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Novel players in the pathogenesis of rheumatic diseases

Subtitle: EPCs in rheumatic diseases

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Abstract

The involvement of the cardiovascular system appears to be an important factor for the outcome of many rheumatic diseases. Vasculogenesis, which is defined as the *de novo* formation of new blood vessels by endothelial progenitor cells (EPCs), plays a crucial role for the maintenance of the cardiovascular system. EPCs have turned out to be key-players of cardiovascular disease. Impaired vasculogenesis with reduced counts or altered function of EPCs have been implicated in the pathogenesis of several rheumatic disorders including systemic sclerosis (SSc), rheumatoid arthritis (RA), systemic lupus erythematodes (SLE), and vasculitides. Moreover, EPCs may represent novel biomarkers of vascular involvement and cardiovascular risk in patients with rheumatic disorders. EPCs might also offer therapeutic potential by direct stimulation of vessel growth or as transport vehicles for drugs to activated endothelia and hypoxic tissues. In this review, we discuss the physiologic functions of EPCs, and highlight the emerging role of EPCs in the pathogenesis of rheumatic diseases.

Introduction

Cardiovascular disease is an integral part of most rheumatic diseases and its impact on the outcome and the prognosis is a major research focus for rheumatologists and cardiologists. In patients with inflammatory rheumatic disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematodes (SLE), the cardiovascular risk is highly increased, even if other cardiovascular risk factors known to promote and accelerate the progression of atherosclerotic lesions are absent. Patients with RA have a two to five fold increased risk of developing premature cardiovascular disease. The increased risk of myocardial infarction and stroke strongly contributes to the increased mortality and to the by 5-10 years shortened life expectancy of patients with RA (1, 2). Other rheumatic disorders, such as systemic sclerosis (SSc), are characterized by a severe vasculopathy. Involvement of the microvascular circulation manifests as Raynaud´s syndrome and ischemic ulcers, and has profound impact on the quality of life (3, 4). In addition, involvement of the pulmonary vasculature leads to pulmonary arterial hypertension (PAH) in 10-20 % of patients with SSc. PAH is known to be a major cause of death in SSc (5, 6). As illustrated by these examples, rheumatic disorders are characterized by a profound cardiovascular disease and therefore may even serve as research models to study the influence of systemic inflammatory processes on the cardiovascular integrity.

Over the past years, endothelial progenitor cells (EPCs) have emerged as crucial regulators of the cardiovascular integrity. Reduced numbers and altered functions of EPCs have been found to be involved in the pathogenesis of cardiovascular disease. The important role of vasculogenesis and EPCs in the adult was discovered late, and the formation of new vessels had long been believed to be exclusively mediated by sprouting of fully differentiated endothelial cells (ECs) from preexisting vessels, a process which is named angiogenesis. This concept was first disproved by Asahara and colleagues in 1997. In a landmark study, they demonstrated that new blood vessels can also be formed by circulating progenitor cells in

adults, independently from the preexisting vasculature (7). In their studies, bone-marrow derived CD34-positive cells could acquire characteristics of mature ECs, express endothelial markers, and incorporate into new vessels at sites of ischemia. Subsequent analysis revealed that these EPCs were not restricted to the bone marrow, but could also be detected in the peripheral circulation of adults (8). Since then, growing evidence has been suggesting that EPCs play an important role for the homeostasis of the physiologic vascular network. EPCs are not only involved in the formation of new vessels in ischemic tissues, thereby contributing to the vascular remodeling, but might also contribute to the repair of preexisting vessels (8-10). This concept is indicated by the inverse correlation of circulating EPC counts with cardiovascular risk factors and it is further supported by the observation that conditions which reduce the cardiovascular risk increase the levels of EPCs (11-15). In addition to the defective EPC compartment observed in primary cardiovascular diseases, reduced numbers and/or altered functions of EPCs have recently been demonstrated in patients with SSc (16-23), RA (24-30), SLE (31-34), and vasculitides (35). In SSc, capillary rarification and vascular alterations might be in part due to the impaired *de novo*-formation of new blood vessels by EPCs. Vasculopathy in SSc is known to cause ischemic manifestations such as fingertip ulcers and gangrene. In patients with RA or SLE, the defective vasculogenesis is likely to contribute to the increased cardiovascular risk. Further studies on vasculogenesis and EPCs may help us to understand vasculopathy and defective vascular repair mechanisms, which are major pathogenic characteristics of many rheumatic disorders

New insights in the role of EPCs in the vasculopathy of rheumatologic disorders might be of interest for several reasons: (I) Alterations in the numbers of circulating EPCs and their functional impairments have been demonstrated in several rheumatic disorders and therefore may be of significant relevance in pathogenic processes. Decreased EPC counts and impaired EPC functions, which result in impaired vasculogenesis, might at least in part explain the often characteristic wide-spread vasculopathy. (II) EPCs might be sensitive biomarkers for

cardiovascular involvement in rheumatic diseases and decreased EPC counts might establish as novel markers for an increased cardiovascular risk. In inflammatory vasculitides, EPCs have already been inversely correlated with disease activity scores. (III) EPCs offer a broad therapeutic potential. Application of autologous *in-vitro* amplified EPCs has already been shown to stimulate vessel formation and to improve the outcome of patients with myocardial infarction or hind limb ischemia. Therefore, injection of EPCs might be a promising approach for the treatment of patients with SSc, who suffer from ischemic fingertip ulcers and gangrene due to progressive loss of capillaries. Alternatively, EPCs might be used as vehicles to transport drugs to activated endothelium and hypoxic tissues. As EPCs home to sites of ischemic tissue injury, this approach would allow targeted therapy of involved organs.

Taken together, there is a rapidly growing interest to elucidate the role of EPCs in the pathogenesis of rheumatic disorders and to develop novel diagnostic and therapeutic applications. This review summarizes the current knowledge about EPCs in rheumatic diseases and discusses open issues about different EPC subsets, their mode of action, and their relevance for vascular repair and integrity, which need to be addressed in further studies.

Subpopulations of EPCs

Two main subpopulations of EPCs with different origin, morphologic characteristics and functions have been identified. These two subpopulations can be differentiated by the expression of the cell surface marker CD14. Short term cultures of seven days or less mainly contain EPCs of the CD14-positive subpopulation. This subset of EPCs is thought to represent transdifferentiated CD14-positive monocytes, which acquire characteristics of ECs under certain culture conditions (36). In vitro, CD14-positive EPCs show little proliferation capacity and undergo apoptosis within a few weeks (37, 38). In contrast to the CD14-positive EPCs arised from short-term cultures, CD14-negative EPCs are harvested from long-term, late out-growth cultures. The CD14-negative subset of EPCs, which sometimes is referred to as “true

EPCs" or "angioblast-like EPCs", possess an extraordinarily high proliferation capacity (39). Besides differentiation by the cell surface marker CD14, the two EPC subsets can also be distinguished morphologically. CD14-positive EPCs are spindle-shaped, whereas CD14-negative EPCs form cobblestone-monolayers (36, 37). Interestingly, both CD14-positive EPCs and CD14-negative EPCs can form capillary tubes *in vitro*, mediate re-endothelialization after vessel injury and improve neovascularization (9, 40). Similar to CD14-negative EPCs, CD14-positive EPCs can be incorporated into vessels after short term-culture under conditions promoting their differentiation into EPCs. In contrast, CD14-positive cells without *ex vivo* differentiation do not promote neovascularization (41). Based on these observations and regarding their different origins, specific functions have been proposed for CD14-positive and CD14-negative EPCs (9, 10). CD14-positive EPCs are supposed to stimulate the formation of granulation tissue and new vessels by releasing inflammatory mediators and vascular growth factors, whereas their integration into vessels is of minor importance. In contrast, CD14-negative angioblast-like EPCs might strongly proliferate and differentiate at sites of vascular injury after integration in the vessel wall. Proliferation and differentiation of EPCs might result in a pool of ECs, which promotes and orchestrates vessel repair and local formation of new vessels via secretion of pro-angiogenic factors. Besides the so far discussed CD14-positive- and CD14-negative EPCs, there is evidence that mesenchymal stem cells and tissue resident stem cells might also differentiate into EPCs. However, their physiological relevance is still unclear (42-44).

Mobilization of EPCs from the bone marrow

Mobilization of EPCs in the bone marrow and their release into the peripheral circulation rely on a complex cascade of signaling events. The translocation of early cKit-positive progenitor cells prompts the release of EPCs from the quiescent bone marrow stromal niche into the vascular zone of the bone marrow. The migration of cKit-positive progenitor

cells is initiated by cleavage of membrane bound Kit ligand to its soluble form by matrix metalloproteinase 9 (MMP-9) (45). The expression of eNOS by local stromal cells seems to be required for the release of EPCs from the bone marrow into the circulation (46). Mice deficient in eNOS showed impaired neovascularization due to the diminished mobilization of EPCs. The defects in neovascularization in mice lacking eNOS were rescued by infusion of wildtype EPCs, but not by bone marrow transplantation. On the molecular level, eNOS may contribute to the activation of MMP-9 (46). Differentiation of primitive cKit-positive cells leads to the formation of bone marrow hemangioblasts, which are early progenitor cells of vascular and hematopoietic cells (47). Activation of VEGFR-2 by VEGF is crucial for further maturation of hemangioblasts and EPCs (48, 49). Besides VEGF, other cytokines are also important for maturation and mobilization of EPCs. Granulocyte-colony stimulating factor (G-CSF) and granulocyte monocyte-colony stimulating factor (GM-CSF) mobilize CD34-positive cells, including EPCs, in the bone marrow (50). Stem cell derived factor-1 (SDF-1) induces a rapid increase in the number of circulating EPCs similar to $VEGF_{165}$, whereas the mobilization of EPCs by angiopoietin-1 is delayed (51).

Neovascularization and re-endothelialization by EPCs

Numerous studies demonstrated that EPCs contribute to vascular homeostasis by inducing neovascularization in ischemic tissues and by stimulating re-endothelialization after vascular injury. EPCs from different sources, isolated by various protocols, have been shown to increase the capillary density and improve neovascularization at sites of ischemia. Kocher and coworkers were first to demonstrate that infusion of CD34-positive cells, isolated from the peripheral blood, stimulated the *de novo*-formation of vessels and the spreading of new vessels from the preexisting vasculature in experimental myocardial infarction (52). Apoptosis of myocytes in the peri-infarct area was reduced in treated animals, scaring of the myocardiac tissue was diminished, and cardiac function was improved. In parallel, Kawamoto

et al. showed that *in-vitro* differentiated, early-outgrowth EPCs accumulated at sites of ischemia and incorporated into foci of neovascularization in experimental myocardial infarction (53). EPCs were also found to improve neovascularization in experimental models of hind limb ischemia. After induction of hind limb ischemia, infusion of *ex-vivo* expanded EPCs into athymic mice or rats increased the capillary density and enhanced the local blood-flow resulting in a reduced rate of limb loss (54, 55). Of note, infusion of mature ECs did not improve vascularization in these models (54, 56). Besides their beneficial effects in the experimental models of myocardial infarction and hind limb ischemia, EPCs also stimulated the re-endothelialization in the rat model of balloon injury of the carotid artery and prevented neointimal thickening (15). Furthermore, EPCs re-endothelialization of vascular grafts was shown to be mediated by EPCs (39). EPCs from the peripheral blood, which had been expanded *in-vitro*, formed confluent monolayers on de-cellularized porcine iliac vessels. EPC seeded grafts exhibited nitric oxide mediated vascular relaxation and contractile activity comparable to native carotid arteries. These grafts remained patent throughout the observation period of 130 days, whereas non-seeded grafts occluded rapidly.

The encouraging results of EPCs in experimental models of tissue ischemia lead to the initiation of clinical trials. Tateishi-Yuyama and coworkers demonstrated that autologous transplantation of mononuclear cells, which contain EPCs among other mononuclear cell populations, can improve critical hind limb ischemia (57). Intramuscular injections of bone marrow-derived mononuclear cells into the calf significantly increased oxygen pressure, ankle-brachial index, and walking distance compared to saline treated control patients. In addition, the authors demonstrated that mononuclear cells derived from peripheral blood were significantly less effective compared to bone-marrow derived mononuclear cells. The number of CD34-positive cells, including EPCs, is about 500-fold higher in the bone marrow compared to the peripheral blood, a fact which might explain the markedly enhanced efficacy of the transplantation of bone marrow-derived mononuclear cells. Tateishi-Yuyama et al. did

not study whether the injected mononuclear cells integrated into the vessels after intramuscular injection. However, the expression of the proliferation marker Ki-67 was found to be increased in ECs, which might have been caused by the release of growth factors, such as VEGF, angiopoietin-1, bFGF, TNFα, IL-1β, and IL-6 from the injected cells (57).

Several randomized controlled clinical trails documented the efficacy of intracoronary injections of blood- or bone marrow derived EPCs for the treatment of acute myocardial infarction (58-61). Patients receiving EPCs had presented with enhanced tissue perfusion, higher ejection fractions, and an increased wall-motion. Of note, one trail was stopped because of a high rate of in-stent stenoses. Although increased in-stent re-stenosis was not reported in the other trials, the infusion of EPCs might potentially lead to uncontrolled neovascularization at unwanted tissue sites (9). Uncontrolled neovascularization could lead to increased vascularization of atherosclerotic plaques, thereby contributing to growth and destabilization of the plaques.

Due to their proliferative and stimulatory nature, EPCs might promote cancerogenesis, a serious side effect which is difficult to study in men. In models of tumor angiogenesis, however, EPCs stimulate the formation of new tumor vessels (62, 63). Of note, the number of cells originating from EPCs in the vessel wall tended to be higher in tumor models than in models for ischemia, suggesting a critical role of EPCs in tumor vascularization.

Molecular mechanisms involved in the neovascularization by EPCs

Neovascularization and re-endothelialization by EPCs are complex multi-step processes, requiring the chemoattraction of EPCs to sites of tissue ischemia or vascular injury, adhesion of EPCs, and their differentiation into mature ECs. The signaling cascades regulating these steps are still incompletely understood and in the focus of intense research. However, several important pathways have been uncovered during the last years.

Chemokines and cytokines: Early studies on mice deficient for VEGF, VEGF-R1, and VEGF-R2, demonstrated the essential role of VEGF-signaling for embryonic vasculogenesis (64-66). Similar to its importance during embryonic development, VEGF and its receptors are also critical for vasculogenesis in the adult. VEGF mobilizes EPCs within the bone marrow, and induces the differentiation of EPCs into ECs in *ex vivo* culture assays (54). Chemokine CXC-motif ligand (CXCL) 12 (SDF-1) also acts as a chemoattractive factor for EPCs and increases the release of EPCs from the bone marrow to the blood (49, 67-69). VEGF and CXCL 12 (SDF-1) are oxygen sensitive cytokines, which are induced by hypoxia (70). VEGF and CXCL12 act as molecular mediators to mobilize EPCs from the bone marrow and to guide them into ischemic tissues (49, 67-69).

Ligands of chemokine (motif CXC) receptor 2 (CXCR2) might also direct EPCs to sites of vascular repair and neoangiogenesis, and increase the adhesion of EPCs. EPCs express CXCR2, and incubation of EPCs with CXCR2 ligands such as CXCL1 (Groα) and CXCL7 (NAP-2) increases adhesion of EPCs to platelet-coated endothelial matrices *in vitro* and to sites of arterial injury *in vivo* (71). Further analysis showed that CXCR2-positive EPCs strongly adhered to injured arteries and that the CXCR2-positive EPCs predominantly expressed the monocytic marker CD14.

A number of CC-chemokines have been implicated in EPC homing. Using a transgenic mouse model of multi-step carcinogenesis, Spring et al. demonstrated that ECs, isolated from tumors, released CCL-2 (MCP-1), CLL-3 (MIP-1α) and CCL5 (RANTES), which directed a subset of CCR2- and CCR5-positive EPCs into the tumor vasculature (72). Inhibition of chemokine receptor signaling by the non-toxic inhibitor pentoxifylline strongly decreased the integration of EPCs into vessels, confirming the functional relevance of CC-chemokines for the integration of EPCs into the tumor vasculature (72).

In addition, pro-inflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin (IL)-1β, and IL-6, which are released by infiltrating leukocytes, might attract

CD14-positive progenitor cells, increase their adherence to the injured endothelium by a β_1-integrin dependent mechanism, and stimulate their differentiation into mature EC (73).

Adhesion molecules: Adhesion of EPCs to de-cellularized vessels might be mediated by adhesion to vitronectin via $\alpha_V\beta_3$- and $\alpha_V\beta_5$-integrins, since re-endothelialization *in vivo* was inhibited by the blocking of $\alpha_V\beta_3$- and $\alpha_V\beta_5$-integrins with cyclic arginine-glycine-aspartic acid (RGD) peptides (74). β_2-integrin is supposed to be particularly important for adhesion of EPCs and for transendothelial migration (75). Although EPCs overexpress mRNA for α_L-, α_M-, α_X- and β_2-integrins compared to HUVECs, only blocking antibodies against β_2-integrin inhibit adhesion and transmigration of EPCs and EPCs from β_2-integrin deficient mice are less capable of homing to ischemic tissues. Finally, incubation of EPCs with β_2-integrin activating antibodies stimulated neovascularization in a model of ischemic hind limb.

Activation of erythropoietin-producing human hepatocellular carcinoma (Eph) receptors might stimulate local adhesion of EPCs and increase their pro-angiogenic potential (76). Induction of EphB4 with an ephrin-B2-Fc chimeric protein enhanced the adhesion of EPCs by upregulating the expression of P selectin glycoprotein ligand-1 (PSGL-1). Inhibition of EphB4 signaling by siRNA or blockade of PSGL-1 with its receptors E selectin and P selectin abolished the stimulatory effects of ephrin-B2-Fc chimeric proteins and decreased the pro-angiogenic potential of EPCs in the nude mouse model of hind limb ischemia.

Matrix degrading enzymes: In an elegant serious of experiments, Urbich et al. demonstrated the importance of cathepsin-L for tissue invasion of EPCs during the formation of new blood vessels (56). Cathepsin-L is expressed at higher levels in EPCs compared to HUVECs, and its activity is significantly increased. Incubation of EPCs with the cathepsin-L inhibitor Z-FF-FMK or cystatin C, a general inhibitor of papain-like cysteine peptidases, reduced the invasiveness of EPCs and decreased their ability for neovascularization *in vivo*. Similar results were obtained with EPCs from mice lacking cathepsin-L. In contrast, inhibition of cathepsin-S, matrix metalloproteinases (MMPs), or elastases showed no effects

on the EPC function. The exceptional role of cathepsin-L for the formation of new vessels can also be demonstrated by overexpression of cathepsin-L in HUVECs and mature aortic endothelial cells. Infusion of mature ECs normally does not improve neovascularization. However, ectopic overexpression of cathepsin-L in HUVECs or aortic endothelial cells was shown to result in the generation of cells that promoted neovascularization at sites ischemia.

Histone deacetylation and Hox proteins: Global histone deacetylation plays a critical role in the *in vitro*-differentiation of embryonic stem cells (77). Differentiation of EPC also depends on the increased histone deacetylation (78). Chemical inhibitors of histone deacetylation block endothelial differentiation of EPCs without affecting adhesion or cell survival. The blocking of EPC differentiation might be mediated by the transcriptional regulator HoxA9. The expression of HoxA9 is increased in EPCs during endothelial differentiation and it regulates the expression of eNOS, VEGF-R2, and probably VE-cadherin. Blockade of histone deacetylation strongly reduced the expression of HoxA9. The importance of HoxA9 for neovascularization was further demonstrated using HoxA9 knockout mice, in which the number of EPCs was found to be significantly lower and which were characterized by impaired postnatal neovascularization. In addition, overexpression of HoxA9 partially reversed the reduction of EPC, provoked by histone deacetylase inhibitors (78). HoxB5, another member of the Hox family of homeodomain transcription factors, also appears to be involved in the *in vitro*-differentiation of embryonic progenitor cells towards the endothelial lineage (79). HoxB5 regulates the expression of VEGF-R2. Therefore, overexpression of HoxB5 in murine embryonic stem cells increased proliferation and stimulated the formation of blood vessels. Finally, the related homeobox gene Hex (or Prh) plays a decisive role in early stages of EPC differentiation (80). Hex is preferentially expressed in hemangioblasts, the primitive progenitor cells of ECs and hematopoietic cells, and it is downregulated during the terminal differentiation into EC. Embryonic stem cells deficient of Hex differentiated normally into hemangioblasts *in vitro*, but further

differentiation of the Hex$^{-/-}$ hemangioblast into ECs, and especially into hematopoitic cells, was significantly reduced.

Vasculogenesis in the pathophysiology of SSc

Recent reports have demonstrated a role for EPCs in the pathogenesis of several rheumatic disorders, including SSc, RA, and SLE (Figure 3). Reduced capillary density and an irregular, chaotic architecture of the capillary network are hallmarks of SSc (3). These changes result in a decreased capillary blood flow, causing a lack of nutrients and severe tissue hypoxia in the affected organs (70). Despite the strong upregulation of pro-angiogenic factors such as VEGF, sufficient vessel formation does not occur (3, 22, 81). Defective angiogenesis and increased apoptosis of mature ECs had been thought to be exclusively responsible for the vascular alterations in SSc. However, several studies, which were published over the last years, suggested that vasculogenesis might also contribute to the vasculopathy in SSc.

Kuwana et al. first investigated whether vasculogenesis might be affected in patients with SSc (22). They defined EPCs as circulating CD34-, CD133-, and VEGFR2-positive mononuclear cells. Using this definition, the absolute numbers of EPCs were found to be lower in patients with SSc than patients with RA and healthy subjects. Comparison of patients with RA and healthy subjects revealed no differences in EPC counts. Numbers of EPCs were stable beyond a 3-month period in all subjects. In SSc patients, EPC counts did not correlate with disease subset, disease duration, or the modified Rodnan Skin Score. However, the numbers of EPCs were lower in SSc patients with pitting scars, and active fingertip ulcers. Additional *in vitro*-experiments demonstrated an impaired differentiation capacity of EPC, which were isolated from the peripheral blood of SSc patients. After five days under standard culture conditions, only few EPCs from patients with SSc expressed the EC marker von-Willebrand factor, indicating impaired differentiation of early outgrowth EPCs into mature

ECs. In a subsequent clinical trial, the same research group could show an increase of circulating EPCs in SSc patients by treatment with atorvastatin, suggesting a beneficial effect of HMG-CoA-reductase inhibitors on vasculogenesis in patients with SSc (20). However, even under treatment, the number of EPCs did not reach the levels of healthy individuals. Furthermore, the capacity of EPC to differentiate into mature EC could not be restored by the application of atorvastatin.

In a follow-up study with increased patient numbers, del Papa et al. identified EPCs via the same cell surface markers as Kuwana et al., i.e. CD34, VEGFR2 and CD133 (19). In contrast to the previous study, the numbers of EPCs were found to be significantly increased in the blood of patients with SSc. Further subgroup analysis revealed a negative correlation between EPCs count and disease duration. Based on this finding, the authors suggested that differences in disease duration might account for the discrepancy between their results and the findings of Kuwana et al. Apart from disease duration, no correlations between EPC counts and clinical parameters, including digital ulcers, could be observed. In addition to the number of circulating EPCs, del Papa et al. determined EPC counts in the bone marrow of SSc patients and healthy individuals (19). The number of CD133-positive cells was significantly decreased in the bone marrow of patients with SSc. The ability of bone marrow-derived, CD133-positive cells to differentiate into endothelial cells *in vitro* was found to be reduced in SSc patients. Finally, the number and the size of colonies were reduced and cells from SSc patients showed morphological signs of cellular senescence. Of note, the same research group has recently reported that the HMG-CoA reductase inhibitor simvastatin increased the levels of EPCs exclusively in SSc patients with hypercholesterolemia, but not SSc patients with normal serum cholesterol levels (18).

Allanore et al. assessed EPC counts in the whole blood in patients with SSc, OA, and RA, and analyzed potential correlations with clinical parameters (16). In contrast to the prior studies, the authors measured the numbers of CD34- and CD133-double-positive cells, but did

not analyze the expression of VEGFR2. The numbers of CD34- and CD133-positive cells were increased in patients with SSc compared to patients with OA. However, EPC counts in SSc patients were lower than in RA. In patients with SSc, CD34/CD133 counts increased in parallel with the European disease activity score. In another study, Allanore et al. used VEGFR-2 and Lineage markers (Lin) as additional markers to identify EPCs. Furthermore, dead cells could be excluded with the use of the viability marker 7-aminoactinomycin (7AAD). EPCs were thus defined as Lin-/7AAD-/CD34+/CD133+/VEGFR-2+ cells. Again, patients with SSc displayed higher circulating EPC counts than healthy subjects (17). Lower EPC counts in SSc patients were associated with higher Medsger's severity scores and with digital ulcers.

Most recently, Zhu and coworkers found decreased EPC counts in different subsets of SSc patients. Patients with limited and diffuse SSc, as well as patients with recent onset and late-stage disease all had reduced numbers of EPCs compared to healthy individuals. EPC counts and EPC function were analyzed by FACS staining for CD34/CD133/VEGFR-2/7AAD and colony-forming unit assays, respectively (23). In addition, the authors proposed a new mechanism, which might explain the reduced EPC counts in their study. They observed an increased rate of apoptosis in freshly isolated EPCs from patients with SSc. Addition of sera from SSc patients to cultured EPCs from healthy volunteers mimicked these findings and substantially induced apoptosis of EPCs. The pro-apoptotic effects of SSc sera were abolished by depletion of the IgG fraction, suggesting the presence of anti-EPC autoantibodies in the serum of SSc patients. Further experiments revealed, that the addition of SSc sera inhibited the phosphorylation of Akt, which prevented the degradation of the forkhead transcription factor FKHRL (FOXO3a). Accumulation of FOXO3a upregulated in turn the expression of the pro-apoptotic protein Bim. Knockdown of FOXO3a and Bim via siRNA strongly reduced the pro-apoptotic effects of SSc serum. (23).

Whether EPC counts are altered in the peripheral blood of SSc patients is currently controversial. The differences in EPC counts between the different studies might be explained by the following reasons: (I) use of different combinations of surface markers resulting in the analysis of different subsets of EPCs; (II) different handling of dead EPCs; (III) differences in the prevalence of cardiovascular risk factors and in the use of medications; (IV) differences in the mean disease duration and severity, which is supported by the inverse correlation of EPC with disease duration and disease activity in some studies (17, 19). In contrast to the number of EPC in patients with SSc, functional defects of EPCs in the peripheral blood as well as in the bone marrow have consistently been reported (19, 21, 22) and indicate a critical role of EPCs in the pathogenesis of SSc, even though numbers of circulating EPC might vary (Table 1).

Drugs that increase the number of EPCs and that may at least in part restore vasculogenesis, might offer new therapeutic options for the treatment of vascular disease in SSc. In this context, patients with SSc might potentially benefit from the treatment with HMG-CoA-reductase inhibitors. Injection of *in-vitro*-amplified, autologous EPCs might be another treatment option for patients with severe vascular disease. In regard to the functional defects of EPCs in patients with SSc, the efficacy of these approaches might, however, be limited in SSc. Further identification of the molecular mechanisms underlying these defects is needed in order to develop specific treatment options and restore functional vasculogenesis in patients with SSc.

EPCs in patients with RA

A growing body of evidence suggests a critical role of impaired vasculogenesis in the pathogenesis of RA, including both reduced numbers and altered functions of EPCs, (Table 1). Grisar and coworkers demonstrated a reduction of circulating EPCs in patients with RA compared to healthy controls (26). As shown by FACS analysis and by colony-forming unit

assays, the number of CD133-, CD34-, andVEGF-R2-positive EPCs was significantly reduced in RA patients with active disease, reflected by a DAS28 ≥ 3.2. Upregulation of erythropoietin and VEGF were insufficient to increase numbers of circulating EPCs in patients with RA. However, no significant differences in EPC numbers and functions could be observed between RA patients with low disease activity (DAS28 < 3.2) and healthy controls. Cardiovascular risk factors influencing the number of EPCs were equally distributed over three groups. Of note, the number of circulating CD133-, CD34- and VEGF-R2-positive EPCs correlated inversely with the disease activity as assessed by the DAS28, in particular with the number of swollen and tender joints, but not with the ESR and global assessment (26). Conventional DMARDs and low doses of glucocorticosteroids had no effects on the levels of EPCs. However, higher doses of glucocorticosteroids increased the numbers of circulating EPCs *in vivo.* Consistent with the increased EPC counts *in vivo,* the numbers of colonies in the colony-forming unit assay were elevated (25). TNFα-antagonist also increased the number of circulating EPCs and had beneficial effects on the differentiation and the adhesion of EPCs (24, 26). Grisha and coworkers reported that EPC counts in patients with RA receiving TNFα-inhibitors were similar to the EPC levels of healthy controls, even when the disease was still active (26). Finally, the number of EPCs was lower in patients with high levels of TNFα compared to those with low serum concentrations of TNFα, which is consistent with the beneficial effects of TNF α-inhibitors.

Silverman et al. reported a pronounced recruitment of putative EPCs to the inflamed synovium in several animal models of RA (30). In the model of collagen-induced arthritis, numerous cells expressing CD117, a stem cell marker that is found on EPC, accumulated in inflamed synovial tissues. In addition, exogenously administered EPCs accumulated preferentially in inflamed joints, but not in control joints, when applied in a model of antibody induced arthritis. Similar results were obtained with human EPCs in the chimeric SCID mouse/ human synovial tissue model. The numbers of CD133- and CD34-positive cells were

increased by approximately 3-fold in RA synovial tissue compared to normal synovial tissue. Silverman and coworkers also observed that blocking antibodies against VCAM-1 and α4-integrin potently inhibited binding of EPCs to activated synovial fibroblasts, suggesting a crucial role of these two receptor molecules for the adhesion process of EPC (30).

In addition to reduced EPC counts, EPC function also seems to be altered in patients with RA (27). Migration of EPCs towards VEGF was reduced in a modified Boyden chamber assay. Furthermore, the adhesion of EPCs to TNFα-treated ECs was impaired compared to EPCs from healthy donors. However, adhesion to resting ECs and to the extracellular matrix proteins collagen type IV, fibronectin and laminin was not affected (29). Furthermore, the generation of endothelial cells from CD34-positive bone-marrow cells after *in-vitro*-stimulation with Kit-ligand and GM-CSF cells was significantly increased in RA compared to OA and healthy controls (28). Interestingly, the generation of ECs from CD34-positive bone marrow cells correlated with the vessel density of the synovium.

Together, these results suggest that many EPCs might get trapped in the synovial tissue in patients with RA, contributing to the increased formation of new vessels in the inflamed joint (82). This might result in a decreased number of circulating EPCs. The bone marrow might not be able to compensate for the emigration of EPCs into the synovial tissue. TNFα seems to be a key player in the impaired vasculogenesis in patients with RA, as it attracts EPCs to the inflamed synovial tissue and induces apoptosis in circulating EPCs. However, the functional defects of EPCs with decreased adhesion and reduced migration of EPCs are not explained by this hypothesis, and therefore have to be investigated by further studies. Although low EPCs counts have not been established as independent risk factors for cardiovascular disease in RA yet, the reduced number along with the functional defects of circulating EPCs might contribute to the increased cardiovascular morbidity and mortality in patients with RA.

Defective vasculogenesis in SLE

SLE is associated with premature and accelerated cardiovascular disease which is not explained by traditional risk factors, such as hypertension, hyperlipidemia, or hyperglycemia. Several groups independently reported reduced numbers of EPCs in the peripheral blood of patients with SLE (Table 1) (31-34). Although triple staining for VEGF-R2, CD34 and CD133 was not performed in any of these studies, double staining for VEGF-R2 and CD133, or for CD34 and CD133 consistently showed a reduction of putative EPCs in patients with SLE, even in inactive stages of the disease. Similar to SSc and RA, EPC may be altered in SLE, as shown by colony forming assays (31, 33). EPCs from patients with SLE also had a reduced capacity to differentiate into mature ECs *in vitro*. Uptake of LDL, binding of agglutinin I, and expression of vWF were found to be reduced. Furthermore, EPCs from patients with SLE released less VEGF and hepatocyte growth factor compared to EPCs from healthy donors (31). EPCs from SLE patients might also be more susceptible to apoptosis, which is indicated by increased levels of caspase-9 protein (33). In SLE, interferon-α (IFNα) may play a central role for the perturbed vasculogenesis, as EPC counts correlated inversely with IFNα serum levels. In this context, the expression of the interferon inducible gene MX-1 in PBMCs was also inversely correlated with EPC counts (32). IFNα induced apoptosis in EPCs and inhibited differentiation into mature ECs. Of note, EPCs themselves produced increased amounts of IFNα *in vitro* after isolation of the peripheral blood of patients with SSc (31). Inhibition of IFNα or type I IFN receptor by blocking antibodies prevented the functional defects and restored a normal EPC phenotype *in vitro*. Similarly, inhibition of Toll-like receptors (TLR) 7 and 9, which are known to regulate the induction of IFNα, had beneficial effects (31). In summary, defective vasculogenesis with reduced numbers and altered functions of EPCs may contribute to the increased cardiovascular risk observed in patients with SLE. Inhibition of IFNα, TLR7 and TLR9 might restore vascular remodeling by EPCs and therefore reduce the cardiovascular risk of patients with SLE.

EPCs for the repair of damaged vessels in vasculitis

De Groot and coworkers analyzed, whether EPCs play a role for the repair of damaged endothelium in anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitis (35). In patients with active, untreated disease, the number of EPCs as assessed by uptake of LDL and binding of agglutinin-I after four days in culture did not differ from healthy volunteers. No correlations with leukocyte counts, thrombocyte counts or kidney functions were observed. With the induction of disease remission, EPC counts increased significantly by almost two-fold. In contrast, circulating ECs were found to be increased during active disease and decreased upon induction of remission. Thus, EPCs might represent markers for vascular regeneration, whereas circulating ECs might reflect disease activity in vasculitides.

Summary and conclusion

Vasculogenesis is not restricted to the embryonic development, but contributes to the vascular homeostasis and integrity in the adult. EPCs induce neovascularization of ischemic tissues and stimulate the repair of damaged vessels by the release of angiogenic factors, integration in the vessel wall, and differentiation into mature ECs. In fact, several cardiovascular risk factors are associated with a reduced number of circulating EPCs, and EPC counts are inversely correlated with the risk for major cardiovascular events, such as myocardial infarction and stroke. So far, EPCs have already been implicated in the vascular pathogenesis of several rheumatic diseases. Reduced levels of circulating EPCs were found in patients with RA and SLE. In SSc, however, the number of EPCs is still unclear. In addition, altered EPC functions with increased apoptosis, decreased production of angiogenic factors, reduced adhesion, and impaired differentiation capacity have been described in patients with RA, SLE, and SSc. Together, these findings suggest that defects in vasculogenesis might

contribute to the increased cardiovascular risk in patients with RA and SLE, and might play a critical role in the vasculopathy observed in patients with SSc.

However, a major problem in EPC research is the lack of clear definitions and common protocols for the isolation and identification of EPCs (83). Due to this fact, comparison of the results of different studies is often difficult, since different EPC isolation procedures might yield different subpopulations and maturation steps of EPCs. The lack of clear definitions and protocols might also explain differences in EPC counts observed in SSc. Guidelines for isolation, identification, quantification and culturing of EPCs, e.g. those compiled by the EUSTAR group (83), will help to unify research within the field and allow better comparison between different studies. Large amounts of blood are needed for the isolation of sufficient numbers of EPCs and protocols for culture and propagation of EPCs are complicated. Consequently, evidence of altered EPC functions in patients with rheumatic diseases is still limited.

Despite great progress in EPC research, many questions remain open. Do different subpopulations of EPCs have distinct functions during vasculogenesis? What is the physiological relevance of mesenchymal stem cells and tissue resident stem cells that can differentiate into EPCs? Although key molecules have been identified, the molecular mechanisms leading to the formation of new vessels and the repair of preexisting vessels are only incompletely understood.

In the field of rheumatology, the levels and function of EPCs have been investigated in SSc, RA, SLE, and vasculitides. However, a number of research questions are still pending: What are the reasons for the observed functional defects of EPCs? Do patients benefit from injections of autologous EPCs or from treatment with drugs that increase the number of circulating EPCs (e.g., HMG-CoA reductase inhibitors) despite functional defects? Are there any long-term side effects of therapeutic injection of EPCs, such as enhanced plaque formation or increased risk for cancer due to uncontrolled neoangiogenesis? These issues

need to be addressed in further studies and might help to improve our understanding about the vascular pathogenesis of rheumatic diseases.

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Overview about the roles of EPCs in the pathogenesis of rheumatic diseases	
SSc	• altered numbers of circulating EPCs in peripheral blood • decreased numbers of CD133 positive precursor cells in the bone marrow • impaired maturation of early outgrowth EPCs into ECs • increased autoantibody mediated apoptosis of EPCs • potential correlation with disease activity (European disease activity score and Medsger's severity score) • potential inverse correlation with disease duration
RA	• reduced EPC counts in peripheral blood in patients with moderate and severe disease activity (DAS28 ≥ 3.2) • functional defects in EPCs with reduced migration towards VEGF and impaired adhesion to activated ECs • pronounced recruitment of EPCs into inflamed joints • inverse correlation with disease activity (DAS28 and swollen joint counts) • TNFα antagonists increase EPC counts (independent from their effects on disease activity)

SLE	• reduced numbers of circulating EPCs in the blood • impaired differentiation of EPCs into ECs • decreased release of angiogenic growth factors such as VEGF and HGF • increased susceptibility to apoptosis • inverse correlation between serum levels of IFNα and EPC counts
Vasculitis	• EPC counts as potential markers for vessel regeneration with increased EPC counts during remission

Table 1: Overview about the different roles of EPCs in the pathogenesis of SSc, RA, SLE and vasculitis