Adenovirus-Mediated Human Tissue Kallikrein Gene Delivery Induces Angiogenesis in Normoperfused Skeletal Muscle

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Abstract—We investigated whether local delivery of the tissue kallikrein gene induces angiogenesis in normoperfused mouse hindlimb muscles. Intramuscular injection of adenovirus containing the human tissue kallikrein gene under the control of a cytomegalovirus enhancer/promoter sequence resulted in local production and release of recombinant human tissue kallikrein, whereas transgene expression was absent in muscles of the contralateral hindlimb. Angiogenesis in infected muscles was documented by histological evidence of increased capillary density. In contrast, no angiogenic effect was seen either in the ipsilateral gastrocnemius or contralateral hindlimb muscles. Neovascularization was associated with a transient increase in muscular blood flow as determined by laser Doppler flowmetry. We also investigated the mechanisms of kallikrein-induced angiogenesis. We found that the angiogenic response to kallikrein was abolished by chronic blockade of the kinin B1 or B2 receptor or by inhibition of nitric oxide synthase. In addition, inhibition of cyclooxygenase-2 by nimesulide significantly reduced kallikrein-induced effects. These results indicate that (1) human tissue kallikrein acts as an angiogenic factor in normoperfused skeletal muscle and (2) nitric oxide and prostacyclin are essential mediators of kallikrein-induced angiogenesis. Our findings provide new insights into the role of the tissue kallikrein-kinin system in vascular biology. (Arterioscler Thromb Vasc Biol. 2000;20:2379-2385.)

Key Words: gene delivery • angiogenesis • kallikrein • kinins • nitric oxide

Tissue kallikrein (EC 3.4.21.35), a glycoprotein of the serine proteinase superfamily, is present in the kidney, pancreas, salivary glands, brain, cardiovascular system, skeletal muscle, and leukocytes.1 This enzyme cleaves low-molecular-weight kininogen to release Lys-bradykinin (Lys-BK). Rapid degradation of kinin peptides by kininase I or kininase II suggests that kinins mainly act as paracrine substances in tissues where they are generated. Presently, 2 receptor subtypes have been recognized with respect to the relative bioassay potency of different kinin analogues.2 The B2 receptor is constitutively expressed in various tissues and is responsible for the majority of BK and Lys-BK effects in vitro and in vivo. In contrast, the B1 receptor has higher affinity for the kinin metabolites desArg9-BK and Lys-desArg10-BK, and its expression is induced by pathological conditions such as tissue damage and inflammation.3 Activation of kinin receptors, through stimulation of nitric oxide (NO)—cGMP and prostacyclin-cAMP pathways,4 modulates a broad spectrum of biological functions, such as regulation of local and systemic hemodynamics, vascular permeability, electrolyte and glucose transport, preservation of muscular energy content, and cell proliferation and migration.5—7 In mice, manipulation of the genes that encode for components of the tissue kallikrein-kinin system (KKS) results in alterations of cardiovascular function and structure.8–10

Circumstantial evidence indicates that angiogenesis may be included among the biological processes that are modulated by the KKS. BK, via activation of the B1 receptor—cAMP pathway, exerts a specific and direct mitogenic effect on coronary postcapillary endothelial cells in vitro.11 In addition, BK in synergy with interleukin-1 enhances the angiogenic response to subcutaneous implantation of a poly-ether sponge in rats.12 Furthermore, activation of kinin B2 receptor signaling might be at least partially responsible for neovascularization induced by angiotensin-converting enzyme inhibitors in skeletal muscle and myocardium.13,14

Administration of angiogenic factors is emerging as a new therapeutic strategy in patients with severe coronary or peripheral vascular disease and in whom traditional revascularization procedures are unsuitable.15–19 Although the presence of an ischemic environment has been considered to be essential for neovascularization to occur,20 recent studies have suggested that gene transfer of angiogenic factors can also promote angiogenesis in normoperfused tissues.21–25 This
issue has fundamental clinical relevance. In fact, generation of new collaterals may raise the threshold of exercise-induced claudication, the only symptom in most patients affected by peripheral atherosclerosis,\(^2,26\) and also limit the consequences of intervening vascular occlusion.

We hypothesized that delivery of the tissue kallikrein gene may induce angiogenesis in normoperfused skeletal muscle by increasing local kinins. To test this hypothesis, we investigated whether intramuscular delivery of an adenoviral vector containing the human tissue kallikrein (\(hKall\)) gene, an efficient method for targeted potentiation of kinin generation,\(^17\) would increase capillary density in the normoperfused mouse hindlimb. In addition, the pathways implicated in kallikrein-induced angiogenesis were studied.

Methods

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) and were approved by the local institutional committee. Male 4-month-old Swiss mice (Charles River, Milan, Italy), B\(_r\)-receptor–knockout mice, and wild-type J129Sv controls (both from Jackson Laboratories, Bar Harbor, Me) were housed at a constant room temperature (24 ± 1°C) and humidity (60 ± 3%).

Delivery of Adenoviral Vectors

Replication-defective adenovirus containing the entire coding sequence of the \(hKall\) gene (Ad.CMV-chKall) or the \(\beta\)-galactosidase gene (Ad.CMV-LacZ) under control of the cytomegalovirus (CMV) enhancer/promoter was prepared as described.\(^2,28\) Ad.CMV-chKall or Ad.CMV-LacZ at 3.6 x 10^10 plaque-forming units in 9 \(\mu\)L was injected in 3 different sites of the left adductor muscle of anesthetized (2,2,2-tribromoethanol, 880 mmol/kg IP) mice.

The effectiveness and localization of infection were evaluated by determining the expression of \(hKall\) mRNA in adductor muscles and the liver at 0, 3, 7, 14, 21, and 28 days after adenovirus injection (n = 3 for each time point). To this aim, tissues were quickly frozen in LN\(_2\) and stored at -80°C until assay (vide infra). Immunoreactive \(hKall\) was measured in plasma and muscle homogenates at the same time points (n = 3 per group) by using an ELISA specific for the active form of the enzyme. The antibody to \(hKall\) does not cross-react with either mouse or rat kallikrein in ELISA.\(^28\) For measurement of muscular immunoreactive kinin levels, hindlimbs were perfused with PBS through a cannula inserted into the abdominal aorta, and blood was allowed to flow out through an incision in the inferior vena cava. Adductor muscles (n = 7 per group) were rapidly dissected and homogenized in a chilled tube containing an inhibitor cocktail (ratio of sample to inhibitor, 9:1 wt/vol). One milliliter of the inhibitor mixture contained aprotinin (10 000 KIU), soybean trypsin inhibitor (800 \(\mu\)g), hexadimethrine bromide (4 mg), 1,10-phenanthroline (10 mg), and EDTA (20 mg). Samples were then sonicated, mixed with 1 mL of 100% ethanol, and centrifuged for 15 minutes at 4°C at 6200g. The supernatants were evaporated to dryness and stored frozen at -80°C until assay. Kinins were measured by radioimmunoassay (Pharmacia) after extraction of the kinin peptide by Sep-Pack C-18 Cartridges (Waters). Protein concentration was determined according to the Lowry method.

Effects of \(hKall\) on Hindlimb Blood Flow and Muscular Capillary Density

Systolic blood pressure and hindlimb blood flow (vide infra) were sequentially measured before and 7, 14, 21, and 28 days after injection of Ad.CMV-chKall (n = 7) or Ad.CMV-LacZ (n = 8). Capillary density (vide infra) was determined in hindlimb muscles at 7, 14, 21, and 28 days after intramuscular injection of Ad.CMV-chKall or Ad.CMV-LacZ (n = at least 6 for each time point). Capillary density in noninjected adductor muscles was also counted for reference (n = 6).

Mechanisms Involved in the Angiogenic Effects of \(hKall\)

Muscular capillary density was counted 14 days after injection of Ad.CMV-chKall or Ad.CMV-LacZ in the left adductor muscles of mice allocated to the following treatments: (1) sterile saline (vehicle; n = 6 for each subgroup), (2) the kinin B\(_r\)-receptor antagonist desArg4[Leu]-6-BK (DAL-BK; 50 nmol/kg per day; Ad.CMV-chKall, n = 7; Ad.CMV-LacZ, n = 9), (3) the B\(_r\) receptor antagonist d-Arg-Hyp, Thi,5-d-Tic, Oic)-6-BK (Icatibant, 1 \(\mu\)mol/kg per day; Ad.CMV-chKall, n = 7; Ad.CMV-LacZ, n = 9), (4) the NO synthase inhibitor L-nitroarginine methyl ester (L-NAME) or the inactive isomer (L-NAME) (both at 1.4 nmol/kg per day; n = 8 for each subgroup), (5) the preferential cyclooxygenase (COX-1) inhibitor indomethacin (5 mg/kg per day; n = 11 for each subgroup), (6) the indomethacin vehicle (10% dimethyl sulfoxide in PBS; n = 6 for each subgroup), or (7) the selective COX-2 inhibitor nimesulide (3 mg/kg per day; n = 6 for each group).

DAL-BK, Icatibant, or their vehicle was delivered by means of osmotic minipumps (Alza Co) inserted into the abdominal cavity at the time of adenovirus delivery. L-NAME, L-NAME, or nimesulide was given in the drinking water, whereas indomethacin was injected subcutaneously. The selectivity of DAL-BK and Icatibant has been previously reported.\(^8,11\) Although a partial agonistic effect of DAL-BK was recognized in the isolated mouse stomach,\(^30\) this compound is devoid of residual agonistic activity in vivo (P.M., unpublished observations, 2000). In preliminary experiments, the dose of DAL-BK indicated above was able to antagonize the hypertensive effect induced by 1 nmol of DAL-BK by 90%, whereas Icatibant prevented the hypotension induced by 1 nmol of BK by 95%. The doses of L-NAME, indomethacin, and nimesulide were chosen according to previous studies.\(^8,31,32\) In additional experiments, the muscular capillary density of B\(_r\)-receptor–knockout mice or wild-type J129Sv controls was measured 14 days after intramuscular injection of Ad.CMV-chKall or Ad.CMV-LacZ (n = 6 per group).

Hemodynamic Measurements

Systolic blood pressure was measured in unanesthetized animals by tail-cuff plethysmography.\(^8\) The animals were then anesthetized and placed on a heating pad at 37°C for 5 minutes for measurements of hindlimb blood flow by laser Doppler flowmetry (Laser Perfusion Imager System).\(^3,14\) After completion of the scanning procedure, a color-coded image representing the microvascular blood flow distribution was captured on the monitor. The perfusion values were then stored for subsequent data analysis. Injected to noninjected hindlimb blood flow ratio was taken as an index of the effect of gene delivery on local hemodynamics.

Analysis of Capillary Density

Hindlimbs of anesthetized mice were perfused with PBS (1 minute), followed by 10% buffered formalin (10 minutes) at 100 mm Hg through the abdominal aorta. Hindlimb muscles were placed in formalin for 48 hours. After paraffin embedding, 3-\(\mu\)m-thick sections were cut from each sample with the muscle fibers oriented in a transverse direction, stained with hematoxylin and eosin, and examined at \(\times 200\) magnification. Analysis of the capillary network was then performed by using an ocular reticle (9604-\(\mu\)m^2 area) at \(\times 1000\) magnification. For each area of tissue section, 25 fields were randomly counted in a blind fashion. The number of capillary profiles (n\(_\text{cp}\)) was used to compute the capillary numerical density per mm\(^2\) of muscle according to the following equation: n\(_\text{cp}\) = n\(_\text{cp}\)/total fields/total field area.\(^25\)

Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was isolated from frozen skeletal muscles and liver with the RNazol B method according to the manufacturer’s instructions (Stratagene). The following primers were used for amplification of the \(hKall\) gene: 5'-primer, 5'-AAC ACA GCC CAG TTT GT-3' and 3'-primer, 5'-CCT CAC ATA AGA CAG CA-3'. Amplification was performed under the following conditions: denaturation at 94°C, annealing at 55°C, and elongation at 72°C for 30 cycles.\(^28\)
Drugs
DAL-BK, L-NAME, d-NAME, nimesulide, indomethacin, and 2,2,2-tribromoethanol were all purchased from Sigma-Aldrich. Icatibant was a kind gift from Aventis Pharmaceutical Co (Frankfurt, Germany).

Statistical Analysis
All results are expressed as mean±SEM. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and the F test indicated significant differences, the statistical value was determined according to the Bonferroni method. Differences within or between groups were determined by using paired or unpaired Student’s t test, respectively. A value of P<0.05 was interpreted to denote statistical significance.

Results

hKall Gene Expression
hKall expression was recognized at the mRNA (data not shown) and protein (Figure 1A) levels in muscles injected with Ad.CMV-chKall, reaching a peak between 3 and 7 days and then declining to undetectable levels at day 28. In contrast, transgene expression was not detected in either the liver and adductor muscle contralateral to the site of Ad.CMV-chKall injection or in Ad.CMV-LacZ–injected muscle. Secretion of recombinant protein from muscles into the circulation was documented by recognition of immunoreactive hKall in the plasma of Ad.CMV-chKall–injected mice (Figure 1B). The presence of hKall in skeletal muscle was associated with increased levels of kinin, a natural product of kallikrein enzymatic action (Figure 1C).

Evidence of Neovascularization in Normoperfused Skeletal Muscle Injected With Ad.CMV-chKall
As shown in Figure 2A, local delivery of the hKall gene induced a transient increase in hindlimb perfusion, whereas delivery of the control virus had no effect. At 7 days after gene delivery, the blood flow in Ad.CMV-chKall–injected hindlimbs was 69% greater compared with that in contralateral noninjected muscles (P<0.01). As shown in Figure 2B, perfusion was augmented in the thigh area around the injection site. Systolic blood pressure was not altered by intramuscular injection of Ad.CMV-chKall or Ad.CMV-LacZ (data not shown).

Histological examination demonstrated a marked angiogenic effect in muscles injected with Ad.CMV-chKall (Figure 3), without any apparent tissue damage or hemorrhage caused by injection. As shown in Figure 4, capillary density was already increased at day 7 after injection (706±19 n cap/mm²) compared with Ad.CMV-LacZ (507±39 n cap/mm²; P<0.05). Kallikrein-induced angiogenesis was still at plateau 28 days after gene transfer.

Mechanisms Responsible for hKall-Induced Neovascularization
Figure 5 shows that chronic blockade of B₁ or B₂ receptors by continuous administration of the selective antagonists, DAL-BK or Icatibant, was able to prevent the hKall-induced increase in capillary density. Because activation of kinin receptor signaling is expected to stimulate the release of NO and prostaglandins, the contribution of these pathways to the angiogenic activity of kallikrein was probed. As shown in Figure 5, L-NAME prevented the increase in capillary density induced by kallikrein, whereas the inactive isomer d-NAME was not effective (data not shown). Administration of indomethacin, a nonselective COX inhibitor with higher affinity for COX-1, resulted in excess mortality. In fact, 5 of 11 mice died within the first few days of treatment. Therefore, muscular specimens were collected on day 7 after Ad.CMV-chKall injection in surviving animals. At this time point, the adductor capillary density of mice given Ad.CMV-chKall in combination with indomethacin or vehicle was similar (695±20 versus 609±15 n cap/mm²; P=NS) and did not differ from that observed in animals given Ad.CMV-chKall alone (706±19 n cap/mm²; P=NS for both comparisons). By contrast, kallikrein-induced angiogenesis was partially reduced by nimesulide, a selective COX-2 inhibitor (737±132 versus 1386±105 n cap/mm² in controls; P<0.01). Nimesulide did not alter the capillary density of Ad.CMV-LacZ–injected muscles (498±60 versus 418±72 n cap/mm²; P=NS). The angiogenic effect of kallikrein was not manifested in mice lacking the kinin B₂ receptor gene (420±25 versus 1121±83 n cap/mm² in wild-type mice; P<0.01).

Discussion
The major findings of the present study consist in the discovery that transfer of the human tissue kallikrein gene
induces angiogenesis in normoperfused skeletal muscles by activation of kinin receptor signaling, with possible consequent stimulation of NO release and induction of COX-2–mediated prostaglandin synthesis.

Administration of angiogenic factors delivered in the form of recombinant protein, naked DNA, or by way of plasmid or viral vectors is emerging as a new therapeutic strategy to accelerate the development of collaterals and blood flow recovery of ischemic tissue. In contrast, the concept that angiogenesis can be induced in normoperfused tissue has been questioned. In fact, chronic infusion of vascular endothelial growth factor (VEGF) into the canine coronary system does not lead to endothelial proliferation. Moreover, endothelial cell growth factor, regarded as an effective angiogenic agent in ischemic tissue, is ineffective in the normoperfused rabbit hindlimb. However, Ad.CMV-VEGF delivery through a gel of basement membrane protein (Matrigel) implanted subcutaneously induced angiogenesis in the nonischemic, subcutaneous tissue surrounding the Matrigel plug and direct injection of this adenoviral vector into the retroperitoneal adipose tissue induced neovascularization. Furt

thermore, Safi et al have shown that acidic fibroblast growth factor gene transfer induces angiogenesis in the nonischemic rabbit heart, providing an anatomic basis for a reduction in the risk region for myocardial infarction on subsequent coronary occlusion. Similar findings have been reported by the same group after gene delivery of VEGF to normoperfused rabbit and rat skeletal muscles. Evidence for successful infection after intramuscular delivery of Ad.CMV-chKall was documented at the mRNA level by reverse transcription–polymerase chain reaction analysis and at the protein level by an ELISA that specifically recognizes the active moiety of hKall. The impact of kalikrein on microscopic angiogenesis was evaluated by analysis of capillary density. This approach revealed a 2-fold increase in vascularity at day 7 after intramuscular injection of Ad.CMV-chKall, with an additional increase at day 14. The newly developed vascular network was still at plateau 28 days after gene transfer, ie, over the duration of transgene 

**Figure 2.** A, Time course of injected/noninjected hindlimb blood flow ratio after intramuscular injection of Ad.CMV-LacZ (hatched bars, n=8) or Ad.CMV-chKall (filled bars, n=7). Measurements of hindlimb blood flow were performed with laser Doppler flowmetry. The perfusion ratio before gene transfer (open bar, time 0) is shown as a reference. Values are mean±SEM. P<0.05 vs time 0; P<0.05 vs Ad.CMV-LacZ. B, Typical laser Doppler perfusion imaging recorded 7 days after intramuscular injection of Ad.CMV-chKall (left) or Ad.CMV-LacZ (right). The abdominal area and ventral parts of limbs and tail are shown. The area represented in red corresponds to increased perfusion around the site of Ad.CMV-chKall injection.

**Figure 3.** Representative pictures (hematoxylin and eosin staining) showing the higher capillary density of adductor muscles injected with Ad.CMV-chKall (B) compared with Ad.CMV-LacZ–injected controls (A). Magnification ×1000.

**Figure 4.** Time course of capillary density in adductor muscles evaluated by light microscopy. Intramuscular injection of Ad.CMV-chKall (filled bars, n=at least 6 per time point) in normoperfused muscle resulted in marked angiogenic effects compared with Ad.CMV-LacZ (hatched bars, n=at least 6 per time point). Capillary density in the adductor of noninjected mice (control, open bar, n=6) is shown as a reference. Values are mean±SEM. *P<0.05 vs control; §P<0.05 vs Ad.CMV-LacZ.
expression in muscle. Our finding that local delivery of Ad.CMV-chKall induces angiogenesis in the normoperfused muscle not only supports the concept expressed by previous studies with other angiogenic factors but also reveals an unknown property of human tissue kallikrein.

hKall mRNA was not detected in the livers of mice receiving gene transfer. Detection of protein in the circulation demonstrates the secreted nature of the gene product from the injected muscle, a property recognized to be relevant for the desired biological action. Concerns regarding undesired effects in remote tissues are obviated by the observation that, strictly consistent with the pattern of mRNA expression, the angiogenic response was localized to the injected adductor.

Various mechanisms, intrinsic to the biological activities of the KKS, may be implicated in hKall-induced angiogenesis: (1) Kallikrein, acting as a proteinase and activating the metalloproteinases type IV collagenase, might favor degradation of the vascular basal membrane and extracellular matrix proteins, thus leading to endothelial cell invasion and migration. (2) Generated kinins may stimulate vascular endothelial cells to proliferate and attract leukocytes. (3) Additional growth factors may be released from migrated leukocytes, thus amplifying the initial angiogenic response.

Previous studies have shown that activation of B1 receptor and cAMP pathways are implicated in the angiogenic action of kinin in vitro, whereas activation of kinin B2 receptor signaling might be responsible for the angiogenic action exerted by angiotensin-converting enzyme (kininase II) inhibitors in skeletal muscle and myocardium. In the present study, chronic blockade of either B1 or B2 receptors nullified the hKall-induced increase in capillary density of normoperfused skeletal muscle. These results suggest that the 2 receptors intervene sequentially in the angiogenic process, so that the functional integrity of both is required for kallikrein to exert its biological effect. The essential role of B2 receptor signaling in neoangiogenesis promoted by tissue kallikrein is confirmed by the finding that this effect was not manifested in knockout mice lacking the B2 receptor gene. Unfortunately, knockouts for the B1 receptor are not available at the present time, thus precluding the possibility of complementing the information obtained with the use of the B1 receptor antagonist.

Previous studies have demonstrated that binding of kinins to their receptors activates NO-cGMP and prostacyclin signal pathways. Because NO and prostaglandins are regarded as angiogenic factors, acting either directly or by stimulation of other paracrine substances, we examined whether inhibition of NO or prostaglandin-forming enzymes impairs hKall-induced angiogenesis. This was the case in mice treated with l-NAME or nimesulide, whereas the angiogenic effect persisted in animals pretreated with indomethacin. Altogether, these results indicate a critical role of NO and COX-2 in angiogenesis in the systemic circulation, suggesting that this effect can be rapidly and transiently induced after exposure to mitogenic stimuli.

Our results indirectly support the opinion that kinin-induced effects may be mediated by induction of COX-2.

Interestingly enough, kinins share important features with the potent angiogenic factor VEGF. Both induce plasma extravasation, vasodilation, and endothelial cell proliferation. On a molar basis, BK proved to be more potent than VEGF in in vitro proliferation assays of human coronary endothelial cells. However, only VEGF is able to stimulate cell migration. In vivo angiogenic activities of hKall and VEGF appear to be superimposable, both depending on stimulation of NO release. However, scrotal edema, a side effect of VEGF angiogenesis gene therapy in rabbits, was not observed in Ad.CMV-chKall–treated mice.

Although less than tissue kallikrein, Ad.CMV-LacZ injection also resulted in increased muscular capillary density. This may be explained by the fact that adenovirus vector transfer is able to recruit monocytes and T cells, which are an important source of growth factors. Nevertheless, as shown here, the angiogenic effect of Ad.CMV-LacZ was physiologically irrelevant, as it was unable to affect local hemodynamics. With regard to positive results seen with Ad.CMV-chKall, it should be cautiously noted that adenovirus infection might have helped unveil the angiogenic effect of kallikrein.

In conclusion, our findings provide new insights into the role of the KKS in vascular biology and reinforce the view that gene transfer of angiogenic factors can induce neovascularization in normoperfused tissue. In perspective, the angiogenic property of kallikrein might be exploited to reduce the occurrence of intermittent claudication in patients with chronic vascular disease and to accelerate functional recovery after acute occlusion.

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In “Adenovirus-Mediated Human Tissue Kallikrein Gene Delivery Induces Angiogenesis in Normoperfused Skeletal Muscle” by Emanueli et al (Arterioscler Thromb Vasc Biol. 2000;20;2379–2385), Figure 3B was provided in error by the authors. The correct figure is now provided.

Representative picture (hematoxylin and eosin staining) showing the capillary density of adductor muscle injected with Ad.CMV-chKall. Magnification ×100.