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Regulation of Endothelial Progenitor Cell Function by Plasma Kallikrein-Kinin System

Yi Wu[1] and Jihong Dai[1,2]
[1]*Sol Sherry Thrombosis Research Center, Temple University School of Medicine, , Philadelphia, PA,*
[2]*Cyrus Tang Hematology Research Center, Sooochow University, Suzhou,*
[1]*USA*
[2]*China*

1. Introduction

Circulating endothelial progenitor cells (EPCs) are a hierarchy of pluripotent cells in peripheral blood capable of differentiating into mature endothelial cells destined for blood vessel formation[1]. These cells have the ability to be mobilized to the site of vascular injury or tissue ischemia, and they differentiate into mature endothelial cells. They are a major determinant of a postnatal mechanism for neovascularization and vascular remodeling, and play an important role in endothelial cell and vessel maintenance. However, in patients with atherosclerosis and cardiovascular disease, EPCs are reduced in number and impaired in function, which are negatively correlated with the atherosclerotic risk factors, and may contribute to vascular dysfunction[2]. For example, the frequency of circulating EPCs is reduced 50% in patients with coronary artery disease, and their EPCs display an impaired migratory response[3]. Moreover, although EPCs successfully restore endothelial function and enhance angiogenesis after tissue ischemia in animal models, the clinical administration of EPCs to patients has had limited efficacy. It is likely that EPCs are targets of endogenous angiogenic inhibitors elaborated in the setting of atherosclerosis. Therefore, understanding the factors and mechanisms that affect EPC function and number may not only provide new insights into the pathogenesis of vasculogenesis, but also promote development of specific therapies to ultimately correct EPC dysfunction and prevent progression of atherosclerosis.

The plasma kallikrein–kinin system (KKS) consists of the proteins factor XII, prekallikrein, and high molecular weight kininogen (HK)[4-5]. This system widely participates in maintenance of the cardiovascular phenotype, and displays multiple physiologic and pathophysiological activities, such as blood pressure adjustment, modulation of thrombosis, regulation of endothelial cell function and angiogenesis. Plasma HK, which is synthesized and released from the liver, is a major component of the KKS and is responsible for the association of the KKS with cell surface. The plasma membrane of endothelial cells is an important site for the assembly and activation of the KKS. Activation of the KKS is triggered *in vivo* by tissue destruction or by thrombus development, and results in cleavage of HK by kallikrein and generation of two-chain HK (HKa) and a nonapeptide bradykinin[6]. HKa

and bradykinin differentially regulate the endothelial cell function, HKa is antiangiogenic, but bradykinin is of proangiogenesis. Unlike HK, HKa exposes its domain 5 to the surface on cleavage, thereby acquiring a function of antiadhesion. This antiadhesive property enables HKa to inhibit endothelial cell proliferation and to induce endothelial apoptosis on extracellular proteins, thus it exhibits potent antiangiogenic activity[7-8]. The inhibitory effect of HKa may result from its inhibition of αvβ3 integrin function[7, 9], and induction of apoptosis via its interaction with uPAR[10]. Because EPCs express high levels of uPAR, which is a HKa receptor, HKa may exerts inhibitory effect on EPCs[11]. In this chapter, we summarize the recent observations that the KKS regulates EPC function.

Human EPCs can be isolated from adult circulation. If EPCs are to be defined as true progenitor cells with postnatal vasculogenic potential, they should give rise to differentiated progeny with the capacity for vessel formation. In a 3D collage gel, EPCs, but not differentiated endothelial cells such as HAECs and HUVECs, exhibit strong capacity to form vacuoles and tubes in the presence of VEGF[11], suggesting that EPCs are unique in their stronger potential for tubular morphogenesis and are more sensitive to physiological growth factor stimulation.

2. HKa inhibits EPC tube formation via suppression of MMP-2 activation

In the process of neovascularization, invasive endothelial cells secrete MMPs to remodel the extracellular matrix (ECM) and the basal lamina – an important physical barrier between the endothelial and connective tissue[12]. Two members of the MMP family, MMP-2 and MMP-9, display the highest enzymatic activities against the ECM components important in angiogenesis. MMP-2 and MMP-9 participate in the mobilization of EPCs. Recently we have demonstrated that MMP-2 is selectively required for VEGF-stimulated vasculogenic differentiation of EPCs[11]. In the conditioned culture media of EPCs embedded in 3D collagen gel, VEGF selectively stimulated the secretion and activation of MMP-2, but not MMP-9, and VEGF-stimulated proMMP-2 expression and secretion are time-dependent, the secreted proMMP-2 was meanwhile converted into active form. As the specific inhibitor of MMP-2 (444244), but not MMP-9 (444278), concentration-dependently attenuated VEGF-stimulated tube formation by EPCs, suggesting the role of MMP-2 activities in tube formation by EPCs[11]. The requirement of MMP-2 and MMP-9 expression for the tube formation by EPCs is examined by specific siRNA oligonucleotides. VEGF-stimulated tube formation by EPCs was almost completely suppressed by gene silencing of MMP-2, but not of MMP-9. [11] Thus, MMP-2 is selectively required for EPC formation of tubular structures. The inhibitory effect of the MMP-2 gene silencing was more potent than that of the enzymatic inhibitor, suggesting that both the catalytic activity and expression of MMP-2 are necessary for the tubular morphogenesis of EPCs. Indeed, pro-MMP-2 possesses enzymatic activity-independent functions; its interaction with cell-surface proteins may mediate intracellular activation signals. Collectively, as found by using pharmaceutical inhibitors and gene ablation, both the enzymatic activity and expression of MMP-2 are required for EPC differentiation. This observation is consistent with a recent study showing that MMP-2 deficiency reduces the functional activities of EPCs, leading to impaired vasculogenesis[12]. Interestingly, HKa markedly inhibits conversion of pro-MMP-2 to active MMP-2 in EPCs without affecting MMP-2 secretion. In a 3D culture system, HKa significantly decreased VEGF-stimulated tube formation by EPCs at the concentrations of 30 nmol /L, 100 nmol/ L and 300 nmol/ L, representing cleavage of HK of 4.5%, 15% and 45%, respectively, which

occurs in experimental inflammatory bowel disease and arthritis[11]. VEGF-stimulated MMP-2 secretion and activation was not detected in the medium of EPCs cultured on collagen-coated surfaces, suggesting that MMP-2 is not involved in EPC activation in a 2D system and HKa did not inhibit endothelial cell function on collagen surfaces[11]. Thus, HKa inhibition of tube formation by EPCs in collagen gel (3D system) reveals a novel mechanism for HKa antiangiogenic activities. In a 3D system MMP-2 activation is very likely a target for HKa. The MMP-2 Inhibitor I (444244) markedly abolished the gelatinolytic activity of both proform and active form of MMP-2. In contrast, HKa treatment only reduced the ratio of active MMP-2 to total MMP-2 (44 ± 7% at 0 nmol/ L vs. 27 ± 6% and 19 ± 3% at 100 and 300 nmol/L, respectively)[11]. Thus, HKa inhibits the conversion of pro-MMP-2 to active MMP-2. In purified systems, HKa at 300 nmol/ L did not have apparent inhibition of catalytic activity of MMP-2 and the conversion of pro-MMP-2 to MMP-2[11]. Collectively, HKa inhibits the conversion of pro-MMP-2 to MMP-2 in EPCs without directly affecting MMP-2 secretion and activity. Because the KKS activation may occur during inflammation and thrombosis, HKa as an activation product of this system may induce EPC dysfunction in the setting of pathological conditions.

3. HKa inhibition of MMP-2 activation in EPCs is dependent on αvβ3 integrin

EPCs expressed a high level of αvβ3 integrin and HKa downregulates αvβ3 integrin ligand binding affinity in endothelial cells, whether HKa inhibition of MMP-2 activation through αvβ3 integrin was examined by an αvβ3CS-1 cell line[11]. In the αvβ3-CS-1 cells, the expression of αvβ3 integrin on the cell surface increased MMP-2 activation and stimulated autoactivation of MMP-2 (conversion of 64-kDa to 62-kDa form)[11]. However, in the presence of HKa, the conversion of proMMP-2 to 64- and 62-kDa forms was inhibited. Correspondingly, HKa blocked the formation of avβ3 integrin-MMP2 complex in EPCs cultured in a 3D gel[11]. HKa disrupts the association between αvβ3 integrin and proMMP-2, which may account for its inhibition of MMP-2 activation[11]. MMP-2-αvβ3 integrin interaction is critical for angiogenesis, HKa inhibition of MMP-2 activation and tube formation by EPCs is, at least in part, mediated through αvβ3 integrin. Therefore, HKa inhibits the vasculogenic differentiation by EPCs via the suppression of MMP-2 activation, which is a novel activity of the KKS system. The inhibitory effect of HKa on MMP-2 is dependent on the presence of αvβ3 integrin and mediated by the dissociation of proMMP-2 from αvβ3 integrin. The αvβ3 integrin is necessary for vascular cell survival, proliferation and invasion during angiogenesis. MMP-2 activation has been suggested to be downstream of αvβ3 integrin activation, and MMP2- αvβ3 binding is required for proper MMP2 function. This is consistent with MMP-2 and αvβ3 integrin being colocalized in a particular membrane fraction caveolae, which enhances cellular protrusive activity. The disruption of MMP-2 binding to αvβ3 integrin blocks angiogenesis *in vitro* and *in vivo*; αvβ3 integrin may serve as an MMP-2 receptor in the process of angiogenesis. In endothelial cells, the primary target for HKa is uPAR and HKa occupancy of uPAR disrupts uPAR-αvβ3 integrin complex formation. HKa seems to indirectly modulate αvβ3 integrin function. In platelets, αIIbβ3 integrin also interacts with the C-terminal hemopexin-like domain of MMP-2, suggesting that MMP-2 is likely to be associated with β3 subunit[13]. In a purified system, αvβ3 integrin did not form a complex with pro-MMP2 or active MMP-2, supporting the observation that MMP-2, which does not have a RGD sequence, indirectly interacts with αvβ3 integrin via a mediatory RGD-bearing molecule. Alternatively, MMP-2 may bind to

αvβ3 integrin in a RGD-independent manner, as cRGDfK failed to completely prevent the association between proMMP-2 and αvβ3 integrin. Strikingly, HKa possesses stronger inhibition of both pathways. Taken together, HKa, by interfering with αvβ3 integrin function, decreases the ligation of proMMP2 or its associated molecule to αvβ3 integrin in EPCs, thereby suppressing MMP-2 activation. The inhibitory effect of HKa on MMP-2, at least in part, accounts for its inhibition of EPC vasculogenic differentiation.

HKa inhibition of the tube forming capacity of EPCs reveals a novel activity of the components of the KKS in vascular dysfunction and expands our understanding of the pathophysiological activities of this system. Local EPCs recruited after early arterial injury incorporate into foci of postnatal vasculogenesis and exhibit strong potential for vascular repair. A study using kininogen-deficient mice indicates that the KKS activation, in the process of vascular injury, induces arterial thrombosis[6]. Thus, developing thrombus in atherosclerotic lesions may form a surface that promotes the assembly of the components of the KKS, leading to a secondary activation of this system and further production of HKa. HKa inhibition of vasculogenesis of EPCs proposes a novel concept that activation of the KKS may contribute to EPC dysfunction. Although it remains to be determined whether and how HKa inhibition of EPCs is involved in the disordered vascular remodeling *in vivo*, recognition of components of plasma KKS modulation of EPC function allows for the beginning of understanding its pathophysiologic activities in atherosclerosis.

4. HKa suppresses clonogenic capacity of EPCs

Clonogenic capacity of EPCs can be analyzed using a single-cell assay. [14]. To test whether HKa affects the clonal expansion potential of EPCs, a single-cell suspension of EGFP-EPCs was seeded into 96-well culture plate precoated with collagen, in order to test whether HKa affects the clonal expansion potential of EPCs. The culture medium EGM-2 was replaced every 2 days. After culture for 14 days, EGFP-EPCs formed large colonies, and ≈85% of colonies contained more than 200 cells (large colonies). However, in the presence of 50 nmol/L HKa, the percentage of large colonies was markedly reduced to <6% (P<0.001), indicating that HKa strongly inhibits clonogenic capacity of EPCs[14].

5. HKa inhibits EPC proliferation without induction of apoptosis

HKa inhibition of EPC colony formation suggested that HKa suppresses proliferation of EPCs. The effect of HKa on proliferative capacity of EPCs was examined using a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. Treatment of EPCs with HKa for 72 hours significantly inhibited vascular endothelial growth factor–stimulated BrdU incorporation into EPCs, and the inhibition was concentration-dependent, whereas the significant inhibitory effect of HKa was detectable only at 100 nmol/L after treatment for 48 hours. In contrast to its significant effects on proliferation, HKa at the same concentrations did not inhibit EPC adhesion to collagen[14], suggesting that HKa inhibition of EPC proliferation does not result from an antiadhesion activity. Because HKa induces apoptosis of differentiated endothelial cells on vitronectin-coated surfaces, the effect of HKa on EPC apoptosis was examined. HKa did not induce EPC apoptosis on collagen surfaces, although its effect on induction of EPCs apoptosis on vitronectin-coated plates was significant. Therefore, HKa inhibition of EPC colony formation and proliferation is not subject to antiadhesive activity and induction of apoptosis.

6. HKa accelerates the onset of EPC senescence and suppresses telomerase activity

Reduction of EPCs in number and activity is associated with EPC senescence. The common features of senescent endothelial cells include the enhanced presence of acidic β-galactosidase activity, an increase in lysosomal mass, and formation of autophagic vacuoles. To determine whether HKa induces EPC senescence, the activity of acidic β-galactosidase was measured, the former referred as senescence-associated-β-galactosidase (SA-β-gal). Although a small portion of EPCs were positive for SA-β-gal staining after culture for 14 days, in the presence of 50 nmol/L HKa the majority of EPCs became positive for SA-β-gal staining[14]. Moreover, EPCs treated with 50 nmol/L HKa displayed a unique flattened and enlarged morphology and formed intracellular vacuoles[14], HKa treatment significantly resulted in an increase in SA-β-gal-positive cells, 17.2±2.6% vs 52.3±3.7% on day 7 and 22.3±2.7% vs 85.5±7.9% on day 14[14]. Acceleration of the onset of EPC senescence is known to be critically influenced by the level of telomerase activity, which elongates telomeres, thereby counteracting telomere length reduction induced by each cell division. Therefore, whether HKa treatment was capable of regulating telomerase activity in EPCs was tested. Treatment of EPCs with 50 nmol/L HKa for 14 days markedly diminished telomerase activity by >60% (P<0.005)[14], serving as additional evidence for HKa acceleration of EPC senescence.

7. HKa increases intracellular ROS production, contributing to EPC senescence

Cell senescence is tightly associated with intracellular ROS production. Whether HKa exposure increases intracellular ROS in EPCs was tested using the 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) labeling assay. In a concentration-dependent manner, HKa significantly increased H_2DCF-DA oxidation level in exposed EPCs[14]. Further whether HKa accelerates EPC senescence via ROS production was tested in an inhibition assay using the ROS scavengers Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and *N*-acetylcysteine (NAC). MnTBAP is a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger. NAC is an efficient free radical scavenger and contributes to production of other antioxidant species. Treatment with either 100 μmol/L NAC or 10 μmol/L MnTBAP significantly attenuated the increase in ROS production in EPCs treated with 100 nmol/L HKa for 12 hours[14]. Moreover, 100 μmol/L NAC or 10 μmol/L MnTBAP significantly reduced both the percentage of senescent cells and ROS production in EPCs treated with 50 nmol/L HKa for 14 days[14]. These observations suggest that HKa generation of ROS is involved in the acceleration of EPC senescence.

8. HKa upregulates p38 kinase phosphorylation and $p16^{INK4a}$ expression

To investigate the mechanism for HKa acceleration of EPC senescence, we measured the levels of p38 kinase activation and prosenescence molecule $p16^{INK4a}$ expression, which are downstream of ROS generation in the process of cellular senescence. Immunoblotting analysis indicated that treatment with HKa at 30 and 100 nmol/L for 7 days increased p38 kinase phosphorylation[14]. Concomitantly, HKa upregulated $p16^{INK4a}$ expression at the protein and mRNA levels. Since 10 μmol/L SB203580, a specific p38 kinase inhibitor,

markedly suppressed HKa-induced $p16^{INK4a}$ expression as well as EPC senescence, HKa upregulates $p16^{INK4a}$ expression via its activation of p38 kinase[14].

The above observation demonstrates novel activities of HKa in regulating several key elements of EPC biology. Aging is associated with an increased risk for atherosclerosis, and insufficient repair of damaged vascular walls by a diminished number or dysfunction in EPC is one of many possible causes. A reduction in EPC number and activity has been associated with EPC senescence. Because senescence limits the ability of EPCs to sustain ischemic tissue repair, a full characterization of the pathophysiological factors leading to EPC senescence, as well as the related underlying mechanisms, is clearly important. Our current study demonstrating HKa acceleration of EPC senescence not only expands our understanding of the KKS activation in the regulation of vascular biology but also reveals a potential novel endogenous inducer of EPC senescence.

The in vivo activation of the KKS and cleavage of HKa has been widely detected in numerous pathophysiological conditions, such as thrombosis, arthritis, inflammatory bowel disease, vasculitis, sepsis, systemic amyloidosis, and preeclampsia[4]. However, how the KKS activation products initiate and regulate downstream effects remains elusive. The plasma concentration of HK is 660 nmol/L. In patients with sepsis and autoimmune diseases, more than 30% of plasma HK was cleaved[15]. Because the minimal concentration of HKa that significantly induced EPC senescence was 30 nmol/L, the circulating levels of HKa in the pathological settings could affect EPC function. This HKa-mediated effect was associated with profoundly impaired EPC clonal expansion potential and resulted in a low overall proliferative capacity of the EPC progeny. Because HK is localized at sites of vascular injury, such as atherosclerotic lesions, it might become cleaved over the abundant negative-charged surfaces to release HKa. The observation that HKa potently inhibited the clonogenic capacity of EPCs suggests that HKa is possibly involved in the vascular dysfunction by blocking EPC aggregation and expansion. Because HKa inhibition of EPC function is not dependent on its antiadhesion activity, HKa in plasma may attack circulating EPCs. Whether the blockade of HK cleavage prevents EPC senescence remains to be determined using an in vivo model.

EPC senescence can be triggered by a variety of factors, such as proinflammatory cytokines, DNA damage, hyperoxia, and hyperglycemia. All these factors increase oxidative stress, by which they induce EPC senescence and suppress telomerase activity. The mechanism by which HKa accelerates the onset of senescence of EPCs seems to be tightly associated with ROS production. NAC and MnTBAP, which quench ROS, significantly attenuated HKa-induced EPC senescence. A previous study has shown that HKa inhibits Akt phosphorylation and endothelial nitric oxide synthase phosphorylation, which may inhibit the production of nitric oxide (NO). Although NO prevents endothelial cell senescence, the NO donor *S*-nitrosopenicillamine did not prevent HKa-accelerated onset of EPCs senescence. Thus, HKa regulation of EPC senescence appears independent of the NO pathway. Accumulation of ROS in most cell types is associated with high expression of $p16^{INK4a}$, which leads to the arrest of the cell cycle at the G_1 phase and accelerates senescence of cells.The expression of $p16^{INK4a}$ increases with age and contributes to age-dependent stem and progenitor cell senescence. It can be deduced that accumulative ROS exposure, caused by various atherosclerotic risk factors, may also increase the expression of $p16^{INK4a}$ in EPCs, contributing to EPC senescence. We found that HKa increased expression of $p16^{INK4a}$ at the mRNA and protein level, demonstrating that HKa accelerates EPC senescence by the regulation of $p16^{INK4a}$ expression. We previously have found that p38 kinase is responsible

for mediating the effect of HKa in other cells. Our observations in this study indicate that HKa enhances phosphorylation of p38 kinase, and the inhibition of p38 kinase prevented both HKa-induced $p16^{INK4a}$ expression and EPC senescence. Therefore, HKa-induced ROS seems to act through p38 kinase to upregulate prosenescence molecule $p16^{INK4a}$ expression, which may further regulate telomerase activity and result in EPC senescence.

9. Bradykinin upregulates CXCR4 mRNA expression and stimulates transendothelial migration (TEM) of EPCs

TEM is an essential step for the EPC homing to sites of inflammatory and ischemic tissues. In a TEM assay using 96-transwell filters, EPCs were cultured on collagen-coated transwell filters until becoming confluent, CFDA-SE-labeled EPCs in endothelial basal medium (EBM-2) containing 2% FBS were placed on top of the lung microvessel endothelial cells (LMEC) monolayer. EBM-2 containing 2% FBS and bradykinin was added to the lower compartment. After incubation at 37°C for 18 hrs, the filters were fixed with 2% PFA. EPCs migrating into the lower side of the filters were visualized with a fluorescent microscope and counted in 6 random microscopic fields. Bradykinin (a B2R agonist) dose-dependently increased the transmigration of EPCs. In contrast, des-Arg9-BK (a B1R agonist) did not have such effect (data not shown). B2R predominantly mediates transmigration of normal EPCs. Treatment with bradykinin dose-dependently increased CXCR-4 mRNA expression in EPCs, but not the expression of other homing receptors such as E-selectin and α4-integrin.

In conclusion, HKa inhibits vasculogenic differentiation of EPCs and accelerates the onset of their senescence, and bradykinin upregulates transendothelial migration capacity. These activities of HKa and bradykinin reveal a novel link between KKS activation and EPC function, and it expands our understanding of the additional pathophysiological activities of this system. Although it remains to be determined whether and how HKa and bradykinin regulate the function of EPCs in the disordered vascular remodeling that occurs in vivo, our novel recognition that components of plasma KKS modulate EPC function allows for focused determination of the pathophysiologic activities of plasma KKS in atherosclerosis in future studies, provides an improved understanding of the contribution of the KKS cascade to EPC dysfunction in vitro and suggest candidate pathways for investigation of EPC dysfunction in subjects with vascular pathology.

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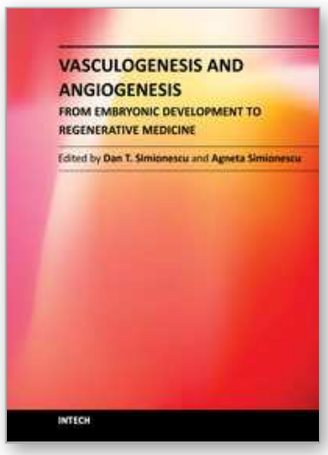

Vasculogenesis and Angiogenesis - from Embryonic Development to Regenerative Medicine

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Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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