation of the culture medium with 10 ng·mL^{-1} or with 20 ng·mL^{-1} of recombinant

VEGF-A_{165} and untreated controls. Inadequate marginal filament bands in association with the formation of stress fibres - which indicate diminished cell-cell and cell-substrate contacts [36, 44] - appearing in HUVEC cultures supplemented with VEGF-A_{165} coincide with the detachment of EC [33]. In addition, Monaghan-Benson and Burridge could show that VEGF alters the vascular permeability via a RAC-mediated production of a reactive oxygen species pathway [26], also involved in cell adhesion and induction of cell apoptosis and necrosis [6, 18, 37]. Endothelial dysfunction was described to be affected by reactive oxygen species [28]. The detachment of cells might also be due to MMP secretion. MMP activity was significantly increased in both HUVEC cultures supplemented with VEGF-A_{165} compared to untreated HUVEC or in HUVEC/aMO2 co-cultures. VEGF is not only facilitating the proliferation of endothelial cells but also angiogenesis [9]. In order to form new vessels, extracellular matrix components (ECM) are degraded by MMPs, whose secretion can be induced by VEGF [10]. In this study, the increased MMP secretion might have induced the degradation of the ECM secreted in the cell culture dishes, resulting in a reduced number of adherent HUVEC. The functionality of HUVEC can be defined - beyond the release of MMPs - by the synthesis of PGI2 and TXA2 from HUVEC. Both are involved in the regulation of the blood flow supplying the tissue and the aggregation/adhesion of platelets. Both mediators were partially affected by the supplementation of HUVEC with VEGF-A_{165} compared to control cells and HUVEC/aMO2 co-cultures. The release of PGl2 was diminished in VEGF-treated HUVEC after 14 and 21 days compared to HUVEC/aMO2 culture. The secretion of TXA2 by VEGF treated HUVEC changed during the performed study. While VEGF-treated HUVEC showed the highest release after d1 and d3 this trend changed after 21 days with a clearly reduced TXA2 synthesis compared to HUVEC/aMO2 cultures. *In vivo* this could contribute to a reduced adherence and aggregation of platelets to the implant material and to an increased blood flow to adjacent tissue.

The investigation of the cytokine release by aMO2 was also of major interest within this study due to the fact that cytokines such as IL-1β and TNF-α can contribute to the failure of vascular prostheses *in vivo* by generating inflammatory conditions or inducing intimal hyperplasia [27, 29]. So far, no data were available about the long-term release of cytokines by aMO2 cultivated over 21 days. However, no inflammatory cytokines were released by aMO2 in monocultures as well as in the co-cultures of HUVEC with aMO2. In contrast to these results, the concentration of pro-inflammatory cytokines was increased in HUVEC cultures supplemented with VEGF-A_{165}, indicating that this growth factor induced the expression of inflammatory cytokines. These results are in line with earlier studies showing

that VEGF is involved in inflammatory events and diseases and can affect the release of pro-inflammatory cytokines [11, 38]. In summary, it seems that aMO2 did not support inflammatory processes and maintained their angiogenic and anti-inflammatory properties presented by the release of VEGF during the whole period of 21 days with no obvious adverse side effects. Thus, aMO2 seem to be suitable to support the endothelialization of biomaterial surfaces over 21 days as was hypothesized by short time investigations from Mayer *et al.* without affecting the morphology or function of the HUVEC [19, 22]. It is getting clear, now, that the addition of VEGF-A_{165} alone cannot replace the cultivation of HUVEC with aMO2, because no functionally-confluent endothelial cell monolayer developed. Single HUVEC or plaques of HUVEC detached leading to cell-free areas on the material surface with a high thrombogenic potential. The secretion of other factors by aMO2 or perhaps their direct cell contact to HUVEC could contribute to these phenomenona and have to be analysed in future studies in transwell chambers.

5. Conclusion

The study revealed that aMO2 in co-culture with HUVEC remained stable in their type 2 state over 21 days, confirming the suitability of aMO2 as biological release system for the enhanced and functional endothelialization of biomaterial surfaces with constant release of angiogenic factors but without secretion of pro-inflammatory cytokines. Therefore, this endothelialization approach seems to be appropriate to improve the hemocompatibility of cardiovascular implant materials *in vitro*. In addition, the study allows to conclude that the use of recombinant VEGF-A_{165} as alternative to aMO2 is not an appropriate approach for the endothelialization of biomaterials *in vitro*. It became obvious also that aMO2, besides the secretion of VEGF, seem to release other factors playing important roles for the endothelialization process of biomaterial surfaces.

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