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REVIEW

The role of the renin–angiotensin–aldosterone system in cardiovascular progenitor cell function

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ABSTRACT

Intervention in the RAAS (renin–angiotensin–aldosterone system) is one of the leading pharmacotherapeutic strategies, among others, used for the treatment of cardiovascular disease to improve the prognosis after myocardial infarction and to reduce hypertension. Recently, regenerative progenitor cell therapy has emerged as a possible alternative for pharmacotherapy in patients after myocardial infarction or ischaemic events elsewhere, e.g. in the limbs. Angiogenic cell therapy to restore the vascular bed in ischaemic tissues is currently being tested in a multitude of clinical studies. This has prompted researchers to investigate the effect of modulation of the RAAS on progenitor cells. Furthermore, the relationship between hypertension and endothelial progenitor cell function is being studied. Pharmacotherapy by means of angiotensin II type 1 receptor antagonists or angiotensin-converting enzyme inhibitors has varying effects on progenitor cell levels and function. These controversial effects may be explained by involvement of multiple mediators, e.g. angiotensin II and angiotensin-(1–7), that have differential effects on mesenchymal stem cells, haematopoietic progenitor cells and endothelial progenitor cells. Importantly, angiotensin II can either stimulate endothelial progenitor cells by improvement of vascular endothelial growth factor signalling, or invoke excessive production of reactive oxygen species causing premature senescence of these cells. On the other hand, angiotensin-(1–7) stimulates haematopoietic cells and possibly also endothelial progenitor cells. Furthermore, aldosterone, bradykinin and Ac-SDKP (*N*-acetyl-Ser-Asp-Lys-Pro) may also affect progenitor cell populations. Alternatively, the variability in effects of angiotensin II type 1 receptor and angiotensin-converting enzyme inhibition on cardiovascular progenitor cells might reflect differences between the various models or diseases with respect to circulating and local tissue RAAS activation. In the present review we discuss what is currently known with respect to the role of the RAAS in the regulation of cardiovascular progenitor cells.

Key words: aldosterone, angiotensin, cardiovascular, progenitor cell, renin.

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibtor; Ac-SDKP, *N*-acetyl-Ser-Asp-Lys-Pro; Ang, angiotensin; AT_1 receptor, AngII type 1 receptor; AT_2 receptor, AngII type 2 receptor; BK, bradykinin; BM, bone marrow; CAD, coronary artery disease; CHF, chronic heart failure; eNOS, endothelial NO synthesis; EPC, endothelial progenitor cell; GM-CSF, granulocyte/macrophage colony-stimulating factor; HIF-1α, hypoxia-inducible factor-1α; hMSC, human mesenchymal stem cell; HSC, haematopoietic stem cell; LDL, low-density lipoprotein; MI, myocardial infarction; MMP, matrix metalloproteinase; MNC, mononuclear cell; MSC, mesenchymal stem cell; pRb, retinoblastoma protein; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species; SDF-1, stromal-cell-derived factor-1; SMC, smooth muscle cell; TNF-α tumour necrosis factor-α; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; VSMC, vascular SMC.

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INTRODUCTION

CAD (coronary artery disease) and CHF (chronic heart failure) are the leading causes of morbidity and mortality all over the world. Despite the improvements brought about by classical pharmacotherapy, the limited success in reducing mortality in these diseases [1] has prompted the development of novel therapeutic strategies, most notably stem cell therapy.

Stem cell therapy research focuses mainly on cardiac repair in CHF, i.e. replacement of scar tissue with cardiomyocytes and stimulation of neovascularization, and on revascularization of ischaemic peripheral tissues such as skin and skeletal muscle of limbs. In the case of CHF, repair of both contractile myocardial tissue and the coronary vascular bed would of course be ideal, but neovascularization is the most commonly addressed strategy for two reasons. First, it is based on the principle that hypertrophy of surviving myocardium combined with insufficient formation of new capillaries leads to reduced capillary supply, which causes a so-called 'relative' myocardial ischaemia that in turn promotes myocardial hypertrophy, contractile dysfunction and sustained ischaemia. Neovascularization might interrupt this vicious circle and improve the prognosis in CHF without the need to restore the scar tissue. Secondly, the isolation and use of EPCs (endothelial progenitor cells), which play an important role in neovascularization and cell therapy, seems relatively easy, and the knowledge on the pharmacological properties of these cells is rapidly growing [2].

Although stem-cell-based therapy may provide a promising approach for treatment of CHF, transplantation of autologous BM (bone marrow) stem cells for cardiogenesis or neovasculogenesis yielded inconsistent results in clinical trials [3–6]. Nevertheless, it is anticipated that with the growing knowledge about progenitor cells and their reaction to pharmacotherapy the chances of reaching an optimal clinical benefit will increase [2].

Pharmacotherapeutical modulation of the RAAS (renin–angiotensin–aldosterone system) is one of the most important treatment modalities in CHF, and the knowledge of the role of the RAAS in the regulation of HSCs (haematopoietic stem cells) and cardiovascular progenitor cells is steadily growing. The effect of Ang (angiotensin) II, the best-studied hormone within the RAAS, on HSCs has been discussed previously [7,8]. HSCs and BM-derived cardiovascular progenitors share a common progeny [9], and it may not come as a surprise that RAAS modulation affects BM-derived cardiovascular progenitor cell function as well. Intriguingly, local angiogenic progenitor cells have been detected in the adventitia and subintimal layer of large arteries [10,11] and in the heart [12], which might serve as the local vasculogenic cells instead of the BM-derived EPCs. Until now the pharmacological features of these local progenitor cells remain largely unexplored, although there is evidence that during aging the RAAS is activated in the local cardiac progenitor cell pool [13]. Considering the lack of studies dealing with the role of the RAAS and modulation thereof in local progenitor cell regulation, the present review will almost exclusively discuss BM-derived cardiovascular progenitor cells, with an emphasis on EPCs.

Besides the fact that progenitor cells are modulated and contribute to revascularization of ischaemic tissues, the function of these cells is deteriorated in various models of hypertension. Hypertension is one of the prime risk factors for CAD and CHF, and pharmacotherapeutical modulation of the RAAS is one of the most important antihypertensive interventions. Most of the knowledge of the role of the RAAS in progenitor cell function comes from studies in models of hypertension that will therefore represent an important part of the work cited herein.

BM-DERIVED EPCs: CHARACTERISTICS AND MODE OF RECRUITMENT

EPCs are of haematopoietic origin and are possibly derived from haemangioblasts. They are found at various stages of differentiation that are featured by the appearance of specific markers. This is often a source for discussion about whether in certain studies (also studies cited herein) the progenitors that are used for cell therapy or that are quantified to reflect the angiogenic potential are truly EPCs. In their fully developed state as progenitor cells capable of endothelial outgrowth and tube or vascular sprout formation, EPCs are characterized as c-Kit$^+$/Sca-1$^+$Lin$^-$/CD133$^+$/CD34$^+$/VEGFR-2 [VEGF (vascular endothelial growth factor) receptor-2]$^+$/CXCR-4 (CXC chemokine receptor-4)$^+$ cells. Apart from the change of markers that occurs during differentiation, a detailed characterization of EPCs would generate practical problems if one is to perform extensive pharmacological studies. Therefore in many studies a simpler cell-typing procedure is used, which suffices as long as effects on EPCs are represented. A commonly used method is the combination of a stem-cell marker (e.g. c-Kit or Sca-1) with an endothelial marker (e.g. VEGFR-2). In many studies other cell-typing methods are chosen, such as the capability of the selected cells to form sprouting colonies, or staining for lectin combined with uptake of acetylated LDL (low-density lipoprotein) [2]. In all of the studies cited in the present review, simplified cell-typing methods were employed.

Although EPCs can be found in the BM and blood at any given time point, the active recruitment and augmentation of EPCs critically depends on events that lead to the release of signalling compounds. We will discuss here ischaemic events. Ischaemic injury provokes the increase in angiogenic factors, most notably HIF-1α (hypoxia-inducible factor-1α), SDF-1 (stromal-cell-derived factor-1) and VEGF, which increase EPC recruitment through

eNOS [endothelial NOS (NO synthase)] activation [14,15]. An important step in the recruitment of EPCs is activation of MMP (matrix metalloproteinase)-9, which increases the cleavage of sKitL (soluble kit ligand; or stem cell factor), thus stimulating the migration of EPCs to proliferation niches within the BM and, subsequently, the mobilization from BM niches to the peripheral circulation [16,17]. Mobilization starts with shedding of the progenitor cells from endosteal or BM stromal niches to the vascular sinusoidal of the BM. The subsequent transmembrane movement to the blood compel BM EPCs to differentiate into circulating EPCs [18].

Studies on the mechanisms of recruitment of cardiovascular progenitor cells are represented by a considerable body of literature. A rather small part focuses on the role of the RAAS, and within that group of publications the attention has almost exclusively been paid to EPCs. AngII has beneficial, as well as deleterious, effects on BM-derived progenitor cells, whereas inhibition of ACE (angiotensin-converting enzyme) affects the activity of a variety of peptide hormones. This explains the paradoxical effects of RAAS intervention of progenitor cells, as will be discussed in the remainder of the present review.

THE RAAS AND ITS KEY PLAYERS

The RAAS contributes significantly to the development and progression of CAD and CHF, and is one of the best-studied hormone systems in experimental and clinical cardiovascular research. The pharmacodynamic and pharmacokinetic pathways in the RAAS that play a role in cardiovascular disease have been intensively studied and reviewed. The production of bioactive angiotensins starts with the cleavage of the prohormone angiotensinogen by renin to form the inactive precursor peptide AngI. AngI is converted by ACE into its active metabolite AngII. AngII exerts its hypertensive, hypertrophic, pro-inflammatory, profibrotic and prothrombotic effects through AT_1 (AngII type 1) receptors, whereas AT_2 (AngII type 2) receptors mostly mediate contrary effects. Another player within the RAAS is the heptapeptide Ang-(1–7). Ang-(1–7) can be formed from both AngI or AngII, involving various enzymes such as ACE, ACE2 and neutral endopeptidase. Ang-(1–7) is considered to be the endogenous counter-regulator of AngII, and displays protective and therapeutic properties [19,20].

AT_1 receptor antagonists and ACEIs (ACE inhibitors) are prescribed in the clinic for various cardiovascular indications [21], including CHF and hypertension. Their beneficial effects on endothelial repair, angiogenesis, inflammation and fibrosis might involve common progenitor cell types, however, both types of RAAS modulation involve very different signalling pathways as will be outlined here. We begin with explaining the role of AngII and its receptors.

ANGII AND ITS RECEPTORS AT_1 AND AT_2

The effect of AngII on EPCs and on angiogenesis has been well-explored. AngII has a dual effect on angiogenesis because it promotes cellular aging (senescence), but meanwhile improves angiogenesis. Effects on both adult cells as well as on EPCs play a role in this paradox, and herein we will mainly focus on EPCs. In addition, effects of AngII have also been observed in vascular smooth muscle and adipocyte progenitors.

Effects on EPCs

AT_1 receptor antagonism is a widely used pharmacotherapeutical modality, notably in patients with hypertension, MI (myocardial infarction) or heart failure. Several studies show a beneficial effect of AT_1 receptor antagonists on EPCs. In relation to the clinical application of AT_1 receptor antagonists, Bahlmann et al. [22] studied the effects on EPCs in patients with Type 2 diabetes. A 12-week treatment with olmesartan or irbesartan selectively increased the EPC subpopulation, but not the $CD34^+$ haematopoietic cells. In agreement with the study in diabetic patients, in salt-loaded stroke-prone hypertensive rats blood EPC number and function was decreased, whereas a 2-week candesartan treatment restored normal EPC levels and function [23]. In further agreement with these two studies, a 2-week losartan treatment restored the angiogenic function of BM MNCs (mononuclear cells) in spontaneously hypertensive rats [24]. In addition, it was shown in Wistar rats that 2 weeks of AngII infusion led to a lowering of EPCs that could be reversed by valsartan treatment [25]. Moreover, it was observed that the angiogenic function of EPCs in the ischaemic hind leg was diminished after AngII infusion [26]. Importantly, the effect in rats was accompanied by an increase in EPC senescence. Therefore prolonged exposure to AngII and AT_1 receptors signalling induces senescence and, hence, impairment of EPCs.

Senescence, or cellular aging, is a situation during which the cell is arrested in a G_0/G_1-phase due to activation of a DNA-damage response. The response to DNA damage starts with activation of the p53 protein and subsequent activation of p16, p21 or p27 cell-cycle proteins. Activation of these proteins leads to hypophosphorylation of the pRb (retinoblastoma protein) through modulation of cyclin-dependent kinase activity. pRb hypophosphorylation suppresses E2F-mediated initiation of the cell cycle [27–29], resulting in G_0/G_1 arrest. Senescent endothelial cells display functional alterations that are believed to play an important role in atherogenesis, as extensively reviewed elsewhere [30,31]. The DNA-damage signal that leads to senescence is provoked either by critical shortening of telomeres, the chromosal ends that shorten during each cell division, or by DNA damage in the remainder of the genome. DNA damage can take place through chemical interaction

with ROS (reactive oxygen species), and indeed exposure of endothelial cells to ROS induces senescence [32]. It is worth noting that the observed beneficial effect of candesartan and losartan on progenitor cell angiogenic function in hypertensive rats was mimicked by antioxidant treatment and was associated with a normalization of increased expression of NAD(P)H oxidase subunits in stroke-prone hypertensive rats [23,24]. Similarly, in cultured EPCs, AT_1 receptor activation induced senescence [33]. This stimulation of senescence took place due to increased ROS/peroxynitrate production involving activation of the NAD(P)H oxidase pathway, as witnessed by the up-regulation of the NAD(P)H oxidase component $gp91^{phox}$. It was accompanied by a down-regulation of the chromosomal telomere-extending enzyme telomerase. It is therefore tempting to speculate that AngII induces telomeric damage in EPCs through NAD(P)H-oxidase-mediated ROS production and a concomitant inhibition of repair by telomerase. However, in adult VSMCs [vascular SMCs (smooth muscle cells)] it was shown that AngII-induced senescence that is mediated by ROS does not necessarily involve telomeres or telomerase activity, and therefore could be associated with global genomic DNA damage rather than telomeric damage [34]. The question as to which of the two mechanisms is of importance in AngII-induced senescence of EPCs remains to be explored.

AT_2 receptors might play an opposite role in EPC senescence. It has been shown that genetic ablation of the AT_2 receptor in mouse aorta and cultured VSMCs enhances AT_1-receptor-mediated oxidative stress and senescence [35]; however, this remains to be confirmed for EPCs.

Apart from mediating EPC senescence, AT_1 receptors might also negatively influence EPC populations through their effect on differentiation of BM MNCs to EPCs. In cultured BM MNCs of spontaneous hypertensive rats, AT_1 receptor blockade increased the percentage of EPCs after a 7-day culture period [24]. Furthermore, it was observed that chronic AT_1 receptor blockade *in vivo* increases EPCs but not $CD34^+$ HSCs in diabetic patients [22], and therefore the results suggest that AT_1 receptor stimulation might hamper differentiation of HSCs into EPCs.

In contrast with the observed senescence and the putative hindrance of EPC differentiation, AngII stimulates the angiogenic function of adult endothelial cells through AT_1 receptors. This process involves VEGF-induced eNOS activation, a pathway that also mediates improvement in angiogenic EPC recruitment in the ischaemic hind limb model by genetic overexpression of SDF-1α, a well-known EPC stimulant [36]. Since EPCs take part in angiogenesis together with adult cells, the direct effect of AngII on EPC proliferation was explored. Both in cultured BM and blood MNCs of rabbits fed with a high-cholesterol diet [37], and in blood MNCs of healthy human volunteers [38], AngII alone does not stimulate EPC proliferation. In women, however, AngII alone can stimulate EPC proliferation in the luteal period of the menstrual cycle, disappearing in the follicular phase and during the course of pregnancy, while strongly re-appearing in pre-eclampsia [39].

Insight into the mechanism of how AngII could promote proliferation of EPCs comes from the study in healthy volunteers [38] (the gender was not reported in this study). Although being without effect on itself, AngII increased VEGF-induced proliferation of human EPCs. Network formation by EPCs was only modestly altered by either AngII or VEGF alone, but both together acted synergistically. It was shown that the synergistic effect of AngII with VEGF in EPCs is caused by AT_1- receptor-mediated up-regulation of the VEGF receptor KDR (kinase insert domain-containing receptor). Therefore AngII can stimulate EPC proliferation as well as angiogenic function through improvement in VEGF signalling. Intriguingly, the stimulatory effect of AngII on EPCs seems to involve ROS signalling. Unilateral clipping of the kidney in mice (2K1C model, a widely used renin-dependent renovascular model of hypertension) caused an increase in EPCs 3 and 7 days after clipping, an effect that was blocked by the AT_1 receptor antagonist telmisartan [40]. EPC recruitment was mimicked by a 5-day AngII infusion. The EPC recruitment after clipping or AngII infusion was accompanied by an increase in various cytokines, and both of these events were prevented by genetic knockout of the NAD(P)H component $p47^{phox}$. At 21 days after clipping, EPC levels were decreased again. Therefore it appears that in the short term RAAS activation activates progenitor cell repair function through the normal physiological AT_1 receptor/NAD(P)H signal-transduction pathway, a process that is possibly started by the release of cytokines; however, chronic AngII/ROS signalling results in senescence, as observed after 2 weeks of AngII infusion [25].

Considering the paradoxical effect of AngII and AT_1 receptor activation on EPCs, it will not come as a surprise that both animal, as well as human, studies display contrasting results with respect to *in vivo* effects of AT_1 receptor antagonism on EPCs. In spontaneous hypertensive rats with ischaemic hind limbs and decreased EPCs as compared with normotensive rats, 3 weeks of losartan was without effect on circulating EPC numbers, although it increased EPC number during culture [24]. Also in patients with essential hypertension, AT_1 receptor blockade did not change EPC levels [24,41]. As a further contrast, in patients with CAD AT_1 receptor blockade was associated with a decrease in EPCs [42]. In addition, in patients that underwent kidney transplantation, AT_1 receptor antagonists decreased EPCs [43]. These contrasting findings might relate to methodological differences, such as the way EPCs were defined or the study models (type of study, co-medication

against graft rejection, etc.) and statistical protocols that were used. On the other hand one could also argue that the outcome depends on the degree and locus of RAAS activation, which determine whether AngII exerts either a physiological function or a pathological effect.

As an example for the importance of the degree of RAAS activation, one can reconsider the aforementioned difference between short-term and long-term AngII infusion [25,40]. In addition, one could place the effectiveness of AT_1 receptor blockade to increase circulating EPCs in renovascular hypertension [40] and salt-loaded stroke-prone hypertensive rats [23], two models with an increase in AngII levels or activity [44–46], and the ineffectiveness in spontaneously hypertensive rats, a model in which AngII does not seem to be elevated [47], opposite to each other. The last example, however, is oversimplified because RAAS activity may vary between (or even within) tissues in these models. Therefore the local RAAS should also be taken into consideration, as will be illustrated towards the end of the present review.

AT_2 receptors act opposite to AT_1 receptors in angiogenesis. Through $G_{i/0}$ activation, AT_2 receptor stimulation with AngII inhibits VEGF-induced adult endothelial cell migration, an effect that involves inhibition of Akt (also known as protein kinase B) phosphorylation [48]. Thus it is believed that, through AT_2 receptors in adult endothelial cells, AngII inhibits Akt-induced eNOS activation and NO release [49], hence inhibiting angiogenesis in ischaemic tissue *in vivo* [50]. It has been shown that in EPCs Akt-mediated eNOS activation can be altered by NEFAs (non-esterified fatty acids) [51], confirming the presence of this part of the pathway in EPCs. It may be that AT_2 receptors therefore inhibit both the pro-angiogenic as well as the senescence-promoting effects of AT_1 receptor activation in EPCs, but this has not yet been confirmed.

Effects on SMC progenitors

Apart from becoming EPCs, BM MNCs can also develop into VSMCs. It was shown that AngII stimulates the differentiation of cultured BM MNCs into VSMCs through stimulation of the AT_1 receptor [52]. By means of femoral artery wire injury in mice harbouring fluorescently tagged BM, the contribution of BM-derived SMCs to neointima formation was investigated. It was found that AngII increased neointima formation. The degree of neointima formation statistically correlated with increased incorporation of fluorescent BM-derived SMCs, and was inhibited by the AT_1 receptor antagonist CV11974. This was in agreement with the finding that in-stent restenosis in hypercholesterolaemic rabbits and monkeys is inhibited by the AT_1 receptor antagonists olmesartan and valsartan. The *in vivo* effect of AT_1 receptor blockade was associated with a decreased *in vitro* differentiation of peripheral blood MNCs into SMCs and a decreased expression of NAD(P)H oxidase subunits $p22^{phox}$ and $gp91^{phox}$ [37], implicating a role for ROS signalling. Strangely, *in vitro* AngII stimulated differentiation of BM cells into SMCs in the mouse, but not in the rabbit model, whereas *in vitro* AT_1 receptor antagonist treatment suppressed differentiation in both cases. A number of factors (culture medium, animal species, type of model, normal compared with high cholesterol, spontaneous AT_1 receptor activity, etc.) might account for the discrepancy, and therefore the outcome of these studies remains to be confirmed and explained. Nevertheless, the AT_2 receptor does not seem to be involved in differentiation of mouse BM MNCs to SMCs [52].

Effects on adipocyte progenitors

Similar to EPCs, the AT_1 and AT_2 receptors also seem to play an opposite role in hMSCs [human MSCs (mesenchymal stem cells)]. hMSCs can differentiate into adipocytes that produce AngII, and it was shown that addition of 1 μmol/l AngII inhibits the expression of adipocyte-related genes and lipid accumulation [53]. AT_2 receptor antagonism abolished, and in some occasions even reversed, the AngII effects. Paradoxically, AT_1 receptor antagonism further decreased the AngII-induced differentiation into adipocytes. But as differentiation of hMSCs into adipocytes progresses, the expression of RAAS components alters. With respect to pharmacokinetics, the changes in expression of metabolic RAAS components favour an increase in AngII production by the hMSCs. With respect to pharmacodynamics, AT_2 receptor expression is augmented, thus causing an increase in the AT_2/AT_1 receptor ratio. Therefore one could hypothesize that, via AT_1 receptors, endogenously produced AngII promotes the differentiation of hMSCs into adipocytes, and that at a certain point the AT_1-receptor-induced changes become counterbalanced through up-regulation of AT_2 receptors. Apparently, AT_2 receptors inhibit hMSC differentiation independently from AT_1 receptor activation because the AT_2 receptor antagonist PD123319 increases differentiation into adipocyte formation despite AT_1 receptor antagonism by valsartan [53]. Thus the observation that there is a role for both receptors in adipogenesis through effects on progenitor cells has opened a new door in research on metabolic disease [54].

Aldosterone

In addition to a direct unbeneficial effect of AngII through AT_1 receptor/ROS-mediated senescence, an increase in aldosterone levels might account for deleterious effects on EPCs. It has been shown that aldosterone reduces formation of EPCs from cultured rat BM MNCs, a process that is associated with VEGFR-2 down-regulation and increased ROS production [55]. Whether this caused senescence was not determined.

In contrast with the findings in rat BM MNCs, there appears to be no relationship between aldosterone activity and human early EPCs. EPC counts and senescence

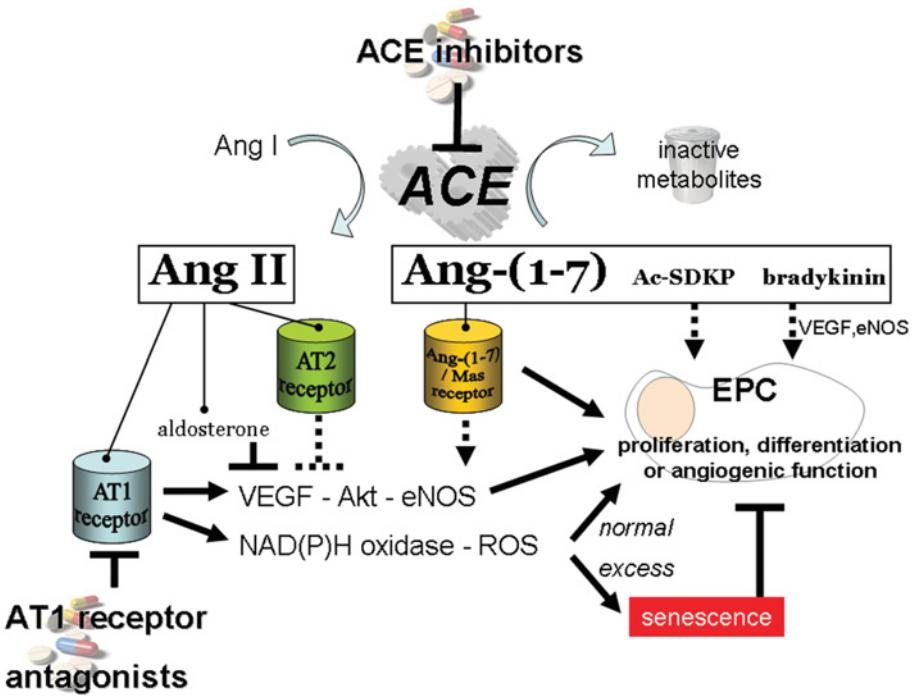

Figure 1 The role of the RAAS in EPC function

Arrows indicate stimulatory effects, while T-shaped lines represent inhibitory effects. Solid lines represent pathways that have been tested in EPCs, whereas effects depicted with broken lines remain to be confirmed in these cells. Through the AT_1 receptor, AngII mediates stimulatory as well as deleterious effects depending on the level of ROS production it invokes. ACE can influence EPCs in multiple ways because of its versatile metabolic function that influences the levels of various hormones. Therefore ACEIs and AT_1 receptor antagonists affect a variety of signalling pathways, and studies in which these drugs are employed report contradictive results with respect to the effect on EPCs.

were not affected in hypertensive patients with primary aldosteronism as compared with healthy normotensive volunteers [56]. In addition, aldosterone did not have any effect on cultured early EPCs and mineralocorticoid receptor mRNA could not be found in these cells.

The differences in both studies might relate to the progenitor cell subpopulation that was studied. Aldosterone might simply affect the more primitive BM MNCs, while being without effect after differentiation into (early) EPCs.

In summary, through AT_1 receptors, AngII has a paradoxical effect on MNCs, on the one hand stimulating angiogenic function of EPCs through VEGF/eNOS signalling involving physiological NAD(P)H-oxidase-mediated ROS signalling, but on the other hand inducing EPC senescence through prolonged ROS production and promoting differentiation into VSMCs. AT_1 receptors on hMSCs might promote adipogenesis. As often observed for AngII effects, AT_2 receptors counter-regulate AT_1 receptors, but this has to be confirmed further in EPCs (Figure 1 and Table 1).

ACEIs AND THEIR MEDIATORS

ACEIs improve prognosis in CAD, which could be related to their ability to attenuate endothelial dysfunction [57]. Endothelial dysfunction is regarded to play a pivotal role in the development of atherosclerosis [31], and it was shown that improvement in EPC function decreases atherosclerosis in ApoE (apolipoprotein E)-knockout mice [58]. Conversely, CAD is associated with lower EPC levels and function [59]. Moreover, endothelial dysfunction is strongly associated with decreased EPC function and increased EPC senescence in humans [60]. Therefore beneficial effects of ACEIs on endothelial function and CAD might relate to effects on EPCs. The fact that ACEIs promote angiogenesis, as seen, for example, in the mouse ischaemic hind limb model [61], further confirms a possible effect on EPCs.

Effects of ACE inhibition on EPCs

In agreement with the hypothesis that ACEI effects involve EPCs, perindopril treatment, especially when given in combination with the diuretic indapamide, improved angiogenesis in the mouse ischaemic hind limb [26]. Indeed, this improvement was accompanied by an increase in EPCs and an improved angiogenic effect of these cells after reimplantation in the ischaemic hind limb. VEGF and eNOS increased upon treatment with perindopril, implying the involvement of this classical angiogenic pathway. In agreement, ramipril treatment

Table 1 A summary of the function of RAAS (-related) components in EPC regulation

RAAS component	Effects on EPCs	Mediators	References
AngII	Acute stimulation:	Acute:	[25,26,35,38,48,49]
	Proliferation ↑	AT_1 receptor stimulation and subsequent VEGF/eNOS signalling. Inhibited by AT_2 receptor (not yet confirmed in EPCs).	
	Angiogenic function ↑		
	Chronic stimulation:	Chronic:	
	Proliferation ↓	AT_1 receptor/NAD(P)H-oxidase-mediated ROS production and DNA damage.	
	Differentiation ↓ (instead: adipocyte ↑)		
	Function ↓	Inhibited by AT_2 receptor (not yet confirmed in EPCs).	
	Senescence ↓		
AT_1 receptor blockade	Differentiation ↑	Inhibits NAD(P)H-oxidase-mediated ROS production and VEGF/eNOS and might promote AT_2 receptor-mediated effects.	[22–24]
	Proliferation ↑		
	Angiogenic function ↑		
	Senescence ↓		
Aldosterone	Differentiation ↓	VEGFR-2 down-regulation and increased ROS production	[55]
ACEI	Differentiation ↑	VEGF/eNOS activation. Proposed mechanisms: increased Ang-(1–7), Ac-SDKP, BK (ROS ↓; NO ↑); MMP-9 activity ↑; activation by CD26.	[26,62,68,69]
	Proliferation ↑	No correlation with VEGF, GM-CSF and TNF-α levels.	
	Migration ↑		
MMP-9	Migration ↑	MMP-9 increases the cleavage of stem cell factor and promotes stem cell mobilization.	[18,68,69]
Ac-SDKP	*In vitro* EPC ↑: promotes angiogenesis?	Unclear	[73]
BK	Not yet reported	BK B_2 receptor stimulation increases VEGFR-2 and eNOS activity and should hypothetically increase EPCs.	[80,81]
Ang-(1–7)	Proliferation and/or differentiation ↑	Stimulates Ang-(1–7) receptor (presumably Mas receptor), leading to autonomous effect through eNOS activation or decrease of AT_1 receptor activity (ROS/senescence ↓).	C. Qian, R. G. Schoemaker, W. H. van Gilst and A. J. M. Roks, unpublished work and [98,99,102,103]
	Can promote as well as inhibit angiogenesis, depending on dose (?)		

leads to increased levels, proliferation, migration, adhesiveness and tube formation of cultured blood-derived EPCs from patients with CAD [62]. These enhanced EPC functions were already observed from 1 week after onset of ramipril treatment, and reached their maximal effect after 3 weeks. Strikingly, the effect of ramipril on EPC function did not correlate with plasma levels of the classical angiogenic factors VEGF, GM-CSF (granulocyte/macrophage colony-stimulating factor) and TNF-α (tumour necrosis factor-α); however, EPC function was associated with NO levels. This suggests that intervention through ACE inhibition acts on EPCs independently from angiogenic factors, and underscores that the angiogenic effect of AngII, which would be in contradiction with a beneficial effect of ACE inhibition, is not involved. In contrast, the ACEI effect could relate to a reduction in AngII-induced senescence caused by increased ROS, which corresponds to the increased NO levels. However, ACEIs reduce AngII levels only for a very short period as AngII levels rapidly increase again to baseline levels through the so-called 'ACE escape' [63–65]: a bypass provided by activation of alternative enzymes. Moreover, ACE is a versatile enzyme that, apart from converting AngI into AngII, metabolizes

various peptide hormones, including BK (bradykinin), Ac-SDKP (*N*-acetyl-Ser-Asp-Lys-Pro) and Ang-(1–7), and displays pleiotropic effects through regulation of MMPs. These mechanisms may account for the effects of EPCs.

MMPs

In the rat MI model it was shown that injection of EPCs combined with ACE inhibition alone or in combination with β-blocker treatment had an additive beneficial result, thus optimizing cardiac angiogenic and antifibrotic effects [66,67]. An effect of the pharmacotherapy alone on EPC levels was not assessed in these studies; however, ACEIs have been shown to promote mobilization of BM EPCs after MI in mice [68], an effect that was ascribed to a pleiotropic effect through activation by CD26. In another study it was shown that EPCs were reduced 3 days after MI, which was associated with increased ROS and decreased MMP-9 activity in the BM [69]. ACE inhibition improved EPC levels, which was accompanied by a normalization of BM MMP-9 activity through ERK (extracellular-signal-regulated kinase) signalling, whereas ROS levels remained unchanged. The participation of MMP-9 is on the one hand logical because MMP-9 is involved in recruitment of EPCs [18]. On the other hand it is surprising in view of the contradicting finding that ACE inhibition decreases migration of adult endothelial cells through inhibition of MMP due to Zn^{2+} scavenging [70], and had no effect or even decreased plasma MMP-9 levels in other studies [71,72]. Although MMP-9 levels are more important in the BM stem-cell niche, where MMP-9 supports recruitment of EPCs [18], rather than in plasma, its involvement in ACEI effects and RAAS inhibition in general remains elusive. This is further emphasized by a study in patients with essential hypertension [41] in which it was shown that telmisartan, an AT_1 receptor antagonist, did not change EPC levels or MMP-9 activity.

Ac-SDKP

Ac-SDKP, which is metabolized exclusively by ACE, is involved in the regulation of haematopoiesis [8], and suppresses the growth of MSCs, fibroblasts and macrophages in BM MNC cultures [73,74]. Thus Ac-SDKP supports the culture of EPC-rich progenitor cell populations [73]. Increased Ac-SDKP levels through ACE inhibition or infusion of the peptide resulted in suppression of cardiac fibrosis in ACE-overexpressing transgenic rats [75] and AngII-infused hypertensive rats [74]. In addition, Ac-SDKP exerts angiogenic effects on cultured endothelial cells [76], and infusion in the rat MI model improved cardiac angiogenesis [77]. Possibly, Ac-SDKP mediates its beneficial effects on cardiac fibrosis through suppression of the differentiation of BM stem cells into inflammatory cell species. Whether this function implicitly leads to increased EPCs *in vivo*, the same way as it occurs *in vitro*, and subsequently to the improved angiogenesis that is observed after chronic ACE inhibition remains to be investigated (Figure 1 and Table 1).

Bradykinin

BK is an antihypertensive hormone that induces endothelium-dependent vasodilation through NO release among other ways. BK is metabolized by ACE to the inactive peptide BK-(1–5), and accordingly ACE inhibition increases BK levels in some studies [78,79]. BK mediates many of its effects on endothelial cell function through BK B_2 receptors, and it has been shown that genetic deletion of this receptor prevents the pro-angiogenic effects of ACE inhibition [61]. The pro-angiogenic activity of BK relates to BK B_2 receptor-mediated transactivation of VEGFR-2, thus stimulating eNOS [80,81]; an important mechanism involved in angiogenic function of adult endothelial cells. Whether this mechanism is of importance in progenitor cells is unknown (Figure 1 and Table 1) because BK effects on EPCs have not yet been explored.

Ang-(1–7)

A fourth mechanism that could account for ACEI effects is an increase in Ang-(1–7) levels. Ang-(1–7) is a bioactive peptide that displays effects that are often opposed to those of AngII [20]. Intriguingly, ACE mediates both the synthesis, as well as the metabolism, of Ang-(1–7). More explicitly, Ang-(1–7) synthesis can occur through sequential metabolism of AngI to Ang-(1–9) by the ACE2 enzyme and the subsequent conversion of Ang-(1–9) into Ang-(1–7) by ACE [82]; however, this pathway has unfavourable pharmacokinetic features. On the other hand, Ang-(1–7) is degraded by ACE to the inactive metabolite Ang-(1–5) [83]. Accordingly, ACE inhibition was shown to increase Ang-(1–7) levels [84]. In addition, Ang-(1–7) itself can act as an ACEI [85]. This has led to the hypothesis that the beneficial effects of ACE inhibition could be mediated by Ang-(1–7).

In addition, there is evidence that Ang-(1–7) is involved in effects of AT_1 receptor blockers. Ang-(1–7) can interact directly with the AT_1 receptor [86,87] leading to their functional or molecular down-regulation [88–91]. Previously, it was discovered that Ang-(1–7) is a ligand for the G-protein-coupled receptor Mas, which mediates effects that can be antagonized by the Ang-(1–7)-specific antagonist A779 [92]. Importantly, some ACEI and AT_1 receptor antagonist effects can also be blocked by A779 or anti-Ang-(1–7) antibodies [93–95]. Therefore the Ang-(1–7)/Mas axis might be involved in therapeutic RAAS modulation and the effect thereof on progenitor cells. The next section will therefore be devoted to this potentially important mediator.

EFFECTS OF ANG-(1–7) ON PROGENITOR CELLS

Ang-(1–7) exerts antihypertensive, antihypertrophic, antifibrotic and antithrombotic effects in the cardiovascular system, as reviewed previously [20]. In previous studies we found that chronic Ang-(1–7) infusion led to preservation of endothelial function in rat models for MI and in-stent restenosis [96,97]. It is therefore tempting to hypothesize that Ang-(1–7) recruits EPCs, and that this accounts for the beneficial effects of ACE inhibition on endothelial function and angiogenesis. The effects of Ang-(1–7) on progenitor cells were hitherto only explored with respect to haematopoiesis, but we have now found evidence that points out that EPCs are also affected.

Effects on HSCs

Ang-(1–7) has effects on HSCs as it accelerates haematopoietic recovery in multiple cellular lineages after myelosuppression. Using mice models subjected to chemotherapy or irradiation, as well as in patients with breast cancer, Rodgers et al. [98,99] showed that daily administration of Ang-(1–7) during and after chemotherapy speeds up recovery of the white blood cell count and the number of various blood cell progenitors in patients with breast cancer. Also, treatment with Ang-(1–7) had a synergistic effect when used in combination with Neupogen, a human G-CSF (granulocyte colony-stimulating factor), already employed in clinics [100]. This beneficial effect on the progenitor cells might not be limited only to the haematopoietic system, but extend to progenitors that are involved in cardiovascular recovery, for example EPCs, which are so closely related to haematopoietic progenitor cells.

Effects on EPCs

The observation that chronic Ang-(1–7) improved systemic endothelial function and improved recovery of haematopoiesis prompted us to investigate the effects of Ang-(1–7) on BM MNCs, and in particular the subpopulation of EPCs. In our recent pilot studies (Table 1 and Figure 2), we isolated rat BM MNCs and cultured them in EGM2 (endothelial growth medium 2; Lonza, Brussels, Belgium) on gelatin-coated culture clusters. After 2 days of attachment, unattached cells were removed and the remaining cells were treated for 7 days with Ang-(1–7) alone or in combination with A779 or the AT_2 receptor antagonist PD123319. Medium was refreshed every 2 days. All cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole), and EPCs were detected by double staining with DiI-acetylated-LDL and BS-1. Preliminary findings show an increase in cultured MNCs after stimulation with Ang-(1–7) (Figure 2). Notably, the effect on the subpopulation of EPCs particularly was much more pronounced than the effect on the entire population of MNCs.

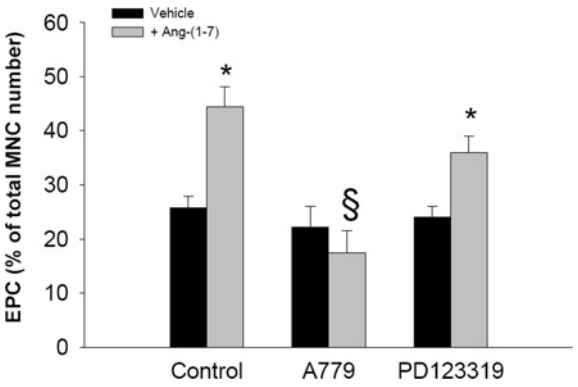

Figure 2 Effect of Ang-(1–7) (10^{-8} mol/l) and the specific Ang-(1–7) receptor antagonist A779 (10^{-7} mol/l) and the AT_2 receptor antagonist PD123319 (10^{-7} mol/l) on the percentage of cultured BM EPCs

Results are means ± S.E.M. of five experiments. $^*P < 0.05$ compared with vehicle as measured using a Student's t test; $\S P < 0.05$ compared with control/Ang-(1–7) as measured using a Dunnett's t test.

Our results show that A779 inhibits the effect on EPCs. This observation implies that Ang-(1–7) promotion of the endothelial regenerative capacity is mediated through Mas receptors; however, this remains to be confirmed in Mas-knockout mice. In these mice it was shown that Ang-(1–7) vasodilator responses are absent [92] and, moreover, decreased mesenteric vasodilator responses to acetylcholine and BK were observed [101], stressing the point that Mas receptors are needed to warrant a healthy vasodilator function of the endothelial cells. Furthermore, PD123319 seemed to lower the Ang-(1–7) effect, although this effect did not reach statistical significance when compared with Ang-(1–7) alone. The involvement of AT_2 receptors therefore remains uncertain.

The pathways through which Ang-(1–7) increases EPCs are still unknown; however, two pathways are eligible (Figure 1 and Table 1). First, in CHO (Chinese-hamster ovary) cells that are stably transfected with Mas and in human aortic endothelial cells, Ang-(1–7) stimulates eNOS activation through Akt [102], the pathway involved in the AT_1-receptor-mediated stimulation of VEGF-induced angiogenesis. Secondly, Ang-(1–7) inhibits AngII/AT_1-receptor-induced ROS production through NAD(P)H oxidase [103]. These Ang-(1–7) effects are again blocked by A779, pointing out an involvement of Mas receptors. Since these pathways play an evident role in EPC angiogenic function and senescence respectively, they can account for the stimulating effects of Ang-(1–7) on EPCs. Moreover, these might also explain the protective effect of chronic Ang-(1–7) treatment on endothelial function observed by us in previous studies [96,97].

Considering the versatility of Ang-(1–7) with respect to stimulation of adult endothelial cells and EPCs, it is tempting to consider this heptapeptide as a regenerative

agent in the cardiovascular systems. Although this is in agreement with our observation that chronic Ang-(1–7) improves endothelial function, in rats with MI we did not observe an effect on cardiac angiogenesis [96]. This might be explained by the study protocol: infusion of Ang-(1–7) started 2 weeks after MI. An extensive angiogenic response that restores basal coronary flow has already taken place within the first week after MI [104], and therefore the effect of Ang-(1–7) on cardiac angiogenesis might have been minimized in our study protocol. Alternatively, paradoxical effects of Ang-(1–7) on angiogenesis may explain the outcome of our former study [96]. Ang-(1–7) was found to inhibit angiogenesis, measured as haemoglobin content of subcutaneous sponges in mice [105], and tended to inhibit *in vitro* tube formation by human umbilical cord endothelial cells [106]. On the other hand, dermal wound repair and blood vessel formation in diabetic mice was improved by Ang-(1–7) [107]. The paradoxical finding may be explained as a dose problem, because we found increased EPC network formation at 10^{-8} mol/l Ang-(1–7), but a decrease at higher doses [108]. Hence the angiogenic effect of Ang-(1–7) is not entirely understood and the effects on EPCs, as well as adult cells, need to be examined more comprehensively.

THE LOCALIZATION OF RAAS COMPONENTS AND THEIR INTERACTIONS WITH CARDIOVASCULAR PROGENITOR CELLS

The finding that AngII and Ang-(1–7) exert effects on isolated EPCs and other progenitor cell species and that these effects can be blocked by AT_1, AT_2 and Mas receptor antagonists suggests that functional RAAS receptor subtypes are present on progenitor cells. Moreover, in BM MNCs isolated from spontaneous hypertensive rats with ischaemic hind limbs ACE inhibition also increased *in vitro* differentiation into EPCs [24], which pleads for the presence of a complete RAAS in these cultures. ACE and AT_1 receptors have been detected at the protein level in the BM and in $CD34^+$ HSCs. In EPCs mRNA coding for AT_1 receptors, AT_2 receptors and cathepsin D were detected, whereas chymase and renin mRNA expression was low [23]; however, detailed studies on the presence and localization of RAAS components in progenitor cell populations are lacking. Moreover, further components, notably ACE and Mas receptors, remain to be determined in EPCs. Nevertheless, RAAS components in the EPCs themselves, as well as those in the non-cardiovascular MNCs and the culture medium that surround them, may both contribute to the effects of RAAS modulation in *in vitro* pharmacological studies. Translating this paradigm to the *in vivo* situation, all of the RAAS components do not have to be present on one and the same cardiovascular progenitor cell type because the surrounding tissue or fluid can provide various components. The tissues comprise the BM as well as the tissue where EPCs home to: the heart and vessels.

There are a few examples that imply an important role for a local BM RAAS. In the case of haematopoietic cells, local BM RAAS activation associates with certain forms of leukaemia and stimulation of $CD34^+$ HSCs [7]. It is very likely that, being related to these cells, EPCs and other cardiovascular progenitor cell types are affected by this local BM RAAS that, as a consequence, plays a role in the beneficial effect of pharmacotherapeutical RAAS modulation. Indeed, it has been shown in hypertensive rats that ACE inhibition increases circulating EPCs [24] implying a role for local BM ACE in recruitment. The local BM or EPC RAAS activity seems to be influenced by remote ischaemic events: the stimulating effect of AT_1 receptor blockade on the differentiation of isolated MNCs into EPCs in cells from hypertensive rats is only observed in animals with ischaemic hind limbs [24]. How this effect of remote ischaemia on the local BM RAAS takes place remains unsolved. It is tempting to speculate that HIF-1α released from the ischaemic tissue stimulates VEGF release from the BM MNCs. Once isolated, these cells sustain VEGF release and proliferate as a consequence of an AT_1-receptor-mediated VEGFR-2 receptor up-regulation [38]. AT_1 receptor blockade would interfere with the proliferative state and lead the MNCs into a differentiating state, which would be detected as an increased percentage of EPCs during the 7 days of culture [24].

To support the notion that the local vascular RAAS is important for progenitor cell function it can be mentioned that the stimulation of angiogenesis by ACEIs in the rabbit hind limb ischaemia model demands an efficient inhibition of the tissue ACE [109], although this observation has not been specifically related to EPCs or other progenitor cells. In further consideration of the role of the local vascular RAAS, the story becomes rather hypothetical. AngII and VEGF are known to increase endothelial and vascular permeability [110,111], which might affect local vascular transmigration of the BM-derived EPCs at the site where angiogenesis occurs. Furthermore, long-lasting local tissue RAAS activation, e.g. through biomechanical stress or after MI, leading to excess AngII formation and NAD(P)H oxidase activity might impede proper repair function through increased senescence of EPCs. Indeed, for local cardiac progenitor cells a relationship between (calendar) aging, RAAS activation and cellular senescence has been proposed [13], but the details and relevance of this relationship need to be explored further.

In summary, it is appealing to suggest that the local RAAS affects EPC transmigration and angiogenic function. It is therefore expected that a tight regulation of local BM, EPC and cardiovascular tissue RAAS activity is needed to warrant a healthy repair function of progenitor

cells and that this local regulation is therefore a target for pharmacotherapy through RAAS modulation. This research topic is seriously underexposed and will be a major challenge for the coming years.

CONCLUDING REMARKS

It has become evident that AngII, Ang-(1–7) and other RAAS mediators have effects on various progenitor cells at various loci. Effects on progenitor cells might play a role in the clinical effects of AT_1 receptor antagonists and ACEIs. Improvement in VEGF/eNOS function and decreased ROS production through inhibition of the NAD(P)H oxidase system are important mechanisms that mediate the effects on EPCs or adult endothelial cells (Table 1). Furthermore, more specific interventions in the Ang-(1–7)/Mas receptor axis could be pursued, as discussed elsewhere [20,112].

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