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Microparticles stimulate angiogenesis by inducing ELR$^+$ CXC-chemokines in synovial fibroblasts

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Abstract

Microparticles (MPs) are small membrane-vesicles that accumulate in the synovial fluids of patients with rheumatoid arthritis (RA). In the arthritic joints, MPs induce a pro-inflammatory and invasive phenotype in synovial fibroblasts (SFs). The present study investigated whether activation of SFs by MPs stimulates angiogenesis in the inflamed joints of patients with RA. MPs were isolated from Jurkat cells and U937 cells by differential centrifugation. SFs were co-cultured with increasing numbers of MPs. The effects of supernatants from co-cultures on endothelial cells were studied *in vitro* and *in vivo* using MTT assays, annexin V and propidium iodide staining, trans-well migration assays and modified matrigel pouch assays. MPs strongly induced the expression of the pro-angiogenic ELR$^+$ chemokines CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 in RASFs. Other vascular growth factors were not induced. Supernatants from co-cultures enhanced the migration of endothelial cells, which could be blocked by neutralizing antibodies against ELR$^+$ chemokines. Consistent with the specific induction of ELR$^+$ chemokines, proliferation and viability of endothelial cells were not affected by the supernatants. In the *in vivo* bio-chamber assay, supernatants from RASFs co-cultured with MPs stimulated angiogenesis with a significant increase of vessels infiltrating into the matrigel chamber. We demonstrated that MPs activate RASFs to release pro-angiogenic ELR$^+$ chemokines. These pro-angiogenic mediators enhance migration of endothelial cells and stimulate the formation of new vessels. Our data suggest that MPs may contribute to the hypervascularization of inflamed joints in patients with rheumatoid arthritis.

Keywords: angiogenesis • microparticles • rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes inflammation and destruction of synovial joints in association with extra-articular manifestations. RA synovial fibroblasts (RASFs) are key players in the pathogenesis of RA. RASFs display an invasive and pro-inflammatory phenotype with invasion into cartilage and aberrant release of pro-inflammatory mediators [1, 2]. RASFs also stimulate the generation of new blood vessels by releasing pro-angiogenic mediators [3, 4]. The enhanced vascularization of the hyperplastic synovium is considered a crucial step in disease progression, since it supports the influx of more immune cells *via* these newly formed blood vessels [5].

Microparticles (MPs) have recently been implicated in the pathogenesis of RA. Leukocyte-derived MPs, which are released from their parental cells upon cellular activation and during apoptosis, accumulate in the synovial fluid of inflamed joints [6, 7]. Indeed, high numbers of leukocyte-derived MPs are found in the synovial fluid of swollen joints from RA patients, whereas MPs counts are low in osteoarthritis (OA) joints with less acute inflammation [8–12]. MPs are small membrane-vesicles that act as signalling elements in coagulation, inflammation and angiogenesis. Once released by cell blebbing and shedding, MPs can transfer

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Table 1 Clinical characteristics of RA patients. RASFs were isolated from patients 1 to 9. MPs from synovial fluid obtained by joint puncture were obtained from patients 10 to 12. All clinical characteristics are from the time-point, when the material was obtained

Patient number	Sex	Age	Rheumatoid factor	Anti-CCP-antibodies	Erosions on x-ray	DAS 28 score	Medication
1	Female	52	+	+	+	5.3	Methotrexate, adalimumab, low-dose corticosteroids
2	Female	56	–	–	+	4.2	Methotrexate
3	Male	48	+	–	+	3.1	Adalimumab
4	Female	44	+	+	+	4.4	Methotrexate, etanercept, low-dose corticosteroids
5	Female	52	+	–	+	4.7	Adalimumab, low-dose corticosteroids
6	Female	39	+	+	+	5.8	Methotrexate, rituximab, low-dose corticosteroids
7	Male	54	+	–	+	3.1	Leflunomide
8	Male	51	–	+	+	4.5	Methotrexate, remicade, low-dose corticosteroids
9	Female	41	+	–	+	4.2	Methotrexate, adalimumab, low-dose corticosteroids
10	Male	32	+	–	–	3.6	–
11	Female	46	–	–	+	3.3	Methotrexate, low-dose corticosteroids
12	Female	51	+	+	+	5.7	Methotrexate, rituximab

lipids, proteins and nucleic acids, as well as activate intracellular pathways in target cells. Content and function of MPs depend on both the paternal cell and on the stimulus for MP release. During MP formation, selective rearrangement processes result in MP subpopulations specific for membrane and intra-vesicular content. Thus, MPs can operate selectively in intercellular communication and orchestrate biological processes [6]. MPs can induce pro-inflammatory and invasive properties in RASF, leading to joint inflammation and destruction. We demonstrated previously that MPs provide a source of arachidonic acids and up-regulate cyclooxygenase 2 (COX-2) and microsomal prostaglandin E synthase 1 (mPGES-1) in SFs, which subsequently convert arachidonic acid to prostaglandin E_2 (PGE2) [13]. MPs from T cells and monocytes stimulate the expression of pro-inflammatory cytokines in SFs, including monocyte chemoattractant protein-1 (MCP-1) as well as interleukins 6 and 8 [14]. Beyond their pro-inflammatory effects in RA joints, MPs can induce the release of matrix metalloproteinases (MMPs)-1, -3, -9 and -13 from SFs, and thus, promote cartilage breakdown and joint destruction [14].

Early studies found that synovial fluids from patients with RA stimulate the formation of blood vessels, suggesting the presence of pro-angiogenic mediators in the joints [15–17]. Based the potent induction of pro-inflammatory mediators and MMPs in RASF by MPs, we hypothesized that MPs might also stimulate angiogenesis and contribute to the hypervascularization of the synovial tissue in inflamed RA joints. We could demonstrate that MPs derived from leukocytes potently induce the expression of ELR+ chemokines in RASF and that MPs induced ELR+ chemokines stimulate angiogenesis by providing a chemotactic gradient for endothelial cells (ECs).

Material and methods

Cell culture

RASFs were isolated from synovial tissue specimens from nine patients with RA [18]. All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA. The clinical characteristics of these patients are summarized in Table 1.

Murine SFs were derived from 8-week-old female C57Bl/6 mice. Paws and joints were minced and digested in 0.5% dispase [19].

Immortalized human microvascular endothelial cells (HMECs) were cultured as described elsewhere [20].

Isolation of MPs and incubation of fibroblasts and HMECs

The release of MPs from Jurkat T cells and U937 monocytic cells was induced by treatment with staurosporine (STS, Sigma Aldrich, Munich, Germany) and TNFα (R&D Systems, Wiesbaden, Germany) as described previously [21]. After 12 hrs of treatment, supernatants were collected and centrifuged at 1500 *g* for 5 min. to remove cells. The cell-free supernatants were centrifuged at 100.000 *g* and 14°C for 40 min. using a Beckman Coulter LE-80K ultracentrifuge with a SW41 rotor. After another washing step, the pellet was resuspended in DMEM/F-12 containing 5% foetal bovine serum (FBS).

For co-culture experiments, 5.0×10^5 fibroblasts were incubated with 1.0×10^4 to 1.0×10^6 freshly isolated MPs. Supernatants of the first ultracentrifugation step served as controls and were therefore processed in the same way as the MP-pellets. Consequently, control supernatants without MPs contained the same concentrations of soluble mediators as the

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Table 2 Primer sequences

Gene	Sequence	
CXCL1	rev	5′- GTC ACT GTT CAG CAT CTT TTC G – 3′
	fwd	5′- CTG CAT CCC CCA TAG TTA AGA A – 3′
CXCL2	rev	5′- ATT TGC CAT TTT TCA GCA TCT T – 3′
	fwd	5′- ATC GCC CAT GGT TAA GAA AAT – 3′
CXCL3	rev	5′- CGG GGG ACC TTA CAT TCA C – 3′
	fwd	5′- GCA TTT AAT TCA CCT CAA GA – 3′
CXCL5	rev	5′- CTA TGG CGA ACA CTT GCA GA – 3′
	fwd	5′- GCA AGG AGT TCA TCC CAA AA – 3′
CXCL6	rev	5′- TCG TTT TGG GGT TTA CTC TCA – 3′
	fwd	5′- GCT GCG TTG CAC TTG TTT AC – 3′

MP suspensions. After 24 hrs of incubation, the supernatant of the co-culture experiments or control experiments was removed and filtered through 0.1 μm pores to remove the remaining MPs. The MP-free supernatants were then used immediately to assess their effects on EC functions and angiogenesis.

Quantification of MPs

To determine the numbers of MPs, freshly isolated preparations were incubated with FITC-labelled antibodies against human CD3 or CD14 and double-stained with PE-labelled annexin V (all Becton Dickinson, Heidelberg, Germany) as described [14].

Quantitative real-time PCR

Total RNA was isolated with the NucleoSpin RNA extraction system (Machery-Nagel, Düren, Germany). RNA was reverse transcribed into cDNA and gene expression was quantified by TaqMan and SYBR Green real-time PCR using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) [22]. Specific primers for each gene were designed with the Primer 3 software. Primer sequences are summarized in Table 2. Samples without enzyme in the reverse transcription (Non-RT-controls) were used to control for genomic contamination. Unspecific signals caused by primer dimers were excluded by dissociation curve analysis and by no template controls. A pre-developed 18S assay (Applied Biosystems) was used as an endogenous control to normalize for the amounts of loaded cDNA. Differences were calculated with the threshold cycle (Ct) and the comparative Ct method.

Transwell migration assays

The effect of supernatants from fibroblasts co-incubated with MPs on EC migration was analysed by transwell migration assays. Cell culture inserts with 8-μm pore-size polyethylene terephthalate (PET) membranes were placed into 24-well cell cultured dishes. The lower chamber was filled with 500 μl of supernatants from fibroblasts co-incubated with MPs in different dilutions. 2×10^4 HMECs in 200 μl DMEM/F-12 containing 5% FBS were seeded. After 24 hrs, the migration of HMECs across the membrane was analysed by counting the HMECs in the lower chamber using flow cytometry (BD FACS-Calibur, Becton Dickinson, Heidelberg, Germany).

To identify stimuli for increased migration, neutralizing antibodies against pro-angiogeneic CXC chemokines were added in a subset of experiments. In these experiments, supernatants were pre-incubated with neutralizing antibodies against GROα/β/γ, ENA78 or GCP-2 (all R&D Systems, Wiesbaden, Germany) for 2 hrs at 37°C.

Quantification of apoptotic and necrotic cells

To analyse potential effects on the viability of ECs, HMECs were incubated for 24 hrs with different concentrations of supernatants from RASFs co-cultured with MPs. HMECs were stained with FITC labelled annexin V and propicium iodide and cell viability was assessed with FACS as described [23].

Microtitre tetrazolium (MTT) assay

Metabolic activity of HMECs incubated with the SF and MPs co-culture supernatants was measured using the MTT [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] method as described [23]. HMECs incubated with 50% dimethylsulfoxide served as positive controls.

In vivo Matrigel-plug assay

The effects of supernatants from SF incubated with MPs on angiogenesis were analysed *in vivo* using a modified matrigel-plug assay [24]. Matrigel chambers were constructed using 3-mm sections of surgical grad tubing (Tygon S-50-HL, Saint-Gobain, Akron, USA) and 100-μm nylon filters that were attached at both ends of the tubes. Chambers were sterilized by UV irradiation. Three ml of supernatant from co-culture experiments of murine SF with MPs was evaporated, re-suspended in 100-μl liquid matrigel and injected into each chamber. Chambers with equally concentrated supernatants from SF cultured without MPs served as controls. For implantation of the chambers, 8-week-old female C57Bl/6 mice were anaesthetized and a 1-cm incision was made along the medial plane in the pectoral region. The chambers were inserted into subcutaneous pockets at the midline in the pelvic region 1.5 cm distal to the incision, which was closed by surgical suture. Eight mice were implanted with two chambers each, one on the left flank and one on the right flank. In each mouse, one chamber contained supernatants from SF co-incubated with MPs and the second chamber contained control supernatants. Ten days after the implantation, the chambers were removed, fixed in 10% paraformaldehyde for 24 hrs and then embedded in paraffin. Two μm thick sections of six different layers of the plaque were stained with haematoxylin and eosin. Numbers of blood vessels were analysed with a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, The Netherlands) at 200-fold magnification.

Immunohistochemistry for CD34

Immunohistochemistry for CD34 was used to visualize ECs in chambers of Matrigel-plug assay. Two-μm-thick sections of plugs were deparaffinized, following by treatment with 1 mg/ml trypsin (Sigma, Munich, Germany) for 1 hr at 37°C. Non-specific binding of antibody was blocked by incubation

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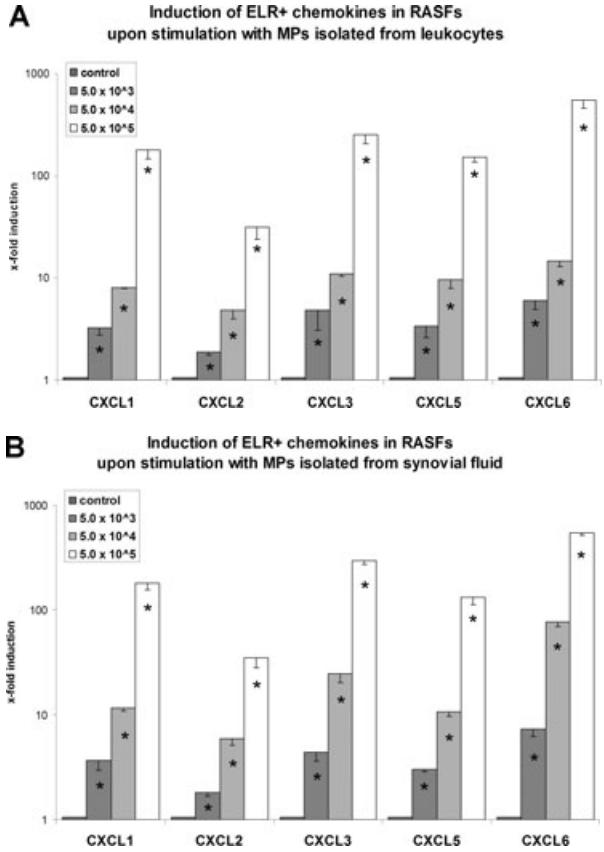

Fig. 1 MPs derived from leucocytes potently induce the expression of pro-angiogenic chemokines in SFs. Co-incubation of RASFs patients with pathophysiologically relevant numbers of MPs derived from U937 cell stimulated the expression of the ELR+ CXC chemokines CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 in dose-dependent manner as analysed by real-time PCR (**A**). * indicates statistically significant differences compared to controls ($P < 0.05$). Comparable effects on the expression of ELR+ chemokines were also observed with MPs isolated from the synovial fluid of inflamed joints of three different RA patients (**B**).

with 5% goat serum in phosphate buffer solution for 30 min. at room temperature and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 10 min. at room temperature. ECs were detected by incubation with polyclonal rat anti-mouse CD34 antibody (Linaris, Wertheim-Bettingen, Germany) for 2 hrs at room temperature. Polyclonal goat anti-rat antibody (Abcam, Cambridge, UK) labelled with horseradish peroxidase was used as secondary antibody for 1 hr at room temperature. Staining was visualized with 3,3′diaminobenzidine tetradydrochloride (DAB, Merck, Darmstadt, Germany).

Statistics

Data are expressed as mean ± standard error of the mean. The Wilcoxon signed rank test for related samples and the Mann–Whitney test for non-related samples were used for statistical analyses. A *P*-value of less than 0.05 was considered statistical significant.

Results

MPs induce the release of pro-angiogenic chemokines in synovial fibroblasts

Co-incubation with MPs from TNFα stimulated U937 cells potently induced the expression of members of the ELR+ subfamily of CXC chemokines in RASFs. The mRNA levels of CXCL1 (GROα), CXCL2 (GROβ) and CXCL3 (GROγ) increased in a dose-dependent manner by up to 180 ± 32 fold, 31 ± 7 fold and 250 ± 42 fold in RASF compared to controls ($P < 0.05$ for all) (Fig. 1A). A significant induction of up to 150 ± 16 fold was also observed for CXCL5 (ENA-78) (Fig. 1A). CXCL6 (GCP-2) was also up-regulated by 551 ± 95 fold in RASF co-incubated with MPs ($P = 0.02$) (Fig. 1A). In addition to MPs derived from U937 monocytic cells, MPs from apoptotic Jurkat T cells also potently stimulated the expression of ELR+ chemokines, indicating a general effect of leukocyte-derived MPs. To confirm the relevance of these findings with MPs from U937- and Jurkat cell lines, MPs were isolated from the synovial fluid of inflamed RA joints. MPs isolated from synovial fluid potently increased the mRNA levels of CXCL-1, CXCL-2, CXCL-3, CXCL-5 and CXCL-6 (Fig. 1B). Indeed, the increased were comparable to those obtained with MPs from U937 and Jurkat T cell lines, indicating that MPs from these cells are a good model to study the effects of leucocyte-derived MPs on RASF.

Supernatants from RASFs co-incubated with MPs are chemotactic for endothelial cells

To investigate, whether the induction of ELR+ chemokines in RASFs by MPs is sufficient to induce EC migration, transwell chamber assays were performed. Supernatants from RASFs co-incubated with MPs induced the migration of ECs. The number of HMECs that migrated into the lower chambers of the transwell towards the supernatants from co-culture experiments with MPs system increased by 7.2 ± 2.3 fold compared to control supernatants from RASFs cultured without MPs ($P = 0.01$) (Fig. 2).

To confirm, the increased migration is indeed due to the induction of ELR+ chemokines, the supernatants from the co-cultured experiments were pre-incubated with neutralizing antibodies against different ELR+ chemokines in a subset of experiments. Neutralizing antibodies against the GRO subfamily (CXCL1, CXCL2 and CXCL 3) reduced the stimulatory effects of supernatants from RASFs co-cultured with MPs by 35 ± 23% ($P = 0.04$) (Fig. 2). Neutralization of CXCL5 and CXCL6 also significantly decreased the stimulatory effects by 57 ± 35% for CXCL5 and 57 ± 35% for CXCL6 ($P = 0.04$ for both) (Fig. 2).

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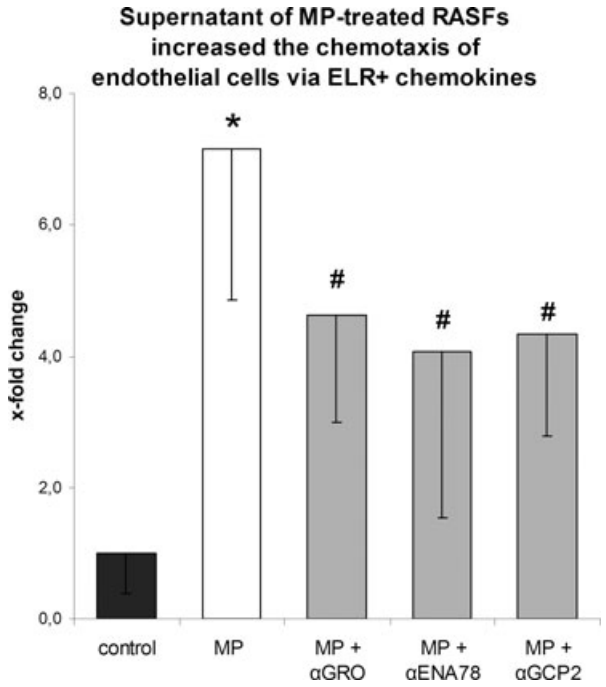

Fig. 2 Supernatants from RASFs incubated with MPs are chemotactic for microvascular ECs. Supernatants from RASFs co-cultured with MPs derived from leucocytes stimulated migration of ECs. The chemotactic effects can be partially inhibited by blocking antibodies against CXCL1, 2, 3, CXCL5 or CXCL6, demonstrating that the increased chemotactic activity is mediated by these ELR+ CXC chemokines. * indicates statistically significant differences compared to controls ($P < 0.05$). # indicates statistically significant compared to supernatants from RASFs co-incubated with MPs with irrelevant, non-blocking antibodies ($P < 0.05$).

Supernatants from RASFs co-cultured with MPs do not affect proliferation or viability of endothelial cells

To investigate whether supernatants from RASFs co-incubated with MPs also alter proliferation or survival in addition to migration, the metabolic activity and the viability of ECs upon co-culture were assessed. Incubation of HMECs with supernatant from RASFs co-cultured with MPs did not alter the proliferation of HMECs (Fig. 3A). Incubation of HMECs with supernatants from RASFs co-incubated with MPs also did not alter the number of apoptotic or necrotic cells as analysed by staining with annexin V and propidiumiodide (Fig. 3B and C), suggesting that co-incubation of RASFs with MPs might specifically stimulate the release of ELR+ chemokines, but not of endothelial growth factors that stimulate proliferation or decrease apoptosis. Consistent with this hypothesis, no induction of major endothelial growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiogenin was observed in RASFs upon co-culture with MPs (data not shown).

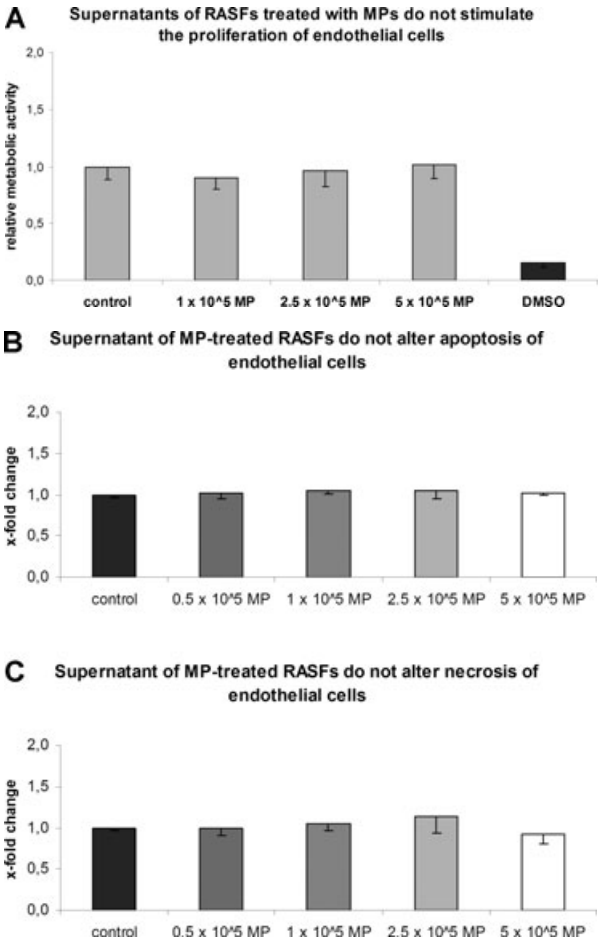

Fig. 3 Supernatants from RASFs co-incubated with microparticles do not alter the metabolic activity or affect the viability of ECs. Incubation of ECs with increasing concentrations of supernatants from RASFs cocultured with MPs did not alter the metabolic activity as analysed with the MTT assay (**A**). The number of annexin V positive, apoptotic ECs (**B**) or the number of PI positive necrotic ECs (**C**) also did not change.

Supernatants from fibroblasts co-incubated with MPs stimulate angiogenesis *in vivo*

To confirm the chemotactic and pro-angiogenic effects of supernatants from murine SFs co-incubated with MPs *in vivo*, modified matrigel chamber assays were performed. The total number of vessels, defined by the presence of erythrocytes in the luminal space lined by CD34 positive ECs, increased significantly by 2.0 ± 0.6 fold in chambers filled with supernatants from murine SFs co-incubated with MPs compared to supernatants from murine SFs cultured without MPs ($P = 0.03$) (Fig. 4). Further sub-analyses demonstrated that the numbers of arterioles and capillaries were both higher, but that the increase in arterioles was more pronounced than in capillaries (2.1 ± 0.7 fold *versus* 1.5 ± 0.5 fold).

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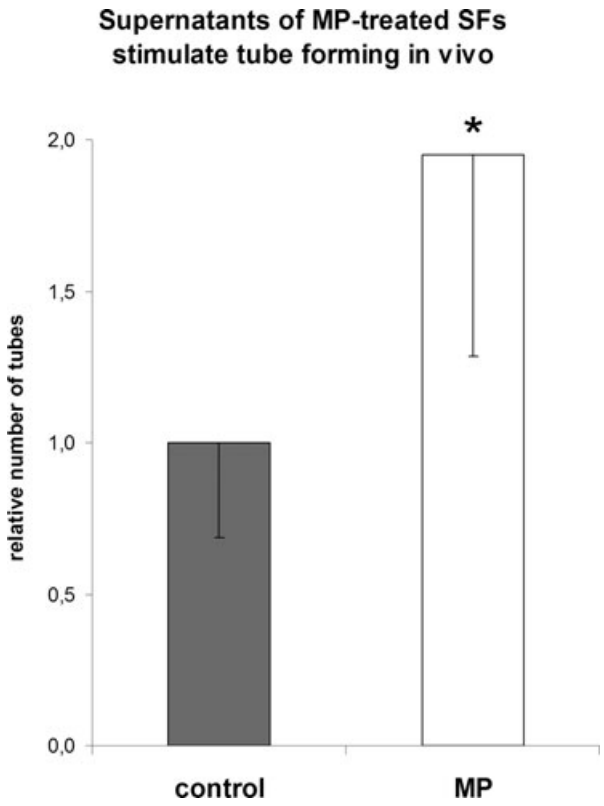

Fig. 4 Supernatants from murine SFs co-cultured with MPs stimulate angiogenesis in the matrigel plague assay *in vivo*. For this assay, chambers filled with supernatants from murine SFs co-incubated with MPs or control supernatants re-suspended in matrigel and chambers were implanted into subcutaneous pockets of C57Bl/6 mice. Blood vessels infiltrating into the matrigel chamber were quantified after 10 days. The number of blood vessels, defined by the presence of erythrocytes in the luminal space lined by CD34 positive endothelial cells, increased significantly higher in chambers filled with supernatants from SFs co-incubated with MPs compared to supernatants from SF cultured without MPs. * indicates statistically significant differences ($P < 0.05$).

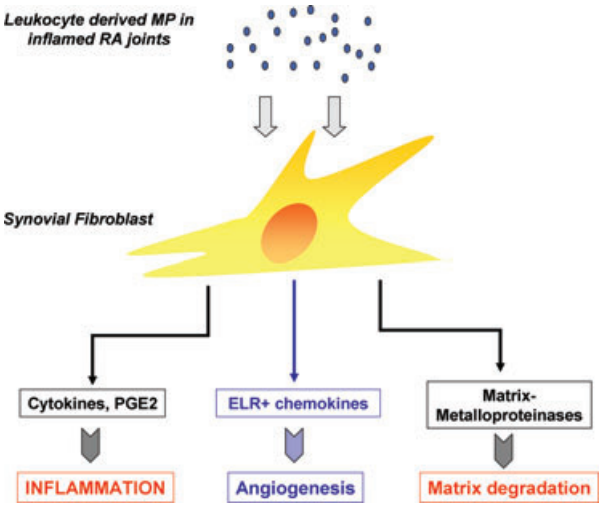

Fig. 5 Microparticles as key-players in the pathogenesis of RA. MPs are released from infiltrating leucocytes upon activation and accumulated in the synovial fluid of inflamed joints. MPs activate SFs and potently stimulate the release of pro-angiogenic ELR+ chemokines, pro-inflammatory CC chemokines and matrix degrading enzymes from SFs. Aberrant activation of SFs by MPs might therefore directly contribute to hypervascularization, inflammation and joint destruction in RA.

Discussion

Our study provides new insights into the mechanisms by which MPs activate RASFs and extend previous results demonstrating that MPs play important roles in the pathogenesis of RA. MPs up-regulate the expression of pro-inflammatory cytokines and prostaglandin E2 by SFs [8, 13, 14]. Moreover, MPs stimulate the expression of matrix degrading enzymes by SF [14]. Besides inflammation and joint destruction, increased angiogenesis with increased formation of new vessels in inflamed joints is a key feature of the pathogenesis of RA [25]. Here, we show that MPs might also stimulate angiogenesis by inducing the release of ELR+ chemokines. MPs are released from activated leukocytes in inflamed RA joints and accumulate in high numbers in the synovial fluid of patients with active RA [9]. In contrast, MP counts are low in patients with degenerative joint diseases such as OA [6, 9, 26]. Thus, the release of pro-angiogenic chemokines and the other actions of MPs on SF are operative only in inflammatory joint diseases such as RA, but not in non-inflammatory degenerative diseases [26]. Together, these findings demonstrate that MPs are novel signalling elements in RA which, along with cytokines and direct cell–cell interactions, can activate SF to promote local inflammation, matrix degradation and angiogenesis in affected joints (Fig. 5).

In the present study, we have demonstrated the MPs potently stimulate the release of pro-angiogenic ELR+ chemokines by SF. MPs up-regulated the expressions of CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 by up to 550-fold in SF. The induction was specific for ELR+ chemokines and other vascular growth factors were not induced. Supernatants from fibroblasts co-incubated with MPs induce angiogenesis in *in vivo* matrigel plaque assays, indicating that the mediators released from SF upon contact with MPs alone are sufficient to stimulate angiogenesis *in vivo*. Thus, the massive induction of ELR+ chemokines might create a chemotactic gradient within inflamed joints that attracts ECs and stimulates the formation of new vessels in affected joints.

In summary, we have demonstrated in this study that MPs stimulate the release of pro-angiogenic ELR+ chemokines in SF by MP. The release of ELR+ chemokines attracts ECs along a chemotactic gradient. The release of ELR+ chemokines upon by

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SF upon contact with MPs is sufficient to stimulate angiogenesis *in vivo* and may contribute to the increased formation of new vessels in affected RA joints. Thus, MPs serve as novel communication devices that contribute to the pathogenesis of RA by inducing an activated phenotype in SF with increased release of matrix degrading enzymes, pro-inflammatory mediators and pro-angiogenic chemokines.

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