Tissue Kallikrein Is Essential for Invasive Capacity of Circulating Proangiogenic Cells

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**Rationale:** Homing of proangiogenic cells (PACs) is guided by chemoattractants and requires proteases to disrupt the extracellular matrix. The possibility that PAC recruitment involves an interaction between proteases and chemotactic factor receptors remains largely unexplored.

**Objective:** To determine the role of human tissue kallikrein (hK1) in PAC invasion and its dependency on kinin receptor signaling.

**Methods and Results:** Human mononuclear cells (MNCs) and culture-selected PACs express and release mature hK1 protein. HK1 gene (KLK1) silencing reduced PACs migratory, invasive, and proangiogenic activities. KLK1-knockout mouse bone marrow–derived MNCs showed similar impairments and were unable to support reparative angiogenesis in a mouse model of peripheral ischemia. Conversely, adenovirus-mediated KLK1 (Ad.KLK1) gene transfer enhanced PAC-associated functions, whereas the catalytically inactive variant R53H-KLK1 was ineffective. HK1-induced effects are mediated by a kinin B2 receptor (B2R)-dependent mechanism involving inducible nitric oxide synthase and metalloproteinase-2 (MMP2). Lower hK1 protein levels were observed in PACs from type 2 diabetic (T2D) patients, whereas KLK1 mRNA levels were similar to those of healthy subjects, suggesting a post-transcriptional defect. Furthermore, B2R is normally expressed on T2D-PACs but remains uncoupled from downstream signaling. Importantly, whereas Ad.KLK1 alone could not restore T2D-PAC invasion capacity, combined KLK1 and B2R expression rescued the diabetic phenotype.

**Conclusions:** This study reveals new interactive components of the PACs invasive machinery, acting via protease- and kinin receptor–dependent mechanisms. (Circ Res. 2011;108:284-293.)

**Key Words:** kallikrein–kinin system ▪ angiogenesis ▪ circulating proangiogenic cells ▪ cell invasion

Circulating proangiogenic cells (PACs), a subset of mononuclear cells (MNCs), formerly referred to as endothelial progenitor cells, cooperate with resident endothelial cells (ECs) in the promotion of reparative neovascularization. Protease-rich PACs, at the forefront of the advancing endothelial bud, drill holes in the extracellular matrix for new vascular structures to expand and liberate extracellular matrix–bound growth factors and cytokines instrumental to tissue remodeling. A reduction in the expression of metalloproteinase (MMP)9 and cathepsin L reportedly contributes to the impaired invasive capacity of PACs in diabetes. The role of serine proteases in PAC-associated functions remains undefined.

Components of the kallikrein–kinin system (KKS), including the serine protease human tissue kallikrein (hK1), the substrate kininogen, and kinin receptors B2 (B2R) (constitutive) and B1 (B1R) (inducible), are expressed in vascular cells and leukocytes. Genetic defects of the KKS result in impairment of reparative neovascularization and attenuation of inflammatory response. Forced hK1 gene (KLK1) expression, but not its loss-of-function polymorphic variant R53H-KLK1, induces capillary and arteriole growth by kinin receptor–dependent mechanisms. Noteworthy, hK1 may also elicit angiogenic and cardioprotective actions through the B2R via a kinin-independent mechanism. Recent studies identified a role for B2R and downstream phosphoinositide 3-kinase (PI3K)/Akt promigratory signaling in the recruitment of distinct populations of proangiogenic cells. Here, we newly show that hK1 and B2R constitute essential cooperative elements of PACs invasive machinery.

**Methods**
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

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Human Studies
Online Table I summarizes diabetic patients and healthy control subjects characteristics.

Experimental Animals
KLK1-knockout mice (KLK<sup>−/−</sup>) were generated by targeted gene inactivation<sup>16</sup> and backcrossed to a pure C57/BL6 genetic background. Six-month-old male KLK<sup>−/−</sup> mice from heterozygous crossbreeding and wild-type (WT) littermates were studied.

Cell Isolation and Characterization
MNCs were isolated and PACs enriched as described previously.<sup>17</sup> Antigenic profile was assessed using a FACS Canto flow cytometer and FACS Diva software (Becton, Dickinson and Company). Migration, invasion, and networking assays were performed as described.<sup>13,18</sup>

Adenoviral Vectors and KLK1 RNA Silencing
Adenoviral vectors carrying KLK1 or R53H-KLK1 were generated as described.<sup>4</sup> Ad.B2R was prepared by subcloning B2R gene into a modified pDC515 shuttle vector (Microbix) followed by site-specific recombination of shuttle and genomic plasmids in 293 cells. For KLK1 knockdown, specific small interfering RNA probes were obtained from Dharmacon.

Reverse Transcription–Polymerase Chain Reaction
RNA was extracted using TRIzol reagent (Invitrogen), reverse-transcribed, and amplified using standard techniques.

Protein Levels and Activity
Immunoreactive hK1 and bradykinin (BK) were measured by ELISA.<sup>9</sup> HK1 enzymatic activity was assayed using colorimetric substrate S-2266 (Kabi Diagnostica). Western blotting (WB) and both in situ- and PAGE-zymographies were performed as described.<sup>18,19</sup>

Immunocytochemistry
Samples were fixed, incubated with appropriate primary and secondary, fluorescence-conjugated antibodies, and microphotographs were acquired by a computer-imaging program.

Statistical Analysis
All results are expressed as means±SEM. Student t test was used for 2-group comparisons and ANOVA, followed by Bonferroni post hoc test, was used for multiple comparisons. A probability value of <0.05 was taken as statistically significant.

Results
Human PACs Express KLK1 mRNA and hK1 Protein
Q-RT-PCR showed that peripheral blood (PB) MNCs and culture-enriched PACs express KLK1 mRNA (Figure 1A). Flow cytometry confirmed hK1 expression on distinct PB-MNC subpopulations, including CD34<sup>pos</sup> cells (30±5%), CD19<sup>pos</sup> B lymphocytes (23±7%) and CD3<sup>pos</sup> T lymphocytes (10±1%) (Figure 1B, i). HK1 was also abundant in "nonclassic" CD16<sup>pos</sup>/CD14<sup>low</sup> monocytes (43±5%), but less in CD16<sup>pos</sup>/CD14<sup>high</sup> (9±3%) and CD16<sup>neg</sup>/CD14<sup>high</sup> monocytes (1.0±0.3%) (Figure 1B, ii). Of note, MNCs cultured in the presence of hK1 inhibitor kallistatin lead to significantly lower PACs yields (P<0.05 versus control; Figure 1C), suggesting a role of hK1 in the acquisition of PAC phenotype by MNCs. However, a cooperative contribution of hK1-negative MNCs in PAC formation cannot be excluded. In fact, immunocytochemistry demonstrated that PACs comprise both hK1 positive (60±19%) and hK1 negative elements (Figure 1D). Flow cytometry identified hK1 expression on both PACs cell membrane and cytosol (Figure 1E). Moreover, PACs secrete hK1 as documented by measuring immunoreactive protein (ELISA) and enzymatic activity (amidolytic assay) in conditioned medium (Figure 1F). We verified that PACs bind UEAI (<i>Ulex europaeus</i> I lectin), take up acetylated low-density lipoprotein (data not shown), and coexpress markers of endothelial/progenitor (CD34, CXCR4, and KDR) and hematopoietic cells (CD45, CD14, CD11b) (Figure 1G).

KLK1 Silencing Reduces PACs Invasive and Proangiogenic Capabilities
To investigate endogenous hK1 functional relevance, human PACs were transfected with a pool of 4 specific small interfering <i>KLK1</i> RNAs (siKLK1) (50 to 100 nmol/L) or scrambled sequences (control). Efficient <i>KLK1</i> silencing was confirmed by reduction of mRNA, protein levels and enzymatic activity (P<0.05 versus control for all comparisons; Figure 2A). Of note, <i>KLK1</i> silencing reduced PAC migration and invasion abilities (Figure 2B) as well as their capacity to stimulate human umbilical vein endothelial cells (HUVECs) networking on Matrigel (Figure 2C; P<0.05 versus control).
for all comparisons). HK1 has been implicated in the activation of MMPs, crucial factors for PACs invasive activity. Using gel zymography, we observed reduced MMP2 gelatinolytic activity in siKLK1-PAC CM compared to control (P < 0.05; Figure 2D).

HK1 Overexpression Enhances the Invasive and Proangiogenic Capacities and MMP2 Activity of PACs

Next, we investigated whether hK1-forced expression enhances PACs functions. HK1 overexpression after Ad.KLK1 infection was confirmed using Q-RT-PCR and WB of PACs lysates and by ELISA and amidolytic assay on PAC CM (P < 0.05 versus Ad.Null for all comparisons; Online Figure I, A through D). Increased hK1 expression did not result however in increased levels of BK, the product of kininogen cleavage by hK1, in Ad.KLK1-PAC CM (Online Figure I, E).

Forced hK1 expression conferred PACs with increased spontaneous motility (1.6-fold), invasive capacity (1.8-fold) and proangiogenic activity (1.4-fold) (P < 0.05 versus Ad.Null for all comparisons; Figure 3A and 3B). In contrast, those functions remained unaltered in PACs infected with the catalytically defective polymorphic variant R53H-KLK1 (P = NS versus Ad.Null; Figure 3A and 3B). Furthermore, KLK1-forced expression did not modify PACs ability to migrate toward SDF-1α and BK (data not shown).

To verify hK1/MMP2 functional association, we used both gel- and in situ-zymography, the latter using a FITC-conjugated gelatin system. Ad.KLK1-PACs showed enhanced MMP2 activity in both assays (P < 0.05 versus Ad.Null or Ad.R53H; Figure 3C and 3D).

PACs Functional Enhancement by hK1 Is Mediated by B2R- and MMP-Dependent Mechanisms

For mechanistic interpretation of hK1-induced effects, we used hK1 and MMPs inhibitors and B2R signaling antagonists. Enhanced Ad.KLK1-PAC migration and invasion were both abrogated by the serine protease inhibitor aprotinin, the specific hK1 inhibitor kallistatin or the B2R antagonist icatibant (P < 0.05 versus Ad.KLK1-PACs exposed to inhibitor vehicle (ctr) and P = NS versus Ad.Null-PACs; Figure 4A, i and ii). Consistent with the data from icatibant, the B2R-selective antagonist MEN16132 (MEN), significantly inhibited the hK1-induced PACs migratory activity (Figure 4A, iii). No inhibition was instead seen after B1R blockade with L-dAL-BK (P = NS versus ctr and P < 0.05 versus Ad.Null-PACs). Of note, both kallistatin and icatibant effectively decreased hK1-dependent MMP2 activation (Figure 4B, i). In addition, hK1-transduced PACs invasive capacity was significantly reduced by MMP inhibitor, GM6001 (P < 0.05 versus ctr and P = NS versus Ad.Null-PACs; Figure 4B, ii).

B2R-dependent activation of PAC migration is reportedly dependent on the PI3K/protein kinase B (PKB/Akt) signaling pathway. We therefore investigated whether hK1-forced...

Figure 1. Human PB-MNCs and PACs express KLK1 gene and hK1 protein. A, Q-RT-PCR KLK1 expression analysis normalized to hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (n = 3 assayed in triplicate). B, Flow cytometric quantification of hk1-expressing MNCs (n = 4 to 7). C, Bar graph shows reduced PACs arising from kallistatin-cultured MNCs (*P < 0.05 vs vehicle, n = 4). D, PACs stain positive for hK1: TRITC-conjugated secondary antibody only (i); and specific anti hK1 antibody plus TRITC-conjugated secondary antibody (ii). Scale bar, 20 μm. E, Flow cytometric analysis of hK1-expressing PACs on cell membrane or cytosol. F, Immunoreactive hK1 in PACs conditioned medium (PAC CM) and nonconditioned medium (NCM) assessed by ELISA (i); and PAC CM hK1 enzymatic activity by amidolytic assay (ii). (*P < 0.05 vs NCM, n = 3). G, PAC antigenic profile: representative scattergrams and average data (n = 3).
expression triggers a similar signaling mechanism. Akt phosphorylation (WB) and activity (ELISA) were both increased in Ad.KLK1-PACs, with this effect being blocked by icatibant (P < 0.05 versus ctr and P < 0.05 versus Ad.Null-PACs; Figure 4C, i and ii). Unexpectedly, we did not find any change in the phosphorylation levels of eNOS (endothelial nitric oxide synthase) (at Ser1177 or Ser1173) in Ad.KLK1-PACs (data not shown). At variance, we found inducible (i)NOS to be increased significantly higher in WT BM-MNC–injected group compared with vehicle- or Ad.Null–treated groups (Figure 5A and 5B). Furthermore, Ad.KLK1 increased KLK−/− PACs spontaneous migration suggesting saturation of the cell migratory capability (P = NS versus Ad.Null; Figure 5B). Finally, Ad.KLK1 increased KLK−/− PACs supporting action on HUVEC network formation, although to a lesser extent when compared to WT PACs (P < 0.05 versus Ad.Null; Figure 5C).

Figure 2. KLK1 silencing impairs PACs function. A, Representative gels and bar graph showing reduction of KLK1 mRNA (i) and protein levels (ii) in siKLK1 RNA (siKLK)-transfected PACs compared with scramble small interfering RNA (scr). Normalization: HGPRT and β-actin. iii, Bar graph showing reduced hK1 amilodicytic activity in siKLK1-PAC CM (P < 0.05 vs scr, n = 3). B, Bar graph showing the effect of KLK1 silencing on PAC migration (i) and invasion (ii): average number of migrated (PACsinv) and invaded PACs (PACsinv) is shown (P < 0.05 vs BSA and # P < 0.05 vs SDF-1α-stimulated scr-PACs, n = 3). C, Reduced HUVEC networking in the presence of siKLK1-PACs (P < 0.05 vs scr-PACs, n = 3). D, Representative gel zymography and bar graph showing reduced MMP2 activity in siKLK1-PAC CM (P < 0.05 vs scr-PACs, n = 3).

KLK1 Gene Deletion Impairs In Vivo Proangiogenic Action of BM-MNCs

Next, we evaluated whether KLK1 gene deletion negatively impacts on reparative angiogenesis. WT or KLK−/− BM-MNCs were intravenously injected in WT mice, 1 day after operative induction of unilateral limb ischemia. We observed a significant treatment-related effect on superficial blood flow recovery as assessed by Doppler flowmetry (P < 0.01, ANOVA with repeated measurements). Post hoc analysis revealed that perfusion was improved by WT BM-MNCs but not by KLK−/− BM-MNCs as compared with vehicle (Figure 6A). Similarly, adductor muscle blood flow measurement using fluorescent microspheres denoted a significant improvement at 3 weeks with WT BM-MNCs, but not with KLK−/− BM-MNCs, over the vehicle (Figure 6B).

Immunofluorescence staining showed that capillary and arteriolar density in the ischemic adductor muscle was significantly higher in WT BM-MNC–injected group compared with vehicle- or KLK−/− BM-MNC–treated groups (Figure 6C).

KLK1 Downregulation in Type 2 Diabetic PACs

PACS functionality is impaired in diabetes, but underpinning mechanisms remain largely undefined.23 Because gene titration studies showed an effect of hK1 on distinct PACs activities, we next asked whether a defect in hK1 expression/activity is implicated in type 2 diabetes (T2D)-PACs dysfunctional phenotype. Consistently, we found that diabetes reduces the abundance of hK1100 cells within the PB-MNC pool, particularly in the CD34100 subfraction. The intensity of the fluorescent signal was diminished in all studied populations (Online Figure II, A). These changes were seen in the context of an overall decrease of specific PB-MNC subpopu-
lations (Online Figure II, B) and were mirrored by a remarkable reduction in EC- and monocyte-specific markers, ie, CD34, KDR, CXCR4, and CD14, on T2D-PACs compared with PACs from age-matched healthy controls (Online Figure II, C). Of note, whereas KLK1 mRNA levels were comparable in T2D- and healthy PACs, hK1 protein levels were significantly reduced in T2D-PACs (−57%, \(P<0.05\) versus healthy PACs; Figure 7A, i and ii), suggesting a post-transcriptional defect in hK1 synthesis. We next investigated microRNA (miR)-637, the only miR

Figure 3. KLK1 overexpression enhances PACs functions. A, Increased migratory (PACsmig) (i) and invasive (PACsinv) (ii) of Ad.KLK1-infected PACs (\(P<0.05\) vs Ad.Null or Ad.R53H; \(n=4\) to 6). B, Representative photographs and bar graph showing increased HUVEC networking in the presence of Ad.KLK1-PACs (\(P<0.05\) vs Ad.Null or Ad.R53H; \(n=4\)). Scale bar, 80 μm. C, MMP2 activity measured by gel zymography in PAC CM: representative experiment and bar graph showing densitometry data (\(P<0.05\) vs Ad.Null or Ad.R53H; \(n=3\)). D, In situ zymography analysis of MMP2 activity: representative microphotographs and bar graph showing abundance of FITCpos PACs (\(P<0.05\) vs Ad.Null or Ad.R53H, \(n=3\)). In negative control (Neg), FITC-conjugated reagent was omitted. Scale bar, 200 μm.

Figure 4. Mechanisms of hK1-induced effects. A, Migration (i) and invasion (ii) is increased in Ad.KLK1-PACs (control, ctr) (\(P<0.05\) vs Ad.Null) and inhibited by aprotinin (Aprot) (10\(^{-9}\) mol/L), kallistatin (Kallist) (10\(^{-9}\) mol/L) or icatibant (aB2R, 2\(^{-10}\) 10\(^{-7}\) mol/L), but not by L-dAL-BK (aB1R, 3\(^{-10}\) 10\(^{-8}\) mol/L) (n=4 in triplicate). iii, B2R antagonist MEN16132 (MEN) (10\(^{-6}\) mol/L) inhibitory effect on PAC migration (\(P<0.05\) vs Ad.Null, n=3 in triplicate). B, MMP2 activity (gel zymography) is increased in Ad.KLK1-PAC CM (\(P<0.05\) vs Ad.Null) and inhibited by kallistatin and icatibant (n=3) (i) and increased Ad.KLK1-PAC invasion is inhibited by MMP inhibitor GM6001 (GM) (15\(^{-10}\) 10\(^{-8}\) mol/L) (n=3) (ii). C, Bar graph showing Akt activity (ELISA) (i) and representative WB of phosphorylated and total Akt in PACs lysates (ii). Ad.KLK1-induced Akt activation (\(P<0.05\) vs Ad.Null) is inhibited by icatibant (2\(^{-10}\) 10\(^{-7}\) mol/L) (n=3). iii, iNOS protein levels in PACs lysates: bar graph showing densitometry data and representative WB (\(P<0.05\) vs Ad.Null; \(n=3\)). iv, Bar graph showing inhibition of Ad.KLK1-induced PAC invasion by the PI3K\(^{\alpha}\) inhibitor AS605240 (10\(^{-6}\) mol/L), the NOS inhibitor (LNIO) (10\(^{-7}\) mol/L), or the NO scavenger (PTIO) (10\(^{-7}\) mol/L) (n=3). D, Enhanced HUVEC networking by Ad.KLK1-PACs (\(P<0.05\) vs Ad.Null) is inhibited by aprotinin and kallistatin but not by icatibant, L-dAL-BK, or GM (n=4).
Figure 5. Deletion of the KLK1 gene results in MNCs and PACs dysfunction. A, Immunomagnetically sorted lineage negative (Lin<sup>-</sup>)-BM-MNCs from kallikrein knockout mice (KLK<sup>−/−</sup>) show reduced migration toward SDF-1α compared to corresponding cells from WT mice (*P<0.05 vs BSA; §P<0.05 vs WT; MNCs from n=7 mice per group were pooled and assayed in triplicate). B, Ad.KLK1 improves spontaneous and SDF-1α-stimulated migration of KLK<sup>−/−</sup> PACs (*P<0.05 vs BSA; §P<0.05 vs Ad.Null; WT stimulated with SDF-1α, #P<0.05 vs corresponding Ad.Null group, n=7 mice per group pooled and assayed in triplicate). C, ECs networking on Matrigel. Same symbols as in B.

Figure 2C, D). In diabetes, neither Ad.KLK1 nor Ad.B2R alone improved PAC invasiveness, but in combination they remarkably rescued the diabetic phenotype (P<0.05 vs Ad.Null+Ad.Null; Figure 8D), thus suggesting a cooperative hK1-B<sub>2</sub>R interaction.

Discussion

Classic chemoattractants/receptors pairs, like SDF-1α/CXCR4 and vascular endothelial growth factor/KDR, have been recently joined by the kinin/B<sub>2</sub>R pair as a potent mediator of PACs chemotaxis.13 Furthermore, PACs use proteases, like MMPs and cathepsins, to disrupt the extracellular matrix in the initiation of reparative neovascularization.4,23 However, the possibility that proteases could cooperate with chemokine receptors during PACs tissue invasion remains largely unexplored. Here, we reveal that hK1 coupling to B<sub>2</sub>R signaling is essential for PACs invasive capacity.

Despite persisting ambiguity about the precise circulating angiogenic/endothelial progenitors characteristics, CD34 is widely used for proangiogenic cells separation in human clinical trials.27 Furthermore, CD34 progenitors level strictly correlates to cardiovascular risk, especially in patients with metabolic syndrome or diabetes.22 Here, we demonstrate for the first time that, among freshly isolated MNCs, CD34<sup>pos</sup> cells represent the subpopulation that most abundantly bears membrane-bound hK1. Furthermore, hK1 was abundantly expressed by nonclassic CD16<sup>pos</sup>/CD14<sup>low</sup> monocytes compared to CD16<sup>neg</sup>/CD14<sup>high</sup> monocytes. The respective role of these 2 populations in postischemic reparative processes is matter of debate.28 The presence of hK1 in CD16<sup>pos</sup> monocytes might add to their angiogenic potential, however this remains to be explored. Interestingly, B<sub>2</sub>R is also highly abundant in CD34<sup>pos</sup> cells,13 and CD16 monocytes.29 HK1/B<sub>2</sub>R coexpression on specific progenitors and monocyte fractions suggests that KKS components might cooperate during recruitment and homing. Importantly, hK1 inhibition by kallistatin results in remarkable reduction of PACs enrichment from cultured MNCs, in line with our previous observation of reduced Flk<sup>10<sup>pos</sup>CD14<sup>high</sup> progenitors production in BM of KLK1<sup>−/−</sup> mice following limb ischemia induction.4 Thus, hK1 might be a potential biomarker of MNCs differentiation into cellular phenotypes implicated in the modulation of vascular functions.

HK1 relevance in PACs functions was strengthened using silencing and viral vector–mediated overexpression approaches. Importantly, KLK1 gene titration data indicate that PAC migration, invasion, and proangiogenic activities concordantly increase with hK1 levels. The most acknowledged pathway mediating PAC migration centers on the kinase Akt. B<sub>2</sub>R and CXCR4 receptors engagement by kinin and SDF-1α on human PACs is associated with this deficit being associated with reduced Akt activity (Figure 8A, ii and iii).

Ad.B<sub>2</sub>R infection resulted in B<sub>2</sub>R increased mRNA and protein expression in PACs (Figure 8B and 8C). Both Ad.KLK1 and Ad.B<sub>2</sub>R increased the invasive capacity of healthy PACs (*P<0.05 versus Ad.Null), with no additive effect after combined infection (Figure 8D). In diabetes, neither Ad.KLK1 nor Ad.B<sub>2</sub>R alone improved PAC invasiveness, but in combination they remarkably rescued the diabetic phenotype (P<0.05 versus Ad.Null+Ad.Null; Figure 8D), thus suggesting a cooperative hK1-B<sub>2</sub>R interaction.
polarized recruitment and activation of PI3Kγ on the cell membrane, phosphorylation/activation of Akt and acquisition of a migratory phenotype. Of note, hK1 overexpression activates Akt in PACs and β2R blockade by icatibant suppresses Akt activation and invasive capacity of hK1-transduced PACs. Surprisingly, neither eNOS protein expression nor activation by phosphorylation on the 2 known regulating serine residues (Ser113 and Ser1177) was modulated by hK1-forced expression. Conversely, we here report for the first time that NOS inducible form, iNOS, is indeed constitutively expressed by PACs and enhanced by hK1 transduction. Furthermore, we show that NOS inhibition or NO scavenge both abrogate the enhanced invasive capacity of hK1-transduced PACs. This data indicates that PACs are equipped with sensors, eg, the β2R, to capture chemokine guidance signals, and enzymatic machinery, eg, hK1, coupled to β2R to enhance PAC motility. Furthermore, PACs-secreted hK1 could modify the extracellular environment and thus facilitate PACs and vascular cells tissue invasion. This latter mechanism is seemingly β2R-independent because kallistatin but not icatibant is able to block hK1-transduced PACs promoting EC networking on Matrigel. It would be interesting to verify whether the same signaling mechanism drives the homing of proinflammatory cells in ischemic tissues.

Furthermore, we found that hK1 overexpression remarkably activates MMP2 through the β2R. Another MMP, MMP9, was shown to act as a downstream modulator of hK1 angiogenic activity in ischemia. It is likely that the interaction of hK1 with different MMPs is context-dependent. For example, unlike MMP9, MMP2 is activated under nonischemic conditions, such as aging-related vascular remodeling.

To gain insight into KLK1 inherited defects, we verified the functional phenotype of BM-MNCs and PACs from KLK1−/− mice. These mutant mice are reportedly unable to mount proper reparative angiogenesis and to mobilize Lin− veg progenitors into the circulation after ischemia.

Our present data show for the first time that KLK1−/− PACs are defective in their proangiogenic function, with such functional defect being ameliorated by hK1 transduction. In a mouse model of unilateral limb ischemia, KLK1−/− BM-MNCs failed to stimulate vascular repair thus highlighting hK1 essential role in promotion of postischemic neovascularization. Furthermore, we found that engineering human PACs with wild type KLK1 improves their invasive and proangiogenic capacities, whereas gene transfer with the loss-of-function polymorphic R53H-KLK1 variant is ineffective. This data complements previous work from our group demonstrating altered angiogenic/arteriogenic capacity of R53H-KLK1 in limb ischemia model. R53H-KLK1 polymorphism is especially common in Afro-Caribbean individuals, but also present as a heterozygous trait in 7% of whites. It would be of paramount importance to verify whether subjects carrying R53H-KLK1 mutation are prone to more complicated postischemic outcomes because of reparative angiogenesis defects.

Finally, we investigated whether hK1 is implicated in T2D-PACs dysfunction. When comparing T2D- and healthy PACs, we found an unexpected diversity in EC-
Figure 7. Reduced hK1 expression in T2D-PACs translates into reduced invasive and proangiogenic activities. A, T2D-PACs show normal KLK1 mRNA (Q-RT-PCR) (i) but reduced protein expression (WB) (ii). Q-RT-PCR data show similar levels of mir637 in healthy and T2D-PACs (iii) (*P < 0.05 vs healthy, n = 5 subjects per group, in triplicates). B, Spontaneous migration (i) and invasion (ii) of Ad.KLK1- or Ad.Null-infected PACs (*P < 0.05 vs Ad.Null-healthy PACs within healthy group, #P < 0.05 vs Ad.Null-healthy PACs, $P < 0.05 vs Ad.KLK1-healthy PACs, n = 3 per group). C, HUVEC networking in the presence of Ad.Null- or Ad.KLK1-PACs. n = 3; same symbols as in B. D, Gel zymography measuring MMP2 activity in Ad.Null- and Ad.KLK1-infected T2D-PAC CM. Representative experiment and average densitometry data (n = 3 subjects).

Figure 8. Combined KLK1-B2R overexpression rescues diabetes-induced PACs dysfunction. A, Bar graph showing similar abundance of B2R expressing cells within healthy or T2D-PACs (flow cytometry) (n = 3) (i); PAC invasion in response to BK (fold increase vs BSA; *P < 0.05 vs healthy PACs, n = 3) (ii); Akt activity in PACs lysates (*P < 0.05 vs healthy PACs, n = 3) (iii). B, B2R-forced expression in PACs. B2R mRNA expression levels normalized to HGPRT (n = 3, *P < 0.05). C, Representative WB of B2R in PACs lysates normalized to β-actin (i) and fluorescence immunocytochemistry of B2R-positive PACs (ii). Neg indicates TRITC-conjugated secondary antibody only; Ad.Null and Ad.B2R, transduced PACs stained with specific anti-B2R antibody plus TRITC-conjugated secondary antibody. Scale bar, 20 μm. D, Effect of Ad.Null, Ad.KLK1, Ad.B2R, or double Ad.KLK1 + Ad.B2R infection on spontaneous invasive activity of PACs from healthy (n = 3) and diabetic subjects (n = 5) (fold increase vs Ad.Null, *P < 0.05 vs Ad.Null + Ad.Null).

Surprisingly, hK1-forced expression could not revert the defective phenotype of T2D-PACs. Investigating possible causes for this failure, we found that the B2R is normally expressed on T2D-PACs, however it remains uncoupled from its downstream effector Akt. Noteworthy, combined hK1-B2R transduction did effectively rescue the impaired T2D-PACs invasive capacity. These data, supporting a key role of B2R in hK1-associated induction of PACs motility, introduces the new concept that implementation of functionally imperfect receptors by gene therapy could provide a means to restore proper coupling between components of the invasive machinery. It remains to be determined whether re-exposing transduced PACs to the diabetic environment may jeopardize their restored invasive function.

Taken together our results show for the first time that human PACs are equipped with active endogenous hK1 and that hK1 is crucial for PAC invasive and proangiogenic activities by both protease- and kinin receptor–mediated mechanisms. Importantly, PACs biological functions are impaired in conditions of hK1 deficit and restored by forced hK1 expression. Finally, this study demonstrates that KKS faulty components need to be repaired to restore the pristine PACs function in diabetes, thus providing the first proof of
concept for multiple gene engineering of a pathway crucially implicated in angiogenesis.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Proangiogenic cells contribute to extracellular matrix remodeling during tissue healing. This mechanism is impaired by diabetes.
- Kinin is a potent chemoattractant for circulating leukocytes and proangiogenic cells.

**What New Information Does This Article Contribute?**

- Distinct subpopulations of circulating mononuclear cells, including CD34<sup>pos</sup> cells and CD16<sup>pos</sup>/CD14<sup>low</sup> monocytes, express kallikrein, a kinin-generating enzyme.
- Gene titration studies indicate that kallikrein levels are relevant for migration, invasion, and promotion of endothelial network formation activities of human proangiogenic cells.
- Enhancement of proangiogenic cell activities by kallikrein overexpression implicates a mechanism encompassing the kinin B2 receptor, phosphoinositide 3-kinase (PI3K), Akt, and inducible nitric oxide synthase (iNOS).
- Proangiogenic cells that express an enzymatically inactive polymorphic variant of the kallikrein gene or an epigenetically modified version of the wild-type gene are defective in their invasive capacity.
- Functionally defective diabetic proangiogenic cells are rescued by combined transfer of kallikrein and kinin B2 receptor genes.

Blood-borne cells participate in the remodeling of an injured tissue by guiding the formation of new vessels. Invading cells use proteases to drill holes in the extracellular matrix for provisional vessels to expand and organize in a network. Findings of this study indicate that human proangiogenic cells are equipped with sensors to capture chemokine guidance signals and with enzymatic machinery, eg, kallikrein, which by coupling to kinin B2 receptor, triggers the PI3K-Akt-iNOS pathway to boost cell motion. We show that engineering human proangiogenic cells with wild-type kallikrein gene improves invasive and proangiogenic capacities, whereas gene transfer with the loss-of-function polymorphic *R53H* variant is unproductive. Finally, this study demonstrates that diabetes induces epigenetic modifications in the motility program and that this requires correction of multiple faulty components for functional rescue.
Tissue Kallikrein Is Essential for Invasive Capacity of Circulating Proangiogenic Cells
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SUPPLEMENTAL MATERIAL

Detailed Methods
Human studies
All procedures were performed in accordance with the Declaration of Helsinki, after obtaining approval of the local ethics committee (Multimedica IRCCS). All the subjects enrolled for this study gave informed consent to participate. Type 2 diabetic (T2D) patients (n=22) were free of retinal, kidney, or cardiac complications. Hypertension (PAOS > 140/85) was not treated at the time of blood withdrawal. Age-matched subjects (n=16) free of evident diseases served as controls. Patients’ characteristics are summarized in Table 1.

Human MNCs isolation and PACs selective culture
Both human and murine cells were isolated as previously described. Briefly, human peripheral blood mononuclear cells (PB-MNCs) were isolated by gradient centrifugation on Histopaque-1077 density medium (Sigma). For enrichment of circulating proangiogenic cells (PACs), 1x10⁷ MNCs/well were plated on fibronectin (Sigma)-coated 6-well plates (BD Falcon) and cultured for 4 days in EBM-2, supplemented with EGM-2 MV SingleQuots and 10% FBS (EGM-2MV; Cambrex).

KLK1⁻/⁻ mice and hindlimb ischemia model
All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, 1996) and were approved by INSERM. KLK1⁻/⁻ mice were generated by targeted gene inactivation, as previously described and the mutation was backcrossed to a pure C57/BL6 genetic background. Six-month-old male KLK1⁻/⁻ mice generated by heterozygous crossing and their wild type (WT) littermates were studied. BM-MNCs cells were isolated by flushing mice femurs and tibiae with 10% FBS-DMEM, and separating the cell fraction by gradient centrifugation using Histopaque-1083 density medium (Sigma). Eight mice were used per group (KLK1⁻/⁻ and WT). PACs were enriched as described above for the human counterpart.

To demonstrate the potential role of hK1 in BM-MNCs proangiogenic capacities, 1X10⁶ PKH26-labeled (Sigma) BM-MNCs from either WT or KLK1⁻/⁻ mice were systemically injected through the tail vein of C57/Bl6 mice (n=10 mice per group), one day after unilateral hindlimb ischemia as described. Blood flow recovery was followed up by laser Doppler (Moor instruments, UK) at days 7, 14 and 21 after ischemia. Neovascularization in the ischemic adductor muscle was assessed by immunofluorescence microscopy on PFA-fixed frozen sections (8 µm) harvested at day 21 after ischemia (n=5 mice per group) using isolectin B4 and α-smooth muscle actin staining to recognize ECs and smooth muscle cells, respectively. Capillaries and arterioles were calculated in at least 5 fields at X50 magnification and the final data expressed as the number of capillaries or arterioles per mm².

Flow Cytometry
PACs (2x10⁵) were stained for surface antigen expression using combinations of the following antibodies: anti-CD133/2-APC (Miltenyi), anti-KDR-PE (R&D Systems), anti-CD45-FITC, anti-CD34-PE, anti-CXCR4-PE-Cy5, anti-CD14-APC and anti-CD11b-PE-Cy7 (all BD Pharmingen). To detect membrane protein expression, the analysis was performed without cell permeabilization, using specific fluorescence-conjugated antibodies as indicated. To analyze hK1 expression, PACs were stained with mouse anti-hK1 antibody (R&D Systems). Briefly, cells were incubated with 1µg of anti-hK1 antibody for 15 min at RT. After incubation, cells were washed and incubated with anti-mouse-PE (Sigma) (1:50 in PBS) for 15min at RT. For AcLDL and UEAI staining, Dil-labelled acetylated lipoproteins (Invitrogen) were added to the PACs culture for 40min, then FITC-labelled UEAI (Sigma) was added for 20min. After incubation, cells were detached with 5mM EDTA and analyzed. Fluorescence was analyzed using a FACS Canto flow cytometer and FACS Diva software (both BD). To control for specificity, an aliquot of cells was stained with secondary antibody only.
Adenoviral vectors

*Ad.KLK1* and *Ad.R53H-KLK1* were prepared as described previously. Briefly, coding sequence was digested from previously described pcDNA3 vectors. Further BamHi-cut fragments were ligated into the adenoviral shuttle vector pDC515 (Microbix Biosystems, Toronto, Canada). Replication deficient adenoviruses were generated by site-specific FLP-mediated recombination of the co-transfected shuttle and genomic plasmids in 293 cells. Production of adenovirus stocks was performed as described, and confirmed by real time PCR using primers that recognized both human KLK1 variants (*KLK1*: TGACAGAGCCTGCTGATACC and *R53H-KLK1*: TCACCCACACAGGTGTCTTT). Results were confirmed by ELISA assay, in which quantification of hK1 protein in the supernatants of infected cells was assessed as previously described. PACs infection was performed using 250 moi adenovirus for 24 hours. For the adenovirus harbouring the human bradykinin B2 receptor (*Ad.B2R*), the coding sequence was excised from a pcDNA3 clone (a gift from Dr. Anne Pizard) and subcloned into a modified pDC515 shuttle vector (Microbix) containing an internal ribosome entry site that allows the B2R and GFP to be translated from a bicistronic mRNA. The adenovirus was generated by site specific recombination of shuttle and genomic plasmids in 293 cells.

**KLK1** RNA silencing

For siRNA protocols, pre-annealed purified siRNA probes were obtained from Dharmacon and were rehydrated prior to transfection following the manufacturer’s instructions. SiRNA used were a pool of four specific RNAs targeting *KLK1* sequence. A non-targeting functional siRNA pool of probes was used as control (scrambled). siRNA probes (50 and 100 nM) were transfected using Gene Silencer (Gene Therapy Systems). After 24 hours of transfection, PACs were harvested for further analysis.

**ELISA**

One 96-well plate coated with anti-human hK1 (1µg/ml) was blocked with 1% BSA (Sigma-Aldrich) and then washed. Human hK1 standard (0.016 ng/mL to 1 ng/mL) or samples in duplicates (dilute 1:5) were added into each well for 1.5h at 37°C. Incubation with biotin-labelled anti-hK1 immunoglobulin G (1 µg/ml) was followed by avidin-peroxidase conjugated antibody (1 µg/mL). After washing, 0.3 mg/mL of 2, 2'-azino-bis (3-ethylbenzthiazoline) sulfuric acid (diluted in 0.1 M citric buffer, pH 4.3) was added to develop the reaction and absorbance was read at 405 nm 15 min later. Standard curve was calculated to extrapolate sample concentration. The assay was carried out in triplicate for *Ad.Null* and...
Ad.KLK1-PACs CM samples. Cellular Akt activity was measured in PACs lysates using Akt/PKB Kinase Activity Assay Kit (Assay Designs) following the manufacturer’s instructions.

**Amidolytic assay**
To measure hK1 activity in PACs supernatant, 30µL of undiluted PACs CM (obtained from 10^6 cultured cells) was added in quadruplicate in a 96-well plate. For each sample, a duplicate was incubated with 100µL of Tris buffer 0.2M (pH 8.2) and a duplicate with the same buffer containing 50U/mL of Trasylol (aprotinin, hK1 inhibitor from Bayer, Leverkusen, FRG). Finally, 50µL of the colorimetric substrate S-2266 (1.5mM, AB Kabi Diagnostica) was added and the reaction incubated overnight at 37°C. Development of the reaction was detected at 405nm.

**Western Blotting and Zymography**
Fifty µg of whole cell lysates were resolved by SDS-PAGE and transferred onto PVDF membrane (Immobilon). The membranes were blocked in Tris-buffered solution containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) BSA for 1 hour and then incubated overnight with anti-hK1 (R&D, 1:250), anti-B2R (BD, 1:1000), anti-iNOS (Cell Signaling, 1:1000), anti-Akt and phosphor-Akt (both Cell Signaling, 1:1000) or anti-β-actin (1:10,000, Sigma) antibody in 5% milk/TBST. HRP-conjugated IgG (Amersham Pharmacia Biotech) were used as secondary antibodies and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). To determine the gelatinolytic activity of MMP, both in situ and PAGE zymographies were performed according to the modified protocol described previously. Briefly, PAGE gel-zymography was performed by separating cells lysates (50µg) or conditioned media (CM, 20µL) on standard SDS-PAGE gels with added 0.1% gelatin. After running, gels were treated with renaturing and developing buffers following the manufacturer’s instructions (Invitrogen). Digested clear bands were visualized by comassie blue staining of the gels. In situ zymography was conducted on cells cultured on glass chamber slides. Zymogram developing buffer (40 µg/mL) and DQ gelatine fluorescein conjugated substrate (Molecular Probes) were added to the cells and incubated overnight at 37°C. Cells were washed and slides mounted using DAPI-containing Vectashield (Vector Laboratories). The reaction was observed using the fluorescence microscope (Axiovision, Zeiss).

**Immunofluorescence staining**
For immunofluorescence and in situ zymography PACs were cultured in fibronectin-coated 8-well chamber slides (Nunc) as described above. After fixation, slides were incubated overnight at 4°C with primary antibodies diluted in accordance with the manufacturer’s instructions. Fixed cells were further incubated with secondary, fluorescence conjugated antibodies, and photomicrographs were acquired by a computer-imaging program (AxioVision Imaging System, Zeiss).

**Migration and invasion assays**
For the invasion assay, modified Boyden chambers were equipped with 5 µm pore-size polycarbonate filters (Costar) coated with Matrigel (BD Biosciences). SDF-1α (R&D) (100 ng/mL), bradikinin (BK) (3X10^{-8}M) or BSA (control) was added to the lower chamber in 220 µL of MM (MM: EBM, 0.1% BSA). PACs (10^6/mL) were placed in the upper chamber in 200 µL of MM. For the migration assay, 5 µm pore-size filter-equipped transwell chambers (Corning) coated with fibronectin were used (100 µg/mL). PACs (5x10^5) were placed in the upper chamber, and allowed to migrate toward SDF-1α or BSA, as described above. The assays were stopped after 16 hours at 37°C, and cells that had invaded the membrane and migrated through the filter were fixed on the lower side of the filter and mounted with Vectashield containing DAPI. Five random fields were counted at 20X magnification for each chamber. Inhibitors or their vehicle were added to both the upper and the lower chamber.

**Matrigel assay**
Briefly, 50X10^3 PHK67-labelled (Sigma) Ad.KLK1-PACs or Ad.Null-PACs were added to 8-well chamber slides pre-coated with 100µL Matrigel (Becton Dickinson), together with 50X10^3 unstained
HUVECs in a total volume of 200µL EBM-2 0.1%BSA. After 12 hours incubation at 37°C, floating cells were removed by washing and capillary-like structures were fixed in 2% paraformaldehyde and mounted with DAPI-containing fluorescence medium. The assays were performed in triplicate wells. Tube formation was measured by counting the number of intersection points (magnification 20X) in 8 random fields of view photographed using a FITC-specific filter setting to concomitantly observe the presence of PACs.

Supplemental References

**Online Table I. Human subjects characteristics.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diabetic Group (n=22)</th>
<th>Control Group (n=16)</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>59.8 ± 9.7</td>
<td>49.2 ± 12.3</td>
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<tr>
<td>Male sex, No. (%)</td>
<td>13 (59%)</td>
<td>5 (31%)</td>
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<tr>
<td>Current smoking, No. (%)</td>
<td>4 (25%)</td>
<td>5 (42%)</td>
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<tr>
<td>Hba1c level (%)</td>
<td>6.6 ± 0.8</td>
<td>N.D.</td>
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<tr>
<td>BMI</td>
<td>30.2 ± 6.4</td>
<td>25.2 ± 3.5</td>
</tr>
<tr>
<td>LDL (mg/mL)</td>
<td>123.6 ± 27.2</td>
<td>114.4 ± 12.5</td>
</tr>
<tr>
<td>Years of diabetes</td>
<td>7.2 ± 7.5</td>
<td>-</td>
</tr>
<tr>
<td>Metformin therapy, No. (%)</td>
<td>18 (82%)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data expressed as mean+SEM.
Online Figure I: Adenovirus-mediated KLK1 infection increases the expression of hK1 in PACs. 
A) mRNA expression levels by Q-RT-PCR normalized to HGPRT. B) Representative western blotting of hK1 in PACs lysates normalized to β-actin. C) Secreted hK1 levels assessed by ELISA in PACs CM (*p<0.05 vs. Ad.Null, n=3). D) hK1 enzymatic activity measured by amidolytic assay in PACs CM. (*p<0.05 vs. Ad.Null, n=3). E) Bradykinin (BK) levels assessed by ELISA in PACs CM (n=3).
Online Figure II: Reduced hK1 expression in PB-MNCs from type 2 diabetic patients. A) Bar graphs showing the percentage of hK1\(^{\text{pos}}\) cells among the indicated PB-MNCs populations (i) and hK1 fluorescence intensity among antigenically defined PB-MNCs (ii). (n=6 subjects per group, *p<0.05 vs. healthy). B) Bar graph showing the antigenic profile of MNCs from type 2 diabetic patients compared to healthy controls. (n=3 subjects per group, *p<0.05 vs. healthy). C) Bar graph showing the antigenic profile of PACs from type 2 diabetic patients as compared to healthy controls. (n=6 subjects per group, *p<0.05 vs. healthy).