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Support of HUVEC proliferation by pro-angiogenic intermediate CD163^{+} monocytes/macrophages: A co-culture experiment

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ABSTRACT

So called intermediate (MO2) monocytes/macrophages possess anti-inflammatory properties and express the MO lineage marker CD163. On a hydrophilic, acrylamide-based hydrogel human intermediate (CD14^{++} CD16^{+}) CD163^{++} monocytes/macrophages (aMO2) which were angiogenically stimulated, maintained a pro-angiogenic and non-inflammatory status for at least 14 days. Here we explored, whether this aMO2 subset can positively influence the proliferation of human umbilical venous endothelial cells (HUVECs) without switching back into a pro-inflammatory (MO1) phenotype.

aMO2 or HUVEC were seeded alone on glass cover slips (0.5×10^5 cells / 1.33 cm^2) in a HUVEC specific cell culture medium (EGM-2) for 3 hrs, 24 hrs and 72 hrs or under co-culture conditions (0.5×10^5 HUVEC + 0.25×10^5 aMO2 / 1.33 cm^2) in EGM-2 for the same time window as well (n=6 each).

Under co-culture conditions the numbers of adherent HUVEC per unit area were significantly higher ($p<0.01$; 525±52 HUVEC/mm^2) compared to control mono-cultures (473±76 HUVEC/mm^2) after 72 hrs of cultivation and showed their typically spread morphology. The aMO2 remained in their subset status and secreted VEGF-A$_{165}$ without release of pro-inflammatory cytokines until the end of the 72 hrs cultivation time period, thereby supporting the HUVEC proliferation.

These *in vitro* results might indicate that this MO subset can be used as cellular delivery system for pro-angiogenic and non-inflammatory mediators to support the endothelialisation of biomaterials like e.g. cP*n*BA.

INTRODUCTION

Morphology, phenotype and function of circulating peripheral blood monocytes/macrophages (MO) are heterogeneous. The majority of the MO are characterized by flow cytometry to be $CD14^{++}$ $CD16^{-}$ and named “classical” (= MO1) [14, 35]. Furthermore two other minor subpopulations were described: $CD14^{++}$ $CD16^{+}$ (“intermediate” = MO2) and $CD14^{+}$ $CD16^{++}$ (“non-classical” = MO3). The latter subpopulation has been well characterised already and is regarded as pro-inflammatory [5, 13].

MO2 were reported to possess anti-inflammatory properties [27] and to express CD163 also [7]. CD163 is known to be restricted to cells of the monocyte/macrophage (MO) lineage [4, 24] and is expressed on pro-angiogenic/anti-inflammatory MO [19, 30], which are involved in the down-regulation of the inflammatory response [7].

Solely this “intermediate” subset of $CD14^{++}$ $CD16^{+}$ $CD163^{++}$ MO (MO2) secrets VEGF-A_{165} in bio-active ranges [1, 23, 34] (>10 ng/ml) after angiogenic stimulation (aMO2) without release of effective pro-inflammatory cytokine levels. This could be shown on a glass surface [20] as well as on elastic polymeric samples [21]. This aMO2 subset might be useful to support the establishment of a functional endothelial layer on body foreign surfaces *ex vivo* as a new strategy in biomaterial-based regenerative therapies [25, 26, 32] or to achieve haemocompatibility [10, 15, 16] on cardiovascular implants.

The formation of a functional endothelium is described not only to be dependent on flow conditions [29] but also on the cytokine milieu of the surrounding environment especially to pro-inflammatory mediators like TNFα [9] mainly secreted by MO1 and pro-angiogenic growth factors like VEGF-A_{165} [1]. The study was aimed to investigate, whether aMO2 can accelerate the formation of an endothelial cell (HUVEC) monolayer on a foreign body surface. The morphology of the HUVEC was evaluated by staining the F-actin skeleton and determining the number and density of adherent HUVEC on the substrate by counting the nuclei stained with DAPI. Additionally a set of cytokines was measured to analyse the function of mono- and co-cultured cells (VEGF-A_{165}, pro-inflammatory cytokines). Cytokines indicating pro-inflammatory processes were measured as markers for a switch of the cultured aMO2 subset back to MO1 or MO3.

MATERIALS AND METHODS

In the framework of a three-armed *in vitro* study it was estimated whether angiogenically stimulated intermediate $CD163^+$ monocytes/macrophages are able to support the endothelialisation of a foreign body surface without releasing pro-inflammatory cytokines.
For each of the three groups n=6 samples were used. The experiments were performed in accordance with the ethical guidelines of Clinical Hemorheology and Microcirculation [3].

Monocyte/macrophage isolation and generating angiogenically stimulated intermediate $CD163^{++}$ MO (aMO2)

Primary peripheral blood MO were isolated and stimulated as previously described [20]. Briefly, peripheral blood was collected from the cubital vein, centrifuged and the majority of plasma and erythrocytes were separated from the buffy coat via apheresis. The buffy coat was diluted 1:2 with phosphate-buffered saline. Subsequently, primary $CD14^+$ MO were isolated from this buffy coat by density gradient centrifugation and indirect magnetic microbead sorting. Latter was based on antibodies directed against non-monocytes, such as T cells, B cells, NK cells, dendritic cells and basophils (CD3, CD7, CD16, CD19, CD56, CD123, CD235a).
Immediately after primary $CD14^+$ MO isolation, the cells were stimulated to become a non-inflammatory subtype by 6 days of incubation with IL-4 and dexamethasone resulting in the intermediate ($CD14^{++}$ $CD16^+$) $CD163^{++}$ MO subset (MO2). This was evaluated as previously described [21] by flow cytometry (MACS Quant, Miltenyi Biotec, Germany) using antibodies against CD14, CD16 and CD163 (BD Biosciences, Germany, dilution 1:20).
Additional angiogenic stimulation was performed with 10 ng/ml $VEGF\text{-}A_{165}$ over 24 hrs.

Cell cultivation of aMO2 and HUVEC

In a pilot study a possible influence of the HUVEC specific cell culture medium EGM-2 (Lonza, Germany) on the phenotype of aMO2 (n=6) was evaluated and showed no switch into MO1 or MO2 and no loss of the defined surface markers CD14 and CD163 (data not shown). Thereby a negative effect of the EGM-2 on the aMO2 functionality can be excluded.
Biological testing was performed on glass cover slips (n=6, 1.33 cm^2 each), which were placed in 24 multi-well cell culture plates made of polystyrene and seeded with 0.5×10^5 aMO2 or HUVEC (Lonza, Germany) each for mono-culture condition. For co-culture condition 0.25×10^5 aMO2 and 0.5×10^5 HUVEC were used. For cell cultivation, standard

incubation conditions (37 °C, humidified atmosphere, 5 vol% CO_2) and a HUVEC specific cell culture medium (1 ml/well, EGM-2) were used. After 3 hrs, 24 hrs and 72 hrs the phenotype and number of the HUVEC cells, which became adherent to the substrates, were analysed by fluorescent staining (n=6). The conditioned cell culture medium at each end point was used for cytokine analysis (n=6) after centrifugation at 8,000 g for 5 minutes and subsequent storage at -20 °C until testing.

Fluorescent staining of aMO2 and HUVEC

Adherent cells on substrate (n=6 each time point) were fixed in 4 wt% paraformaldehyde for 30 minutes on ice and made permeable with Triton X-100 (0.5 vol%) for 10 minutes at room temperature. F-actin was visualised using phalloidin coupled with the fluorescent dye AlexaFluor555® (Molecular Probes, Germany), CD14 and CD163 with specific monoclonal antibodies (BD Pharmingen, Germany) and dsDNA/nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Five different fields of view were analysed per sample using confocal laser scanning microscopy (LSM 510 META, Zeiss; Germany) and image analysis software AxioVision (Zeiss, Germany).

Cytokine secretion analysis of aMO2 and/or HUVEC

Conditioned cell culture medium was analysed after 3, 24 and 72 hrs (n=6 each) for pro-angiogenic growth factor VEGF-A_{165} and immune mediators (IL-1ra, IL-1β, IL-6, IL-10, IFNγ, TNFα) using Multiplex technique (Bio-Plex200®, BioRad, Germany). VEGF-A_{165} was present in culture medium. This measured background amount was subtracted from the measured quantities to determine the secreted quantities.

Statistics

Mean values and standard deviations are given for continuous data. Gaussian distribution was tested according to Kolmogorov Smirnov. Paired two samples tests were performed using the t-test for Gaussian distributed samples and the Wilcoxon-test for non-Gaussian distributed samples. For time courses, a variance analysis for repeated measures was performed. A one-factorial variance analysis was used for group comparisons. $P<0.05$ was considered significant. Because of the explorative character of the study, a Bonferoni adjustment was disclaimed.

RESULTS

Cell density and morphology of HUVEC under mono- and co-culture conditions

After 3 hrs of cultivation the HUVEC were homogeneously distributed on the samples. The cell numbers did not differ for both culture conditions (mono-culture: 154±63 cells/mm^2; co-culture: 142±84 cells/mm^2, n=6 each). The cells started to exhibit their typically spread morphology and expressed a physiological actin skeleton (red, Fig. 1) after 24 hrs of cell growth. Also the number of adherent HUVEC was increasing but not different between mono-culture (232±78 cell/mm^2) and co-culture (226±64 cells/mm^2). After 72 hrs the majority of the cells were completely spread and the morphology of the HUVEC in the co-culture was comparable to that in the control culture. However, a significantly higher number of HUVEC was detectable when co-cultured together with aMO2 (525±52 cells/mm^2, n=6, p<0.01) as when cultured alone (473±46 cells/mm^2; n=6 each). After 72 hrs cultivation the aMO2 were still expressing CD14 but CD163 expression was fading (Fig. 1).

Cytokine secretion analysis of aMO2 on cP*n*BA

The mean VEGF-A_{165} concentration of aMO2 cells (2.62±0.66 ng/ml) cultivated for 3 hrs was significantly higher compared to VEGF-A_{165} in HUVEC mono- and co-culture (p<0.01, 0.11±0.03 ng/ml HUVEC alone, 0.17±0.9 ng/ml HUVEC co-culture). However, 24 hrs after seeding VEGF-A_{165} concentration was significantly increased in aMO2 mono-culture (p<0.05, 7.88±0.72 ng/ml) and remained stable over 72 hrs (8.10±0.49 ng/ml) as shown in table 1. 24 hrs after seeding the VEGF-A_{165} concentration was significantly higher (0.76±0.17 ng/ml, p<0.05) in the supernatant of HUVEC, which were cultured alone compared to the amount in the supernatant of HUVEC and aMO2 cultured together (0.16±0.05 ng/ml). Nevertheless, 72 hrs after seeding in both HUVEC cultures (with and without aMO2) the measured VEGF-A_{165} concentration was comparable to each other and significantly less than in the supernatant of aMO2 alone (p<0.05, HUVEC mono-culture: 7.6±0.2 pg/ml, HUVEC/aMO2 co-culture: 6.1±0.6)

The secretion levels of the cytokines IL-1ra, IL-1β, IL-6, IL-10, IFNγ and TNFα were low (0 – 150 pg/ml, table 1) in all three culture conditions. The values of those cytokines were comparable for both HUVEC cultures over time. Interestingly, the IFNγ concentration in the supernatant of aMO2 was 3 hrs after seeding 3-fold higher (see table 1) than in the HUVEC cultures but decreased over time similarly strong. Vice versa in both HUVEC cultures the IL-

6 concentration was increasing over time and significantly higher than in the supernatant of the aMO2.

DISCUSSION

In the present study, it could be shown that the aMO2 remained in their pro-angiogenic non-inflammatory status and secreted VEGF-A_{165} in biologically effective ranges [20], while the levels of pro-inflammatory cytokines as TNFα or IL-1β were very low, not increasing and clearly below biologically effective ranges [2, 11, 12, 18, 33]. Thereby the functionality of the aMO2 as cellular VEGF-A delivery system for supporting endothelialisation without inducing inflammatory processes in the HUVECs could be proven.

The VEGF-A_{165} concentrations of the HUVEC cultures were comparable after 3 hrs and 72 hrs of cultivation. But, 24 hrs after seeding, in the HUVEC/aMO2 co-culture 49% ($p<0.05$) more VEGF-A_{165} was detected than in the HUVEC mono-culture. This might be due to the presence of the aMO2, which might have induced an increased HUVEC proliferation resulting in a significantly higher number of cells after 72 hrs of cultivation. The occurring VEGF-A_{165} during the proliferation [22] entailed a further stimulation of the aMO2 to secrete even more VEGF-A_{165} thereby again stimulating the HUVECs and resulting in a positive feedback loop.

The amounts of all other evaluated cytokines (TNFα, IL-1β, IL-1ra and IL-10) beside IFNγ and IL-6 were comparable for the two mono-cultures and the co-culture and clearly below biologically effective thresholds [2, 11, 12, 18, 33]. Thus a back switch of the aMO2 into MO1 or MO3 phenotype can be excluded and also that the aMO2 induced stress reactions in the HUVECs. Although there are differences in the secreted amounts of IFNγ for the three different cultures, under all three cultivation conditions the detected amounts of IFNγ were low and decreasing over time. The initially elevated values were most probably due to the trypsination [6]. An inhibition of proliferation is unlikely based on the low values which decreased to zero.

The IL-6 concentration of aMO2 remained stable over time but was clearly below biologically effective ranges [17, 28], which likewise proved that the aMO2 did not switch back. In the cultures with HUVEC the measured amounts of IL-6 were increasing over time. Although IL-6 is mainly secreted by endothelial cells under inflammatory circumstances [8, 31] in both cultures (co-culture as well as mono-culture) the measured amounts of IL-6 were comparable and thereby not due to the presence of the aMO2. This indicates that the aMO2 did not induce this HUVEC secretion profile but might be the natural secretion range of endothelial cells lacking further pro-inflammatory stimuli [8].

CONCLUSION

Angiogenically stimulated intermediate CD163$^+$ monocyte/macrophages (aMO2) increased the number of adherent cells over a time period of 72 hrs significantly thereby accelerating the formation of an endothelial cell monolayer. In addition neither the aMO2 nor the HUVEC gave indications of an inflammatory switch on cytokine level. Future work will test, whether the aMO2 will also accelerate the formation of an HUVEC monolayer of other body foreign surfaces e.g. cP*n*BA.

Figure 1: HUVEC mono-culture and HUVEC/aMO2 co-culture on glass after three hrs, 24 hrs and 72 hrs of cultivation, n=6, 20-fold magnification, blue = nucleus, red = F-actin skeleton.

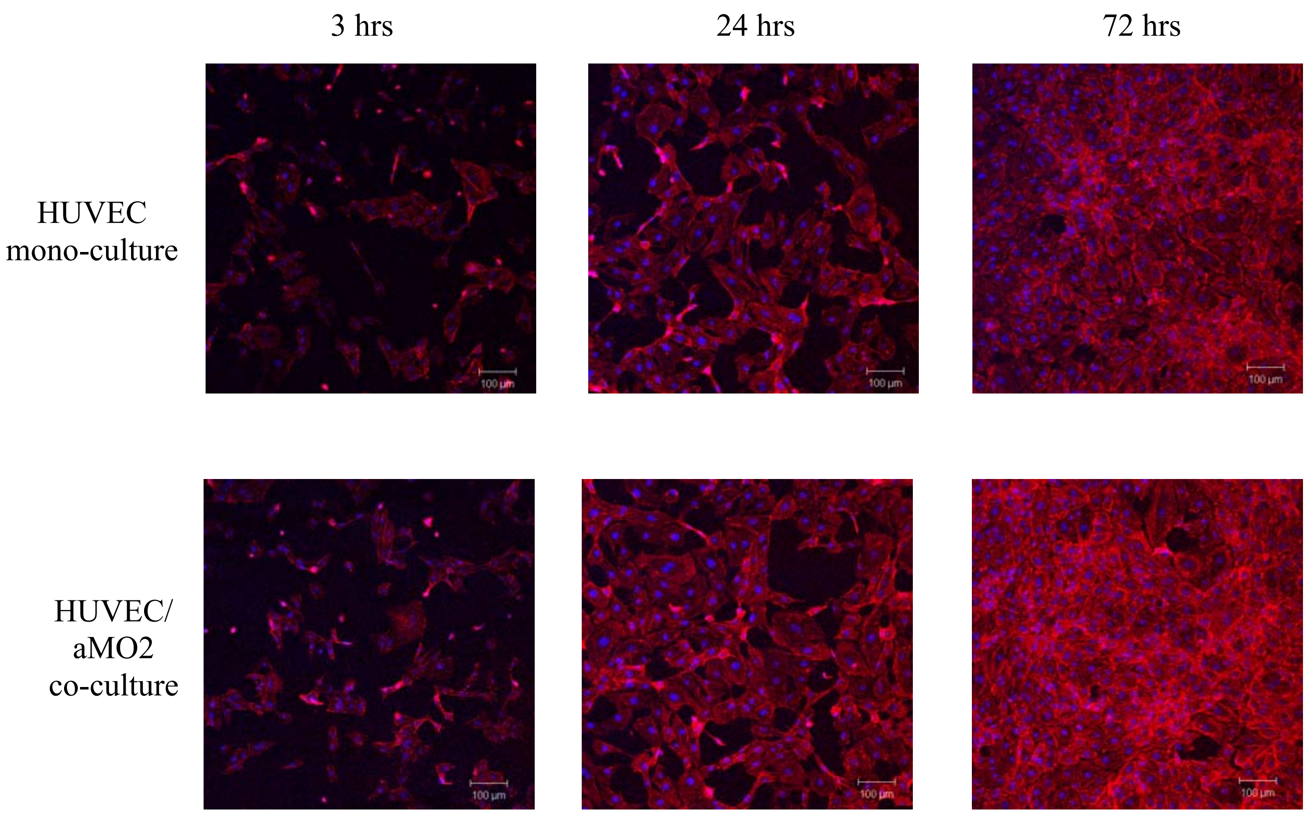

Table 1: Cytokine secretion of aMO2 and HUVEC alone as well as in co-culture after 3 hrs, 24 hrs and 72 hrs of cultivation on glass cover slips in pg/ml, means ± standard deviation, n=6, one symbol: p<0.05, doubled symbol: p<0.01, + vs. aMO2, # vs. HUVEC, ● vs. co-culture, ■ vs. 3 h, ◊ vs 24 h.

	Cytokine secretion [pg·ml^{-1}]		
	3 h	24 h	72 h
HUVEC mono-culture			
IL-10	47,5 ± 3,6	8,4 ± 6,3 $^{\blacksquare}$	1,9 ± 0,1 $^{\blacksquare}$
IFNγ	84,5 ± 24,4 $^{++}$	35,2 ± 3,4 $^{+}$	0,2 ± 0,1 $^{+,\blacksquare,\lozenge\lozenge}$
TNFα	19,4 ± 0,5	3,5 ± 1,3 $^{\blacksquare}$	2,4 ± 1,1 $^{\blacksquare}$
IL-6	24,3 ± 1,8 $^{++}$	44,0 ± 9,7 $^{+}$	84,9 ± 10,4 $^{++,\blacksquare}$
IL-1β	2,1 ± 0,2	1,8 ± 0,1	1,8 ± 0,1
IL-1ra	7,2 ± 0,9	2,4 ± 0,5	1,2 ± 0,5
VEGF-A165	90,1 ± 18,0 $^{++}$	105,3 ± 10,5 $^{++,\bullet}$	7,6 ± 0,2 $^{++,\blacksquare,\lozenge\lozenge}$
HUVEC/aMO2 co-culture			
IL-10	45,8 ± 4,5	11,9 ± 9,0 $^{\blacksquare}$	2,2 ± 0,2 $^{\blacksquare}$
IFNγ	89,9 ± 19,3 $^{+}$	39,5 ± 6,2 $^{+,\blacksquare}$	0,0 ± 0,0 $^{+,\blacksquare}$
TNFα	16,7 ± 2,7	2,4 ± 0,2	1,7 ± 0,1
IL-6	21,5 ± 3,5 $^{++}$	43,3 ± 12,5	82,8 ± 14,0 $^{++}$
IL-1β	1,9 ± 0,1	1,8 ± 0,2	1,9 ± 0,1
IL-1ra	5,8 ± 1,1	2,6 ± 0,3	2,4 ± 0,9
VEGF-A165	96,5 ± 27,0 $^{++}$	156,9 ± 22,6 $^{++,\#}$	6,1 ± 0,6 $^{++,\blacksquare,\lozenge}$
aMO2 mono-culture			
IL-10	53,8 ± 2,9	47,3 ± 7,8	47,5 ± 2,7
IFNγ	143,5 ± 38,1	8,1 ± 1,8 $^{\blacksquare}$	15,4 ± 5,1 $^{\blacksquare}$
TNFα	20,1 ± 0,8	15,4 ± 2,4	16,3 ± 0,4
IL-6	12,4 ± 0,3	11,4 ± 0,6 $^{\#}$	11,1 ± 0,5 $^{\#\#,\bullet\bullet}$
IL-1β	1,9 ± 0,1	1,8 ± 0,1	1,8 ± 0,2
IL-1ra	8,0 ± 0,7	7,3 ± 1,7	6,5 ± 0,4
VEGF-A165	2618,9 ± 657,4 $^{\#\#,\bullet\bullet}$	7886,4 ± 329,8 $^{\#\#,\bullet\bullet}$	8102,1 ± 489,1 $^{\#\#,\bullet\bullet,\blacksquare}$

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