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Critical Role of Tissue Kallikrein in Vessel Formation and Maturation:

Implications for Therapeutic Revascularization

Oliver A. Stone, **Christine Richer**, **Costanza Emanueli**, **Vincent van Weel**, **Paul H.A. Quax**, **Rajesh Katare**, **Nicolle Kraenkel**, **Paola Campagnolo**, **Luciola S. Barcelos**, **Mauro Siragusa**, **Graciela B. Sala-Newby**, **Danila Baldessari**, **Marina Mione**, **Marie P. Vincent**, **Andrew V. Benest**, **Ayman Al Haj Zen**, **Julien Gonzalez**, **David O. Bates**, **Francois Alhenc-Gelas**, and **Paolo Madeddu**

Microvascular Research Laboratories, Bristol Heart Institute, Department of Physiology and Pharmacology (O.A.S., A.V.B., D.O.B.), University of Bristol, UK; INSERM U652/U872, Centre de Recherche des Cordeliers (C.R., M.P.V., F.A.-G), Paris, France; Experimental Cardiovascular Medicine (C.E., R.K., N.K., P.C., L.S.B., M.S., A.A.H.Z., P.M.) and Vascular Biology Division (G.B.S.-N), Bristol Heart Institute, University of Bristol, UK; The Gaubius Laboratory TNO Quality of Life (V.v.W., P.H.A.Q.), Leiden, the Netherlands; Department of Surgery (V.v.W., P.H.A.Q.), Leiden University Medical Center, Leiden, the Netherlands; IFOM, Fondazione Istituto FIRC di Oncologia Molecolare (D.B., M.M.), Milan, Italy; and I2MR INSERM U858 (J.G.), Toulouse, France

Abstract

Objective—Human Tissue Kallikrein (*hKLK1*) overexpression promotes an enduring neovascularization of ischemic tissue, yet the cellular mechanisms of *hKLK1*-induced arteriogenesis remain unknown. Furthermore, no previous study has compared the angiogenic potency of *hKLK1*, with its loss of function polymorphic variant, rs5515 (R53H), which possesses reduced kinin-forming activity.

Methods and Results—Here, we demonstrate that *tissue kallikrein* knockout mice ($KLK1^{-/-}$) show impaired muscle neovascularization in response to hindlimb ischemia. Gene-transfer of wild-type *Ad.hKLK1* but not *Ad.R53H-hKLK1* was able to rescue this defect. Similarly, in the rat mesenteric assay, *Ad.hKLK1* induced a mature neovasculature with increased vessel diameter through kinin-B_2 receptor-mediated recruitment of pericytes and vascular smooth muscle cells, whereas *Ad.R53H-hKLK1* was ineffective. Moreover, *hKLK1* but not *R53H-hKLK1* overexpression in the zebrafish induced endothelial precursor cell migration and vascular remodeling. Furthermore, *Ad.hKLK1* activates metalloproteinase (MMP) activity in normoperfused muscle and fails to promote reparative neovascularization in ischemic $MMP9^{-/-}$ mice, whereas its proarteriogenic action was preserved in $ApoE^{-/-}$ mice, an atherosclerotic model of impaired angiogenesis.

Conclusions—These results demonstrate the fundamental role of endogenous Tissue Kallikrein in vascular repair and provide novel information on the cellular and molecular mechanisms responsible for the robust arterialization induced by *hKLK1* overexpression.

Correspondence to Prof Paolo Madeddu, Experimental Cardiovascular Medicine, Bristol Heart Institute, Level 7, Bristol Royal Infirmary, Upper Maudlin Street, University of Bristol, Bristol, BS2 8HW, United Kingdom. madeddu@yahoo.com.

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Keywords

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Peripheral artery disease (PAD) is a major global economic healthcare burden, with dissatisfactory treatment strategies available to date. Pleiotropic agents, such as proteases able to interfere at various levels of the angiogenic program, could represent a valuable option for the treatment of ischemic disease. The catalytic class of serine proteases, which encompasses trypsin-like enzymes, has recently been implicated in angiogenesis.1,2

Our group has proposed another member of this class, human tissue kallikrein 1 (*hKLK1*), as a potent factor for promotion of therapeutic neovascularization, as indicated by studies in animal models of ischemia.3,4 *hKLK1* can exert its effects via kininogen cleavage, thus generating kinins (eg, bradykinin - BK or kallidin - kDa).5 Kinins, such as BK, activate kinin-B_2 receptors (B_2R), which are constitutively expressed on endothelial cells (ECs), and kinin-B_1 receptors (B_1R), whose expression is induced under stress conditions.5 Overexpression of *hKLK1* promotes a robust and persistent neovascularization through kinin-mediated activation of the Akt-eNOS pathway, and has recently been shown to display tissue specific VEGF dependency.4,6 It is not known, however, whether *hKLK1* could promote the activation of other proangiogenic enzymes like metalloproteinases (MMP). Furthermore, although studies on *B_2R* or *B_1R* knockout mice have established the role of kinin receptors in reparative angiogenesis,7-9 no information is available on the importance of endogenous kallikrein in postischemic vascular healing. This issue is relevant especially in the light of recent demonstration that function-defective variants of the *hKLK1* gene are frequent in the general population. In man, a common missense polymorphism in exon 3 of the *hKLK1* gene (rs5515), replacing an active site arginine at position 53 with a histidine (*R53H-hKLK1*), results in a major loss of kinin-forming activity.10 The mutation is associated with inward remodeling of the brachial artery, which is not adapted to a chronic increase in wall shear stress, indicating a new form of arterial dysfunction.11

Here, we provide evidence that deletion of the murine *KLK1* gene is deleterious for reparative neovascularization. Moreover, the *R53H-hKLK1* variant leads to an immature neovascularization. Furthermore, we demonstrate that levels of *hKLK1* are relevant for the promotion of developmental vasculogenesis, arteriogenesis in the rat mesenteric assay, and postischemic neovascularization in the mouse ischemic hindlimb muscle.

Methods

For supplemental methods please see http://atvb.ahajournals.org.

Animal Models

Unilateral Ischemia was induced in male age-matched *tissue kallikrein* knockout (*$KLK1^{-/-}$*), *$MMP9^{-/-}$*, *$ApoE^{-/-}$*, and appropriate wildtype (WT) control mice. Limb blood flow recovery was assessed by Laser Doppler flowmetry. Capillary and arteriole densities were evaluated postmortem by immunohistochemistry.

The rat mesenteric angiogenesis assay was used to investigate the cellular mechanisms of *hKLK1*-induced angiogenesis. Male rats were anesthetized and a laparotomy performed under sterile conditions. The mesenteric panels were imaged intravitally. Adenoviruses of interest were injected into the mesenteric fat pad. The animal was sutured and allowed to recover. Six days later the same mesenteric panel was located and imaged. After in vivo fixation, staining for blood vessel cell markers (isolectin B4 for endothelium; NG2 for

pericytes; αSMA for vascular smooth muscle cells [VSMCs]) was imaged by confocal microscopy.

The zebrafish was used to test the effect of *hKLK1* on prenatal vasculogenesis. *Tg(fli1:egfp)*^{y1} zebrafish embryos were injected with 10 to 50 pg of *hKLK1* mRNA at the one-two cell stage. *Tg(fli1:egfp)*^{y1} zebrafish embryos were first examined under a fluorescence microscope at 20 hours after fertilization (hpf) and then regularly every 2 hours until they reached 42 hpf. Half of the injected embryos were fixed in 4% paraformaldehyde at 24 hpf, and the remainder were allowed to progress until 48hpf. Fixed embryos were stained with anti-GFP serum and imaged. To study the fate of supernumerary GFP$^+$ cells, ectopic GFP$^+$ cells were visualized in living embryos and subjected to time-lapse analysis with a Leica SP2 confocal microscope as previously described.

In Situ Zymography

Gelatinolytic in situ zymography was performed as previously described.12

Pericyte Wound Healing Assay

Bovine retinal pericytes (BRP) were grown to confluence on fibronectin/gelatin-coated 48-well plates in complete medium (DMEM supplemented with 1g/L glucose, L-Glutamine and 20% FBS). BRPs were subjected to the scratch wound assay as previously described and then incubated in the conditioned medium of bovine retinal endothelial cells (BREC) infected with *Ad.hKLK1, Ad.Null*, or control medium (250 MOI). Conditioned medium was diluted 1:100 in DMEM with 2% FBS and 2 mmol/L hydroxyurea (to block cell proliferation) with or without the addition of B_2R antagonist Icatibant (5×10^{-7} mol/L). After 24 hours gap closure was calculated.

FACS Analysis of Mononuclear Cells

Mononuclear cells (MNCs) from peripheral blood (PB) and BM of *KLK*$^{-/-}$ and WT mice were analyzed for the expression of cKit, Sca-1, and lineage antigens by flow cytometry (FACS Calibur).

Statistical Analysis

Results are expressed as distribution of sample value or mean±SEM. $P<0.05$ was considered statistically significant. Comparisons between means of data were performed using the Student *t* test, or ANOVA when comparing more than 2 groups. Posthoc Mann–Whitney tests were used when ANOVA showed overall $P<0.05$.

Results

Impaired Reparative Neovascularization in Kallikrein Knockout Mice: Rescue by Wild-Type hKLK1 but not Mutant R53H-hKLK1 Gene Transfer

To evaluate the importance of endogenous *KLK1* in reparative angiogenesis, we applied the classical model of unilateral limb ischemia to *KLK1*$^{-/-}$ mice and WT littermates.4 In previous studies, *KLK1*$^{-/-}$ mice showed impaired flow-dependent vasodilatation of large arteries,13 without obvious alterations in vascular structure.14 Consistently, vascular density was similar in normoperfused muscles from *KLK1*$^{-/-}$ and WT mice (data not shown). However, the neovascularization response to limb ischemia was profoundly altered at capillary (Figure 1A) and arteriole (Figure 1B) level, which led to delayed perfusion recovery of the ischemic limb of *KLK1*$^{-/-}$ mice (Figure 1C and 1D). Peripheral ischemia promotes the activation and mobilization of bone marrow Lin$^-$Sca1$^+$cKit$^+$ hematopoietic progenitor cells with proangiogenic capacity15 and Flk1$^+$cKit$^+$ vascular progenitor cells.16

We found that $Lin^{-}Sca1^{+}cKit^{+}$ and $Flk1^{+}cKit^{+}$ progenitor cell activation by ischemia is blunted in *KLK1*$^{-/-}$ mice (supplemental Figure I). Altogether, these results indicate that *KLK1* is important for the activation of postischemic reparative responses at both local and bone marrow levels.

We then evaluated whether *Ad.hKLK1* and the mutant *Ad.R53H-hKLK1* are equipotent in promoting reparative angiogenesis in WT and *KLK1*$^{-/-}$ mice. Gene expression was confirmed by RT-PCR (supplemental Figure II). As shown in Figure 2, *Ad.hKLK1* gene therapy was able to improve the blood flow to the ischemic limb especially in *KLK1*$^{-/-}$ mice, whereas *Ad.R53H-hKLK1* failed to produce any benefit. In both WT and *KLK1*$^{-/-}$ mice, *Ad.hKLK1* increased capillary density (Figure 2B and 2E and supplemental Figure II; $P<0.01$ and $P<0.001$ versus *Ad.Null*, respectively) and arteriole density (Figure 2C and 2F; $P<0.001$ versus *Ad.Null* for both comparisons), whereas *Ad.R53H-hKLK1* did not affect arteriole density, only increasing capillary:myocyte ratio in the *KLK1*$^{-/-}$ ($P<0.01$ versus *Ad.Null*).

hKLK1 Promotes Arteriogenesis in the Rat Mesentery

We then chose to characterize the cellular mechanisms of *Ad.hKLK1*-induced angiogenesis in a nonischemic model, the mesenteric angiogenesis assay (supplemental Figure IIIa). Successful transduction of the mesentery was demonstrated by immunohistochemistry and ELISA (supplemental Figure IIIb).

Neovascularization of the mesenteric connective tissue panel was assessed by intravital microscopy (supplemental Figure IV). Quantification of the functional vessel area (FVA) on day 6 versus day 0 demonstrated that *Ad.hKLK1* gene transfer increases tissue perfusion as compared with *Ad.Null* ($P<0.001$, supplemental Figure IVa). Icatibant, a selective B_2R antagonist, did not block the angiogenic effect of *Ad.hKLK1*, however neovessels at the apical edge displayed signs of microhemorrhaging. Importantly, the polymorphic variant *R53H-hKLK1* displayed a lower angiogenic capacity as compared with the wild type form ($P<0.05$), at both 5×10^5 and 5×10^6 pfu (supplemental Figure IVb).

We then used confocal microscopy to characterize *hKLK1*-induced neovascularization, with focus on vessel diameter and length, and pericyte and VSMC recruitment. Figure 3A shows composite Z-stack confocal images of whole-mount mesentery after *Ad.hKLK1* or *Ad.Null* injection. Morphometric analysis indicates that *hKLK1* overexpression increases vessel density and diameter (Figure 3B and 3C). The increase in vessel density was mainly ascribable to conduit vessels (16 to 35 μm diameter, $P<0.001$ versus *Ad.Null,* Figure 3D), leading to a rightward shift of vessel distribution toward larger calibre vessels (Figure 3E). Furthermore, the *Ad.hKLK1*-treated mesentery showed more proliferating vessels (Ki67 positive, 36±2 versus 2±1% of total vessels in *Ad.Null, P*<0.01). Importantly, *Ad.hKLK1* induced a striking increase in pericyte and VSMC fractional coverage (57±5 versus 32±2% and 4±1 versus 0, respectively, versus *Ad.Null, P*<0.001 for both comparisons, Figure 3F and 3G). Further analysis indicated that *Ad.hKLK1* produces vessels with high branch point density (270±25 versus 150±10 n/mm^2 in *Ad.Null, P*<0.01, supplemental Figure Va), but low sprout density (7.5±1.5 versus10.5±3.0 n/mm^2 in *Ad.Null,* supplemental Figure Vb). We then explored the contribution of B_2R to *hKLK1*-induced vascular remodelling. We found that B_2R colocalizes with pericytes in *Ad.Null* and *Ad.hKLK1* injected mesentery (supplemental Figure VIa). In addition, the B_2R antagonist icatibant inhibited the stimulatory action of *Ad.hKLK1* on the formation of larger vessels (Figure 3D and 3E) and recruitment of pericytes and VSMCs (Figure 3F and 3G). Hence, after B_2R blockade, *Ad.hKLK1*-induced neovascularization consisted of small-size sprouting vessels (sprout point density: 18±4 *Ad.hKLK1* plus icatibant versus 8±2 n/mm^2 in *Ad.hKLK1* alone,

P<0.05, supplemental Figure Vb). These data indicate that *hKLK1* overexpression induces the formation and growth of mature vessels through a B_2R-mediated mechanism.

The polymorphic variant *Ad.R53H-hKLK1* was unable to increase vessel density and diameter to the levels observed with wild-type *Ad.hKLK1* (Figure 4A through 4C) and also displayed reduced capacity in recruiting pericytes and VSMCs (Figure 4D and 4E).

hKLK1 Induces In Vitro Pericyte Migration

To further confirm that pericytes are activated by *hKLK1*, we performed an in vitro scratch assay in which bovine pericytes were stimulated with the conditioned medium of ECs infected with *Ad.Null* or *Ad.KLK1*. Results indicate that the conditioned medium from *hKLK1*-infected ECs stimulated pericyte migration (gap closure, 75±7 versus 58±3% in *Ad.Null, P*<0.05), with this effect inhibited by icatibant (gap closure, 58±2%, *P*=N.S. versus *Ad.Null*).

Vascular Remodeling by hKLK1 Gene Delivery in Zebrafish

We then evaluated the effects of *hKLK1* overexpression in a complex in vivo developmental system, the zebrafish. Components of the kallikrein-kinin system are expressed in zebrafish including homologues of B_1R, B_2R and Kininogen.17 We injected *hKLK1* and *R53H-hKLK1* mRNA into 1 to 2 cell stage zebrafish embryos of the transgenic line *tg(fli1:egfp)*^{y1}, which express GFP in hemangioblasts, ECs, and migrating leukocytes.18 Antibodies against *hKLK1* demonstrated ubiquitous expression in *hKLK1* mRNA injected embryos and expression of a zebrafish homologue in noninjected embryos (supplemental Figure VII). Injecting *R53H-hKLK1* mRNA or up to 1 ng of mRNA encoding for *hKLK1* did not affect vasculogenesis. Injecting 1.5 ng or 2 ng of *hKLK1* mRNA led to a reproducible phenotype, which becomes visible just before the sprouting of intersegmental vessels (ISV) from the dorsal aorta (DA), ie, around 24-somite stage, in approximately 35% of injected embryos. Some ECs (1 to 4 per embryo) were found in ectopic positions along the trunk, detached from the ISV sprouts and from the DA (supplemental Figure VII, supplemental movies I and II). These cells (which were twice as large as leukocytes/tissue macrophages found on the ventral side of the embryos) do not migrate from the position where they appear, but behave like endothelial tip cells (when observed by confocal microscopy), ie, extend several filopodia and protrusions that sense the environment. Later, they are reached by the ISV sprouts and become incorporated in the growing vessels. The ectopic *fli1:egfp* expressing cells did not express VE-cadherin before being incorporated in the nascent vasculature, thus suggesting that they might be undifferentiated hemangioblasts or macrophages that later transdifferentiate to ECs.

Involvement of MMP9 in hKLK1-Induced Neovascularization

We delivered *Ad.hKLK1* and *Ad.Null* to normoperfused adductor muscles. Three days after injection, *Ad.hKLK1* increased the cleavage of DQ MMP substrate compared with *Ad.Null* and this increase was blocked by the Zn^{2+} chelator, EDTA and the specific inhibitor of MMP activity, Galardin (Figure 5A). *Ad.hKLK1* delivery to ischemic muscles accelerated the recovery of WT control mice but not of *MMP9*$^{-/-}$ mice (Figure 5B). Furthermore, *Ad.hKLK1* promoted neovascularization at both the capillary and arteriole level in WT but not in *MMP9*$^{-/-}$ mice (Figure 5C and 5D).

To exclude that failure of Ad.hKLK1 to promote angiogenesis in MMP9$^{-/-}$ mice is unspecific (eg, ascribable to a generic unresponsiveness to exogenous stimulation), we evaluated the healing action of hKLK1 gene transfer in ApoE$^{-/-}$ mice, which share with MMP9$^{-/-}$ mice the same genetic background and reduced capacity to build arterial collaterals in response to ischemia. To test this, intramuscular injection of Ad.hKLK1 or

control Ad.LacZ was performed at the time of operative femoral artery occlusion. Because we had previous evidence that collateral artery development is inversely correlated to plasma cholesterol levels,19 the study was conducted in ApoE$^{-/-}$ mice fed on a cholesterol-enriched high-fat diet (cholesterol levels were 39±8 mmol/L and 37±5 mmol/L, for Ad.LacZ and Ad.hKLK1 group, respectively; P=N.S.). RT-PCR confirmed no difference in hKLK1 or murine B_2R mRNA between MMP9$^{-/-}$, ApoE$^{-/-}$, and WT mice (supplemental Table I). Laser-Doppler perfusion in the ischemic limb was 2.2-fold and 1.6-fold increased in Ad.hKLK1-treated mice as compared with Ad.LacZ-treated mice, at 3 and 7 days after femoral artery occlusion (P<0.01 and 0.02, respectively; Figure 6A).

We then conducted postmortem angiography studies to determine whether the improved perfusion recovery after *Ad.hKLK1* treatment correlates with an increased score of visible collateral arteries. Angiography was technically successful in 7 *Ad.hKLK1*-treated and 8 *Ad.LacZ*-treated mice. Of the *Ad.hKLK1*-treated mice, 7/7 mice (100%) demonstrated well-developed collateral arteries with moderate to good distal filling of the femoral artery, whereas only 4/8 of the *Ad.LacZ*-treated mice (50%) showed collateral arteries with distal filling. The 4 remaining mice in the *Ad.LacZ* group showed no filling of the femoral artery distal to the occlusion (Figure 6B). The angiographic Rentrop score of collateral arteries was 1.9-fold increased in *Ad.hKLK1*-treated mice as compared with control mice (P<0.05, Pearson Chi-square test; Figure 6C). These data were further supported by immunohistochemical studies indicating that *Ad.hKLK1* treatment improves collateral artery development in this model (Figure 6D, P<0.01 versus *Ad.LacZ*), leading to a 1.5-fold increase in vascular area (28.4±3.9 versus 18.7±3.2×10^3 μm^2 in *Ad.LacZ*, P<0.05). Furthermore, *Ad.hKLK1* treatment increased vessel pericyte coverage (Figure 6E and 6F, P<0.001 versus *Ad.LacZ*).

Discussion

Delivering therapeutic genes into the cardiovascular system represents an attractive approach for the cure of ischemic disease. Although a large number of preclinical studies around the world have highlighted the potential of master angiogenic factors such as VEGF and FGF family members, clinical studies have shown disappointing results. Pleiotropic agents like *hKLK1* have shown potential, but their therapeutic activity has mainly been validated in single laboratories. In the present study, different groups were engaged in validating the arteriogenic action of *hKLK1*. Results consistently demonstrated that *hKLK1* overexpression stimulates a well-organized and functional neovascularization, whereas the polymorphic variant, *R53H-hKLK1*, is devoid of such capacity.

This study newly demonstrates the importance of endogenous kallikrein in vascular repair. We document that *KLK1*$^{-/-}$ mice display an altered neovascularization response to limb ischemia resulting in profoundly delayed hemodynamic recovery. *KLK1*$^{-/-}$ mice also show an impaired postischemic activation of bone marrow cKit$^+$Flk1$^+$ vascular progenitor cells. Stem cells of the BM are reportedly activated after ischemia by the cooperation of multiple mechanisms involving proteases and nitric oxide (NO) bioavailability.20 We recently demonstrated that kinins exert potent chemoattractant effects on human CD133$^+$CD34$^+$ and murine Lin$^-$cKit$^+$ progenitor cells through a PI3K/Akt/eNOS-mediated mechanism.21 We also know from pilot studies that human EPCs express *hKLK1* and that inhibition of *KLK1* by aprotinin or kallistatin results in reduction of EPC invasive capacity (Spinetti et al, unpublished results). Thus, the endogenous kallikrein-kinin system might play a role in multiple steps of postnatal vasculogenesis. Delivery of *hKLK1* mRNA to the developing zebrafish resulted in transitory acceleration of vasculogenesis, confirming that this process might be manipulated for therapeutic purposes by overexpressing *hKLK1*.

The altered arteriogenesis of *KLK1*$^{-/-}$ mice was rescued by local *Ad.hKLK1* gene therapy, whereas the *R53H-hKLK1* mutant was ineffective. The R53H mutant of *hKLK1* has 1% kininogenase activity of the wild-type and is present, at the heterozygote state, in 14% of black and 7% of white subjects.10 The *R53H* allele carriers have reduced urinary kallikrein activity, decreased levels of plasma *KLK1*, and display a mild form of endothelial dysfunction in resting condition.11 The present study shows that the *R53H-hKLK1* variant has impaired angiogenic/arteriogenic activity compared to wild-type *hKLK1* in normoperfused and ischemic models. The residual angiogenic activity of the R53H mutant could be hypothesized to result from the large enzyme excess, cleavage of thus far unidentified substrates or direct kinin-independent activation of B_2R, as recently described. 22 It would therefore be of interest to determine whether patients carrying the *R53H* mutation and suffering from coronary or peripheral artery disease are at a worse prognosis because of impairment in postischemic collateralization similar to that observed in *KLK1*$^{-/-}$ mice.

To clarify the cellular basis of *Ad.hKLK1*-induced arteriogenesis, without the confounding necrosis and inflammation background typical of ischemic tissues, we next sought to test the candidate angiogenic factor in a normoperfused tissue. To this aim, we delivered 2 doses of *Ad.hKLK1, Ad.R53H-hKLK1* or control *Ad.Null* to the rat mesentery, a model uniquely suited to obtain a detailed cellular characterization of neovessel phenotype.23 Results indicate that the wild-type variant stimulates the formation of functional conduit vessels (16 to 35 μm diameter in rats), developing through vessel branching and perivascular cell recruitment. The absence of sprout points but increased branching and increased vessel density either suggests that the sprouts form and are rapidly remodeled to vessels because of recruitment of supporting cells, or that vessel growth occurs via other means (such as intussusceptive growth).

Pericytes and VSMCs not only provide a logistic support to vascular endothelium, but also dynamically modulate the phenotypic change from a proliferative angiogenic sprout to a mature microvascular conduit within a quiescent endothelium.24 Several signaling pathways govern perivascular cell recruitment and specification, including Ang1/Tie2, PDGFB/PDGFR-β, and Jagged-δ-like/Notch signaling pathways.24 We demonstrate for the first time that pericytes express B_2R. Under B_2R blockade, *hKLK1*-induced neovasculature is converted to an inflammatory-type sprouting capillarization with reduced pericyte coverage, no VSMC coverage and microhemorrhages, resembling the picture seen after VEGF-A overexpression in the same model.23 Furthermore, we found that cultured bovine pericytes, via B_2R, are chemotactically activated by the conditioned media of *Ad.hKLK1*-infected ECs. Altogether, these results illustrate the following possible scenario: overexpression of the *hKLK1* transgene generates kinins, leading to EC proliferation and sprouting, which is then arrested and remodeled by kinin-recruited pericytes and VSMCs. The failure of *R53H-KLK1*, a variant with markedly reduced kinin-generating activity,11 in promoting neovascularization and perivascular cell recruitment further supports our hypothesis.

We then determined whether activation of MMP contributes to vascular repair by *hKLK1*. Zymography studies verified the capacity of hKLK1 to process pro-MMP into active MMP. Furthermore, intramuscular *Ad.hKLK1* gene therapy was severely impaired in ischemic *MMP9*$^{-/-}$ mice, suggesting that MMP9 is fundamental for *hKLK1*-induced angiogenesis in vivo. The therapeutic value of *Ad.hKLK1* gene therapy was then challenged in *ApoE*$^{-/-}$ mice, which were subjected to limb ischemia. Angiographic demonstration of improved collateralization accounts for the accelerated blood flow recovery observed in hypercholesterolemic *ApoE*$^{-/-}$ mice. These results indirectly discount the possibility that failure of *Ad.hKLK1* gene therapy in *MMP9*$^{-/-}$ mice is attributable to intrinsic defects of

collateralization. The pleiotropic mechanisms implicated in KLK1-induced neovascularization are summarized in supplemental Figure VIII.

In conclusion, this preclinical study opens new avenues for the therapeutic application of *hKLK1* gene therapy and also supports the possibility that loss of function mutations of the *hKLK1* gene might be detrimental for proper reparative arteriogenesis.

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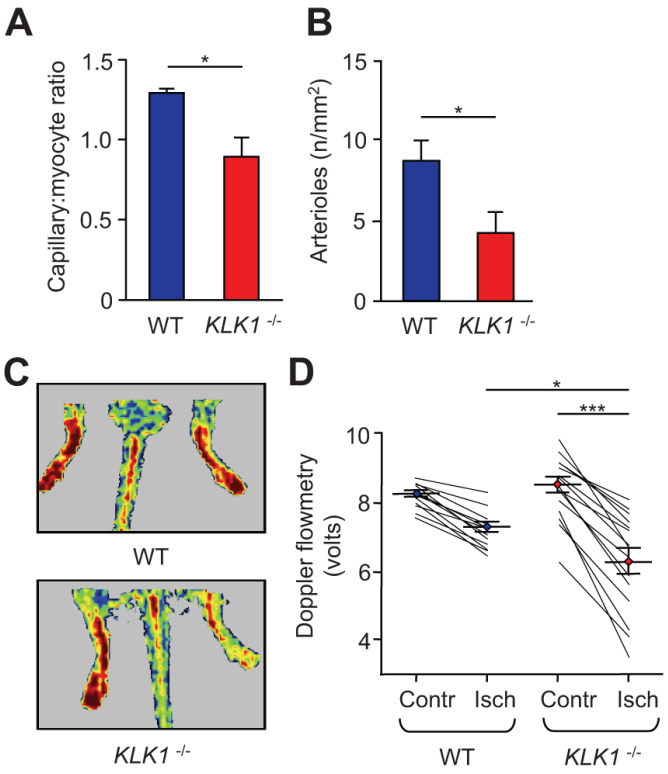

Figure 1.

Reparative neovascularization is impaired in *KLK1*$^{-/-}$ mice. A and B, Capillary:myocyte ratio and arteriole density in adductors harvested 14 days postischemia. C, Representative laser Doppler images collected at 14 days postischemia. D, Comparison of blood flow in ischemic and contralateral limbs. (All data: mean±SEM. Histology: n=6. **P*<0.05; ****P*<0.001).

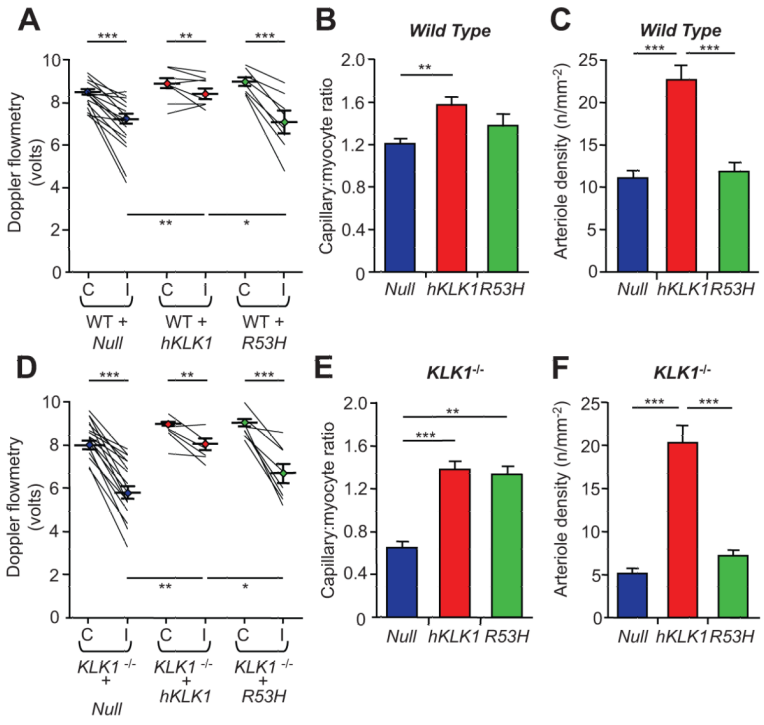

Figure 2.

R53H leads to impaired postischemic reparative neovascularization. Comparison of blood flow to ischemic (I) and contralateral (C) limbs 14 days postischemia in WT (A) and *KLK1*$^{-/-}$ mice (D). Capillary:myocyte ratio (B and E) and arteriole density (C and F) in adductors harvested 14 days postischemia. (All data: mean±SEM. Histology: n=6. **P*<0.05; ***P*<0.01; ****P*<0.001).

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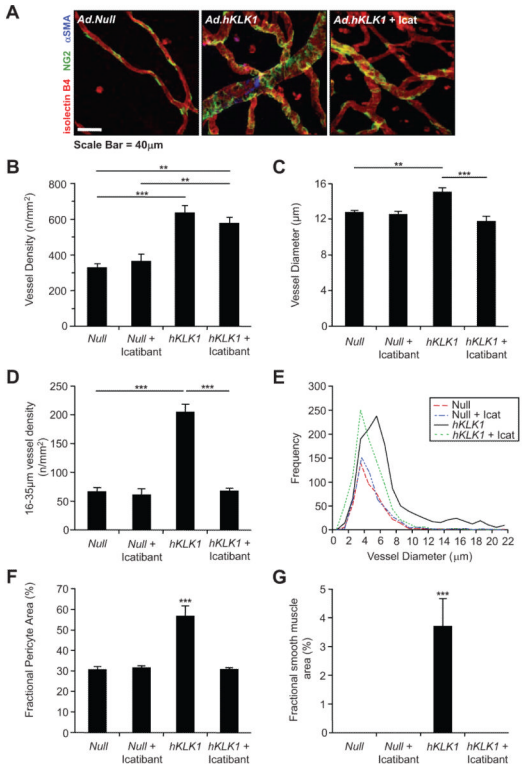

Figure 3.

hKLK1-induced arteriogenesis. Confocal-stack images (A) from rat mesentery 6 days after treatment. *hKLK1* induces arteriogenesis via kinin–B2 receptor activation as demonstrated by comparison of vessel density (B), diameter (C), conduit vessel density (D), distribution (E), and perivascular cell coverage (F and G). (All data: mean±SEM, n=6. ** $P<0.01$; *** $P<0.001$).

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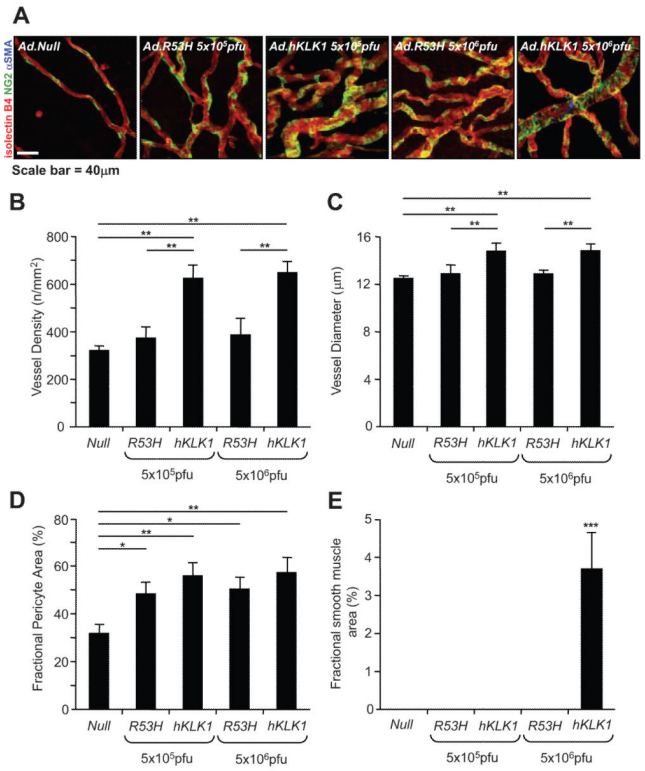

Figure 4.

R53H impairs the arteriogenic ability of *hKLK1*. Confocal-stack images (A) from rat mesentery 6 days after treatment. Two viral doses were used. Comparison of vessel density (B), diameter (C), and perivascular cell coverage (D and E) indicates a reduced capacity to induce vessel maturation. (All data: mean±SEM, n=6. **P*<0.05; ***P*<0.01; ****P*<0.001 vs *Ad.Null*).

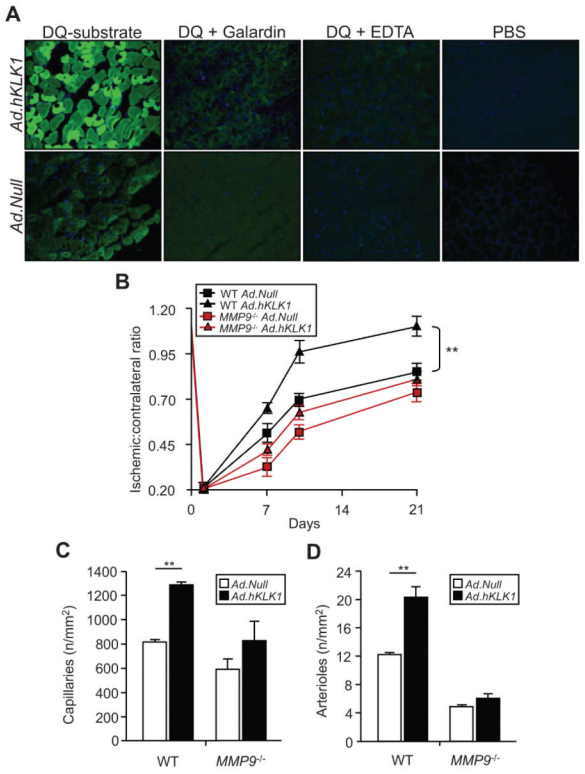

Figure 5.

Involvement of MMP-9 in *hKLK1*-induced neovascularization. A, Gelatinolytic in situ zymography on normoperfused adductors. B, Comparison of hemodynamic recovery after ischemia. *Ad.hKLK1* increased capillary (C) and arteriole density (D) in adductors from WT but not $MMP9^{-/-}$ mice, 21 days postischemia. (All data: mean±SEM n=8. $^{**}P<0.01$).

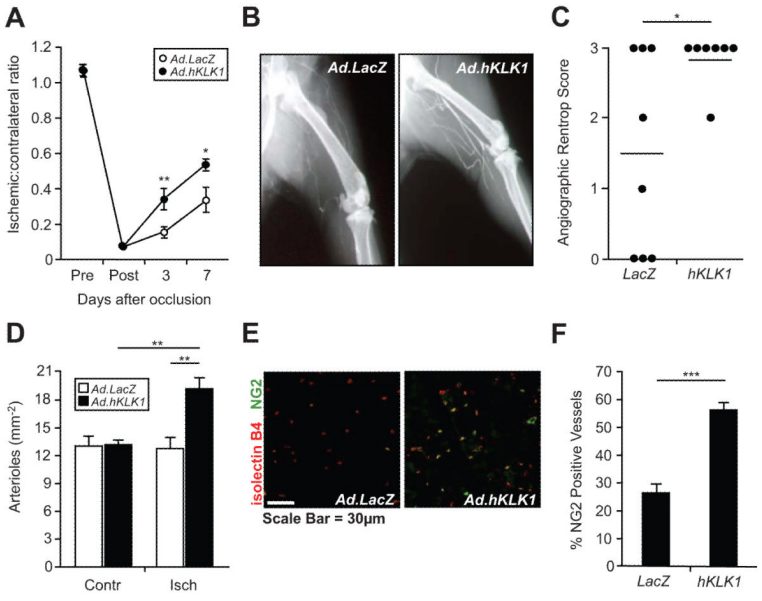

Figure 6.

Ad.hKLK1 promotes postischemic arteriogenesis in ApoE$^{-/-}$ mice. A, Comparison of hemodynamic recovery after ischemia. B, Representative postmortem angiograms 7 days after hindlimb ischemia. *Ad.hKLK1*-treated mice show an increased angiographic Rentrop score (C) and arteriole density (D). *Ad.hKLK1* increased pericyte coverage (E and F). (All data: mean±SEM, n=8. **P*<0.05; ***P*<0.01; ****P*<0.001).

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