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Modulation of angiogenic functions in human macrophages by biomaterials ☆

Güngör Dagtekin[a,*], Ruth Schiffer[a], Birgit Klein[a], Willi Jahnen-Dechent[a], Gabriele Zwadlo-Klarwasser[b]

[a] *Interdisciplinary Center of Clinical Research "Biomat.", RWTH Aachen, Pauwelsstraße 30, Aachen 52074, Germany*
[b] *Department of Dermatology, Medical Faculty, RWTH, Aachen, Germany*

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Abstract

We examined the ability of polyvinylchloride (PVC), polytetrafluorethylene (PTFE) and tissue culture polystyrene (TCPS) to affect angiogenic functions in human monocyte-derived macrophages by measuring the mRNA expression of genes encoding angiogenic and anti-angiogenic molecules including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and thrombospondin-1 (Tsp-1). The angiogenic activity of the corresponding macrophage conditioned media (CM) was measured by the proliferation of endothelial cells and the sprouting of new capillaries from fragments of human placental blood vessels. We determined that bFGF was not expressed in macrophages while VEGF and Tsp-1 mRNAs were expressed constitutively. Ang-1 was expressed in macrophages cultured up to 7 days on PTFE and TCPS independent of the culture stage. In contrast, macrophages cultured on PVC did not produce detectable amounts of Ang-1 mRNA after 7 days. CM from macrophages cultured either on PTFE or TCPS stimulated angiogenesis whereas CM from macrophages cultured on PVC inhibited it. The results demonstrate that polymers can cause differential expression of the angiogenic molecule Ang-1 in macrophages. They also induce different phenotypes of macrophages, which can either stimulate or inhibit angiogenesis suggesting a material-dependent influence on neovascularization.

Keywords: Angiogenesis; Biomaterials; Macrophages; Angiogenic growth factors

1. Introduction

Angiogenesis is an important process involved in the development of organs during embryogenesis, in wound healing and in the female reproductive cycle of the adult organism. There are a variety of pathological conditions like retinopathies, rheumatoid arthritis, psoriasis, tumor growth and chronic inflammation in which angiogenesis is implicated [1]. Chronic inflammation is a major cause of implant failure [2]. Surface modification of biomaterials and tissue-engineering approaches with modified native tissues all aim at a reduction of the potentially damaging effects of inflammation and at the augmentation of angiogenesis [3]. In certain situations, inflammation may be beneficial in that it triggers angiogenesis. For instance, the successful application of tissue-engineered implants requires the growth of blood capillaries for the supply of essential nutrients and oxygen [1]. Presently, the level of angiogenesis for a given implant is not well controlled. Likewise, the influence of inflammatory cell interactions on angiogenesis is largely unknown [4].

Macrophages play a central role in angiogenesis and in the integration of biomaterials into the tissues in which they have been implanted [2,5]. Macrophages are known to produce several potent angiogenic factors including vascular endothelial growth factor (VEGF) [6], basic fibroblast growth factor (bFGF) [7], transforming growth factor-β (TGF-β) [8] and interleukin-8 (IL-8) [9]. Activated macrophages are suspected to initiate the degradation of implants [2]. Thus it is necessary to determine the quality and quantity of the

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*Corresponding author. Tel.: +49-241-8088561; fax: +49-241-8082573.
E-mail address: gdagtekin@ukaachen.de (G. Dagtekin).

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macrophage response in biomaterials since this response critically determines the fate of biomaterials and tissue-engineered implants.

One potent signalling molecule produced by activated peripheral blood monocytes/macrophages is VEGF, also known as vascular permeability factor, a glycoprotein of approximately 45 kDa [6]. VEGF is a prototypic angiogenic factor because it is secreted in a biologically active form, its receptors are found at sites of angiogenesis, and it is the most specific endothelial cell growth factor known to date [10]. Besides directly stimulating angiogenesis, VEGF also induces vascular permeability, regulates production of proteases and their inhibitors, and promotes endothelial cell differentiation, movement and survival [11], all of which are involved in angiogenesis.

Macrophages activated by tumor cells in vitro produce the heparin binding bFGF [7], a potent inducer of endothelial cell migration, proliferation and tube formation in vitro and which is highly angiogenic in vivo [12].

Recently, Yuan et al. have shown that tissue macrophages express angiopoietin-1 (Ang-1) [13]. Ang-1 is a ligand of the Tie-2 receptor [14]. In vitro experiments have shown that Ang-1 potently induces network formation [15], chemotactic response [16], and survival during apoptosis [17,18]. Ang-1 also causes sprouting of endothelial cells in fibrin gel, but does not stimulate proliferation [19].

Besides activators of angiogenesis, endogenous inhibitors of angiogenesis also exist [20]. One inhibitor of angiogenesis, thrombospondin-1 (Tsp-1) is produced by many cells including macrophages [21]. Tsp-1 is a large, trimeric matricellular protein, which inhibits the angiogenic response of endothelial cells both in vivo and in vitro [22].

In this study, we analysed the expression of the above-mentioned stimulators and inhibitors of angiogenesis in human monocyte-derived macrophages cultured on chemically and structurally different polymers. Furthermore, we examined the angiogenic potential of the macrophage culture supernatants using human umbilical vein endothelial cells (HUVECs) and an in vitro vessel culture system as models of angiogenesis.

2. Materials and methods

2.1. Polymer materials

Three different polymers were tested: polyvinylchloride (PVC) medical grade (Rehau AG, Hanau, Germany) polytetrafluorethylene (PTFE) foil (Heraeus, Düsseldorf, Germany) and tissue culture polystyrene (TCPS) (Sarstedt, Nümbrecht, Germany) as a hydrophilic cell culture material. The materials were placed into a 6-well culture plate and sterilized with ethylenoxide.

2.2. Monocyte and macrophage isolation and culture

Monocytes were obtained from buffy coats of healthy donors by a two-step density gradient centrifugation using Ficoll-Paque (Pharmacia, Freiburg, Germany) and Percoll (Sigma, Deisenhofen, Germany). The viability of the macrophages was assessed by trypan blue dye exclusion. We routinely measured viabilities of greater than 90% in all preparations. We noted no difference in viability if macrophage cultures on different biomaterials were analysed, although the cell number decreased over time in all cultures. The cells were cultured on the biomaterials mentioned above at a density of 2×10^6 cells/ml in RPMI 1640 (Sigma) containing 100 μg/ml penicillin and 100 μg/ml streptomycin. For subsequent testing of cell proliferation, the culture medium also contained 10% heat-inactivated human serum. For the production of conditioned media (CM) for in vitro angiogenesis assays, however, a serum-free medium formulation was chosen to exclude the contribution of serum-derived angiogenic factors in this test. To this end, monocytes were isolated as above and subsequently cultured for 7 days in serum-free Iscove's modified DMEM (Life Technologies, Eggenstein, Germany) supplemented with 500 μg/ml bovine serum albumin, 50 μg/ml soybean lipids, 33 μg/ml human transferrin, 17 μg/ml aprotinin (Trasylol, Bayer, Leverkusen, Germany), 2×10^{-5} M dithiothreitol (DTT), fungizone (2.5 μg/ml), penicillin and streptomycin on the respective polymers. Total protein concentration of CM was determined by the Bradford dye assay.

After 2, 4 and 7 days in culture the cells, referred to as monocyte-derived macrophages, and their CM were harvested and the mRNA expression of angiogenic factors was examined using RT-PCR. The biological activity of the macrophage-CM was screened by a proliferation assay using endothelial cells (HUVECs) and ^{3}H-thymidine incorporation. Tube formation and the capillary sprouting were examined using an in vitro angiogenesis assay.

2.3. Human umbilical vein endothelial cells (HUVECS)

HUVECs were isolated from umbilical cord veins by collagenase digestion as described previously [23] and grown on gelatin-coated tissue culture flasks in RPMI 1640 (Sigma) containing 10% human serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. HUVECs were used from passage number 2.

2.4. Proliferation assay

The activity of conditioned medium of macrophages on the proliferation of HUVEC was assessed by determining ^{3}H-thymidine incorporation. Confluent cultures were detached and resuspended in RPMI 1640

containing 10% human serum, 100 μg/ml penicillin and 100 μg/ml streptomycin to a final concentration of 1×10^4 cells/ml. Twenty-four-well plates were coated with gelatin and 1 ml cell suspension was added per well. Cells were allowed to settle and attach for 2 h. The medium was then replaced either with medium conditioned by monocyte-derived macrophages cultured on different biomaterials or control medium (RPMI 1640 with 10% human serum) or in some experiments with medium cultured on the biomaterials in the absence of macrophages (toxicity control). For adaptation, HUVECs were pre-cultured in these media for 2 days. All cell cultures remained sub-confluent during this period. ^{3}H-thymidine (1 μCi/ml) was added to each well for 24 h of the culture. After incubation, the medium was removed and the cells were washed three times with PBS. 1 ml of Optiphase Supermix reagent (Wallac, Turku, Finland) was added to solubilize the cells and ^{3}H-thymidine incorporation was measured using a Microbeta liquid scintillation counter (Wallac). Each of the six independent experiments were performed as quadruplicate. In additional experiments, the viability of HUVECs cultured for 3 days with CM from macrophages cultured for 7 days on PVC was assessed by trypan blue dye exclusion. Total protein concentration of CM was estimated by the Bradford assay.

2.5. Preparation of blood vessel fragments

Superficial vessels, approximately 1–2 mm in diameter and 5–7 cm in length, were excised from the apical surface of human placentas within 6 h of delivery. The vessels were placed in Hank's balanced salt solution containing 2.5 μg/ml of fungizone (Life Technologies) and cut into 1–2 mm fragments using fine dissecting forceps and iridectomy scissors. Vessel fragments were freed of residual clots and soaked in Hank's balanced salt solution before use [24].

2.6. Angiogenesis assay

Assays were performed as described previously [24]. Briefly, fibrinogen from human plasma (Sigma) was dissolved in Medium 199 (Life Technologies) at a final concentration of 3 mg/ml. To prepare fibrin gels, 14 μl of thrombin (50 NIH μg/ml, Sigma) was added to each well of a 48-well plate followed by 500 μl fibrinogen. Thrombin and fibrinogen were briefly mixed and one vessel fragment was quickly placed in the center of the well before clot formation. After gel formation, 500 μl/well of the macrophage CM or control medium (serum-free Iscove's DMEM) with or without dexamethasone (Sigma), 10^{-7} M, was added. The culture plates were then placed in a 95% O_2–5% CO_2 humidified incubator for 21 days and microvessels were counted on day 9 and 11 using an inverted microscope. Twenty individual samples were prepared for each treatment and experiments were repeated with two individual placentas and macrophage supernatants of two different donors.

2.7. RNA isolation and RT-PCR

mRNA was extracted from cells using the Oligotex Direct mRNA purification kit (Qiagen, Hilden, Germany). Total RNA was isolated using the Peq Gold RNA Pure reagent (Peq Lab, Erlangen, Germany) following the manufacturer's protocol.

Reverse transcription and PCR was performed with the GeneAmp RNA PCR kit (Perkin Elmer, Überlingen, Germany) according to the manufacturer's instructions. The expression of specific mRNAs for angiogenic factors was achieved using following intron-spanning primer pairs; Ang-1: forward, 5′-GGGGGAGGAAGGACTGTAAT-3′; reverse, 5′-AGGGCACATTTGCACATACA-3′; VEGF: forward, 5′-CCGGTGGACATCTTCCAGGAGTACC-3′; reverse: 5′-GAAGCTCATCTCTCCTATGTGCTGGC-3′; bFGF: forward, 5′-AAGCGGCTGTACTGCAAAAA-3′; reverse: 5′-CATGTTAAACCTCCTTAGTG-3′; Tsp-1: forward, 5′-ACCGCATTCCAGAGTCTGGC-3′; reverse: 5′-ATGGGGACGTCCAACTCAGC-3′; β-Actin: forward, 5′-ATCTGGCACCAACTTCTACA-3′; reverse: 5′-GTTTCGTGGATGCCACAGGACT-3′. Amplification was carried out with 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 62°C and 1 min extension at 72°C. Amplification was terminated with an extention step of 4 min duration after the last cycle. PCR products were separated on 1.8% agarose gels (1 × TAE) and stained with ethidium bromide.

2.8. Statistical analysis

Statistical analysis of the data was performed using the unpaired single-sided Student's *t*-test.

3. Results

3.1. mRNA expression of angiogenic factors

To determine if biomaterials cause the production of angiogenic or anti-angiogenic factors in macrophages, we cultured monocyte-derived macrophages on PTFE biofoil, medical grade PVC and TCPS and analysed the production of mRNA for various angiogenic factors using RT-PCR and β-actin mRNA expression as an internal control. We designed the RT-PCR strategies in such a way that amplicons were clearly distinguishable by fragment length from genomic DNA. Using this method, we detected β-actin mRNA in all samples analysed (Fig. 1). In the identical samples, we detected no bFGF expression in any biomaterial and time

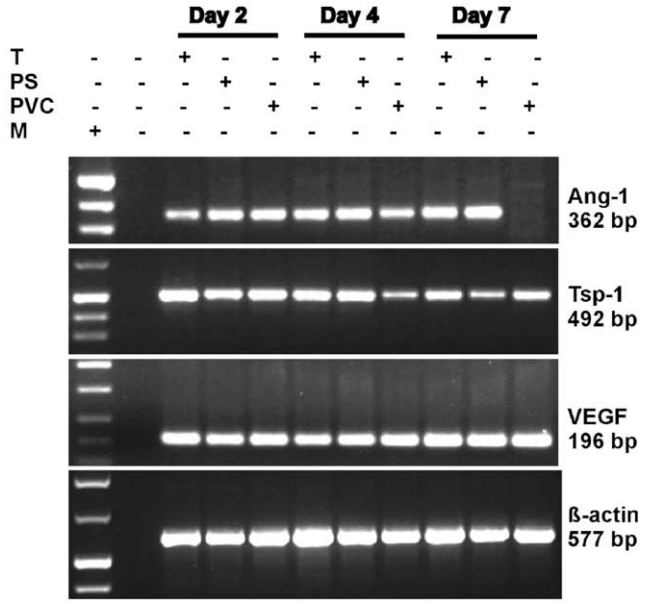

Fig. 1. RT-PCR analysis of Ang-1, VEGF and Tsp-1 mRNA expression in human monocytes and monocyte-derived macrophages cultured on PTFE biofoil (PTFE), TCPS and PVC. Cells were harvested after 2, 4 and 7 days in culture. mRNA from cells was reverse transcribed and amplified by PCR with the primer pairs specified in methods. The PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. M, DNA molecular weight marker. (–), negative control, RT-PCR performed with mRNA template but without reverse transcriptase. The amplification of β-actin was used as an internal control (lower panel). Results were similar in six independent experiments.

combination tested although induction of bFGF mRNA was readily detected in macrophages after stimulation with concanavalin A or lipopolysaccharide (data not shown). This finding indicates that the macrophages did not produce bFGF when contacting biomaterials. Fig. 1 illustrates that both VEGF and Tsp-1 amplicons were readily amplified by RT-PCR of mRNA of macrophages either cultured on PTFE (T), PVC or TCPS (PS), indicating that VEGF and Tsp-1 mRNAs were produced by macrophages cultured on these biomaterials. Ang-1 expression varied in that Ang-1 mRNA was produced by macrophages cultured up to 7 days on PTFE and TCPS. Macrophages cultured on PVC ceased to produce detectable levels of Ang-1 mRNA after 7 days (Fig. 1). These results were consistently obtained with macrophages from six independent donors. We analysed freshly isolated blood monocytes by the identical RT-PCR procedure described in Fig. 1 and consistently detected β-actin amplicon amounts similar to Fig. 1, but no amplicons for bFGF, Ang-1, Tsp-1 and VEGF. We conclude that these angiogenic factors are produced only after the monocyte-derived macrophages contact the biomaterials. Because RT-PCR is a qualitative measure of mRNA production, but does not necessarily indicate protein production or even biological activity of the gene product we asked if these factors conferred biological acitvity.

3.2. Effect of macrophage conditioned media on the proliferation of HUVECs

To this end, we cultured HUVECs in the presence of CM obtained from macrophages and measured cell proliferation by ^{3}H-thymidine incorporation. Fig. 2 illustrates that CM from macrophages cultured on PTFE stimulated endothelial cell proliferation two-fold ($p<0.01$). Similar results were obtained with CM from macrophages cultured on TCPS (not shown). CM from macrophages cultured on PVC inhibited the proliferation of HUVECs increasing with culture time (Fig. 2). This result was consistently obtained in six independent experiments.

The inhibitory effect was macrophage-dependent, because the cell-free control media exposed to PVC for 7 days did not inhibit HUVEC proliferation (not shown). This result also rules out the possibility that the inhibition of HUVEC proliferation was caused by toxic compounds leaching from the PVC foil. We determined by trypan blue dye exclusion that HUVEC viability was unchanged by both CM from macrophages cultured on PVC or by cell-free control medium from PVC. Macrophage numbers decreased during the 7 days of culture from initially 40×10^6 cells per culture dish (10 cm diameter) to $24.9\pm2.6\times10^6$ (PVC) and $27.3\pm6.1\times10^6$ for PTFE, respectively ($N=6$ in each case). This indicates that the macrophage viability was not compromised by any biomaterial tested. Total protein content of the CM was also similar at all time points and material combinations tested (i.e. 0.72 mg/ml

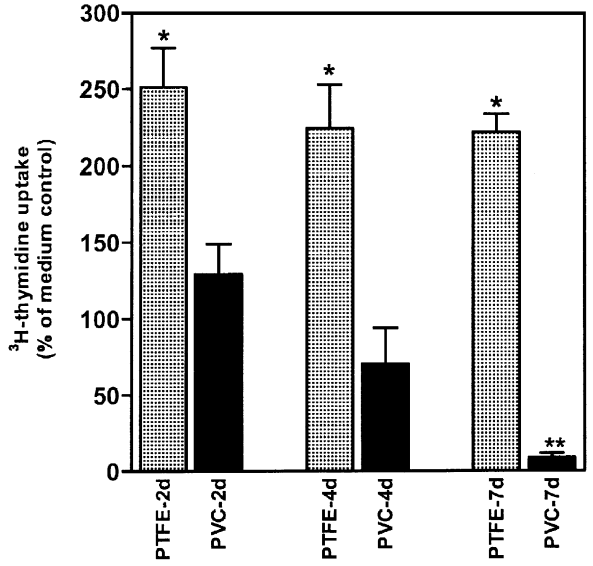

Fig. 2. Effect of macrophage-CM on HUVEC proliferation. Data (mean $\pm$ SEM) are presented as percentage of medium control ($n=6$). *$p<0.01$ vs. control, ** $p<0.01$ vs. control and vs. PTFE.

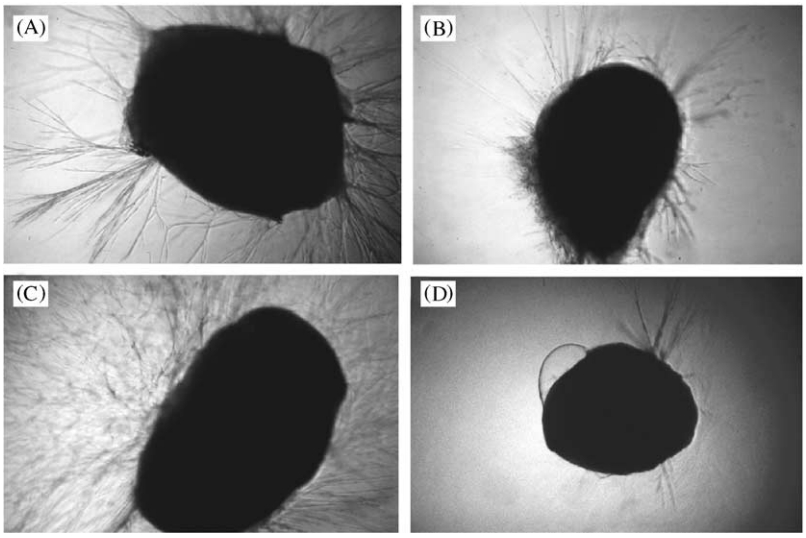

Fig. 3. Placental vessel fragments were embedded in fibrin gel and cultured for 9 days with control medium (A), 10^{-7} M dexamethasone (B), with conditioned medium of macrophages cultured on PTFE (C), and with conditioned medium of macrophages cultured on PVC (D) under serum-free conditions, as described in methods (original magnification, $\times 25$). Note that the addition of conditioned medium from macrophages cultured on PTFE biofoil for 7 days stimulated the formation of microvessels, whereas conditioned medium from macrophages cultured on PVC inhibited microvessel sprouting. The serum-free control medium produced a basal sprouting activity (control) and dexamethasone (10^{-7} M) caused a moderate decrease in the number of microvessels.

for PTFE and 0.67 mg/ml for PVC at day 7). We concluded that different biomaterials can induce in macrophages specific proliferative activity towards HUVECs. In biological terms, this would indicate control of endothelial proliferation but not necessarily (neo)vascularization. To test this second important aspect of angiogenesis we employed a vessel-sprouting test.

3.3. Effect of macrophage-conditioned media on capillary sprouting

We employed an established angiogenesis model [24], capillary sprouting from placental vessel fragments in fibrin matrix, to estimate the combined effect of macrophage culture supernatants on proliferation, migration and differentiation of endothelial cells. Fig. 3 shows the results obtained with serum-free CM collected after 7 days of continuous culture of macrophages on biomaterials. Medium conditioned by incubation with macrophages cultured on PTFE induced capillary sprouting, determined as the number of capillary sprouts in vessel cultures, to a greater extent than control, whereas medium conditioned by macrophages cultured on PVC showed inhibitory activity (Fig. 3). The serum-free control medium produced a basal sprouting activity (median 36, $n = 20$) most likely due to the endogenous production of aFGF and bFGF by parent vessels [24]. As anticipated, dexamethasone (10^{-7} M) caused a moderate decrease in the number of microvessels (13, $n = 20$). Conditioned medium from macrophages cultured on PTFE produced a 2.9-fold higher sprouting activity (116, $n = 20$) compared to medium control. In contrast, conditioned medium from macrophages cultured on PVC caused a drastic reduction of sprouting activity (0, $n = 20$) (Fig. 4). This inhibition was more pronounced than that of

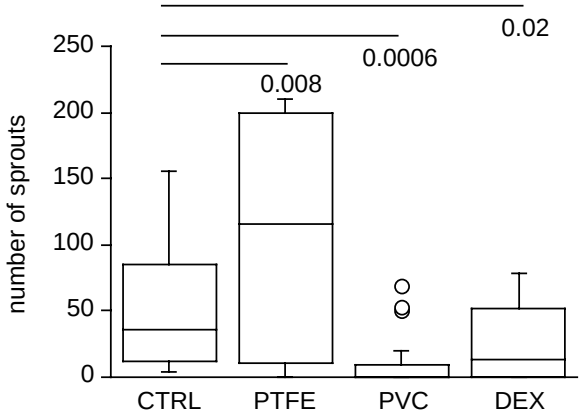

Fig. 4. Quantitation of microvascular growth in serum-free fibrin gel. Cultures treated for 9 days with control medium (CTRL), with CM of macrophages cultured on PTFE foil (PTFE), with CM of macrophages cultured on PVC and 10^{-7} M dexamethasone under serum-free conditions. Data are displayed in a box plot of 20 individual measurements for each macrophage/material combination. Each box encloses 50% of the data with the median value of the variable displayed as a line. The top and bottom of the box mark the limits of $\pm 25\%$ of the variable population. The lines extending from the top and bottom of each box mark the minimum and maximum values within the data set that fall within an acceptable range. Any value outside of this range, called an outlier, is displayed as an individual point. Outliers are included in all calculations. p-values of Student's t-test are indicated on top of each column.

dexamethasone, which caused only a moderate suppression of capillary sprouting. The protein concentration in the CM was similar regardless of the material on which the macrophages had been cultured at 0.72 mg/ml for PTFE and 0.67 mg/ml for PVC. This rules out the possibility that a grossly distorted protein synthesis or viability of the macrophages on one, but not on the other material caused the differential biological activity of the CM.

4. Discussion

Function or failure of biomaterial implants depend on a successful healing of the implantation site. Macrophages activated by biomaterial contact are critically important during the initial inflammatory stage of implant healing [25]. Here, we investigated whether biomaterial-primed macrophages also influence later stages of the implant healing, especially tissue vascularization. It is known that macrophages produce a number of angiogenic factors [8]. Whether or not this is influenced by previous contact with biomaterials is presently unknown. We therefore investigated the expression of Ang-1, VEGF, bFGF and Tsp-1 in monocytes and monocyte-derived macrophages cultured on different polymers.

The present study demonstrates the expression of Ang-1 in human monocytes and macrophages in vitro, which so far has only been shown in tissue macrophages during chronic inflammatory processes [13]. Our data clearly show that monocytes and monocyte-derived macrophages express the angiogenic molecule Ang-1 in a material-dependent manner, while the mRNA expression of VEGF as well as the anti-angiogenic molecule Tsp-1 is material-independent.

Although macrophages cultured for 7 days on PVC produced the mRNA for VEGF, one of the most potently acting angiogenic factors, surprisingly, CM from these macrophage cultures proved to be anti-proliferative on endothelial cells and anti-angiogenic in our in vitro angiogenesis assay. At the same time, PVC inhibited the Ang-1 mRNA expression, suggesting that the absence of Ang-1 may contribute to the inhibition of capillary sprouting in vessel culture system, since Ang-1 has been shown to induce sprouting activity in fibrin gels [19]. It is however unlikely that the inhibitory effect of supernatants from macrophages cultured on PVC is due to the absence of Ang-1, because it is well established that Ang-1 has no proliferative effect on endothelial cells [14,19]. Tsp-1 is a naturally occuring inhibitor of angiogenesis [22,26]. Therefore, elevated expression of Tsp-1 mRNA could explain the anti-angiogenic activity of supernatants from macrophages cultured on PVC. However, macrophages cultured on PTFE also expressed Tsp-1 mRNA but showed high angiogenic properties, a finding which has also been reported for LPS-activated macrophages [27]. The presence of Tsp-1 mRNA in both, angiogenic and anti-angiogenic macrophages, therefore suggests that Tsp-1 is not a key regulator of the PVC-mediated inhibition of angiogenesis.

Our demonstration that VEGF-producing macrophages act anti-angiogenic seems paradoxical, but macrophages are known to release pleiotropic factors with opposing effects on endothelial cells like TGF-β, TNF-α and IL-1 [8]. Recently, it has been shown that macrophages cultured on PVC express IL-1β while macrophages on PTFE do not [28]. Thus, IL-1β might also be involved in the PVC-induced anti-angiogenic activity of macrophages. Most likely, the inhibition of the angiogenesis by supernatants of macrophages cultured on PVC is due to decreased production of angiogenic factors and increased production of anti-angiogenic factors. Regardless of the exact mechanism, we consistenly demonstrated that macrophages cultured on PVC are anti-angiogenic and therefore provide an excellent model to analyse the gene expression profile associated with the anti-angiogenic phenotype. Studies to this effect are ongoing.

It is currently unknown, which properties of biomaterials used in our study are responsible for pro- and anti-angiogenic phenotypes of peripheral blood-derived macrophages and if these properties could potentially be engineered. It is known that PVC, TCPS and the PTFE biofoil differ in their hydrophilicity (contact angle, PVC: 74.5°; TCPS: 18.7°; PTFE: 113.2°, unpublished data from Klein et al.). However, the chemical composition and surface properties of the polymers, such as hydrophilicity, do not seem to correlate with the expression profile of angiogenic factors in macrophages cultured on different materials. Many macrophage-derived angiogenic factors are synthesized or released only by activated macrophages. The bacterial product LPS is a potent inducer of angiogenic activity, but does not appear to be a stimulus of angiogenesis per se [29,30]. It is known that alternatively activated macrophages have angiogenic properties [31]. Alternative activation of macrophages can be induced by IL-4 and glucocorticoids as well as by other cytokines such as IL-10, IL-13 and TGF-β [32,33]. In summary, we have demonstrated differential regulation of angiogenic properties in macrophages triggered by previous biomaterial contact. Our study provides a model system for the assessment of macrophage–implant interaction, which may ultimately improve implant healing and performance.

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