Local Delivery of Human Tissue Kallikrein Gene Accelerates Spontaneous Angiogenesis in Mouse Model of Hindlimb Ischemia

Costanza Emanueli, PhD; Alessandra Minasi, PhD; Antonella Zacheo, BS; Julie Chao, PhD;
Lee Chao, PhD; Maria Bonaria Salis, BS; Stefania Straino, BS; Maria Grazia Tozzi, PhD;
Robert Smith, BS; Leonardo Gaspa, MD; Giuseppe Bianchini, MD; Francesco Stillo, MD;
Maurizio C. Capogrossi, MD; Paolo Madeddu, MD

Background—Human tissue kallikrein (HK) releases kinins from kininogen. We investigated whether adenovirus-mediated HK gene delivery is angiogenic in the context of ischemia.

Methods and Results—Hindlimb ischemia, caused by femoral artery excision, increased muscular capillary density ($P<0.001$) and induced the expression of kinin B_1 receptor gene ($P<0.05$). Pharmacological blockade of B_1 receptors blunted ischemia-induced angiogenesis ($P<0.01$), whereas kinin B_2 receptor antagonism was ineffective. Intramuscular delivery of adenovirus containing the HK gene (Ad.CMV-cHK) enhanced the increase in capillary density caused by ischemia (969 ± 32 versus 541 ± 18 capillaries/mm^2 for control, $P<0.001$), accelerated blood flow recovery ($P<0.01$), and preserved energetic charge of ischemic muscle ($P<0.01$). Chronic blockade of kinin B_1 or B_2 receptors prevented HK-induced angiogenesis.

Conclusions—HK gene delivery enhances the native angiogenic response to ischemia. Angiogenesis gene therapy with HK might be applicable to peripheral occlusive vascular disease. (*Circulation*. 2001;103:125-132.)

Key Words: gene therapy ■ angiogenesis ■ bradykinin ■ ischemia ■ muscles

L imb ischemia is a major health problem. Because of the absence of effective pharmacological treatment, this disabling condition follows an inexorable course, and at the end stage, amputation is undertaken as a unique solution to unbearable symptoms. Delivery of angiogenic factors as recombinant protein or by gene transfer proved to be effective in animal models of ischemia[1,2] and is now emerging as a new therapeutic strategy in peripheral vascular disease not susceptible to revascularization.[3–5]

Circumstantial evidence suggests that kinins cleaved from kininogen by tissue kallikrein may participate in embryonic vasculogenesis[6] and stimulate angiogenesis in vivo and in vitro.[7,8] Kinins interact with G protein–coupled B_1 and B_2 receptors on the cell surface.[9,10] Constitutively expressed B_2 receptors mediate most biological effects of bradykinin (BK) and Lys-BK. In contrast, B_1 receptors, which preferentially bind des-Arg^9-BK and des-Arg^{10}-kallidin, are induced by tissue damage and inflammation.[11] Kinin-mediated stimulation of NO-cGMP and prostacyclin-cAMP pathways modulates a broad spectrum of biological functions,[12–17] including vasodilation and endothelial cell proliferation. These proper-

ties led us to speculate that human tissue kallikrein (HK) may exert angiogenic effects, utilitarian in the context of ischemia.

To test this hypothesis, we investigated whether an intramuscular injection of replication-defective adenovirus containing the HK gene, a new approach for targeted potentiation of kinin generation in tissues,[18–20] increases capillary density and facilitates functional recovery in a mouse model of hindlimb ischemia. In addition, the pathways implicated in HK-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). Male 4-month-old Swiss mice (Charles River, Comerio, Italy) were housed at constant room temperature ($24\pm1°C$) and humidity ($60\pm3\%$).

Induction of Ischemia and Adenoviral Vector Injection

With mice under the effect of 2,2,2-tribromoethanol anesthesia (880 mmol/kg body wt IP, Sigma-Aldrich), the left femoral artery was exposed, dissected free, and excised.

Gene Primers for RT-PCR Analysis

	Sequences
Human tissue kallikrein	
5′-Primer	5′-AACACA GCCCAGTTTGT-3′
3′-Primer	5′-CCTCACATAAGACAGCA-3′
Mouse tissue kallikrein	
5′-Primer	5′-GCTTCACCAAATATCAATGTCGGGGTATC-3′
3′-Primer	5′-ATGAATACCCAGATGAGCTCCAGTG-3′
Mouse BK B_1 receptor	
5′-Primer	5′-TTCCTTTTGCCTTGGCGACG-3′
3′-Primer	5′-GTTGAAGTAGAGGATGGCAGC-3′
Mouse BK B_2 receptor	
5′-Primer	5′-GAACATCTTTGT CCTCAGCG-3′
3′-Primer	5′-CCGTCTGGATCTCCTTGAAC-3′

Adenovirus containing the HK gene (Ad.CMV-cHK) or the β-galactosidase gene under the control of the cytomegalovirus (CMV) enhancer/promoter (Ad.CMV-LacZ) was prepared as described.[18] A total of 3.6×10^8 plaque-forming units (in 9 μL) of Ad.CMV-cHK or Ad.CMV-LacZ or 9 μL vehicle was injected in 3 different sites of the adductor muscle of anesthetized mice.

Experimental Protocols

Effects of Ischemia on Muscular Capillary Density and Gene Expression

Mouse tissue kallikrein, B_1 receptor, and B_2 receptor mRNA levels were determined by reverse transcription (RT)–polymerase chain reaction (PCR) (see below) in both adductors at 1, 2, 3, and 7 days after femoral artery excision (n=3 per group). Capillary density (see below) was determined in ischemic adductors at 7, 14, and 21 days after surgery (at least 6 mice for each time point). Sections from nonoperated mice were examined for reference. In separate experiments, capillary density was measured 21 days after the induction of ischemia in mice given the B_1 receptor antagonist des-Arg[9]-[Leu[8]]-BK (DALBK from Sigma-Aldrich, 50 nmol/kg body wt per day, n=7), the B_2 antagonist D-Arg-[Hyp[3],Thi[5],D-Tic[7],Oic[8]]-BK (Icatibant, a gift from Aventis Pharmaceutical Co, at 1 μmol/kg body wt per day; n=6), or saline (vehicle) intraperitoneally via miniosmotic pumps (Alza Co). Selectivity of antagonists has been reported
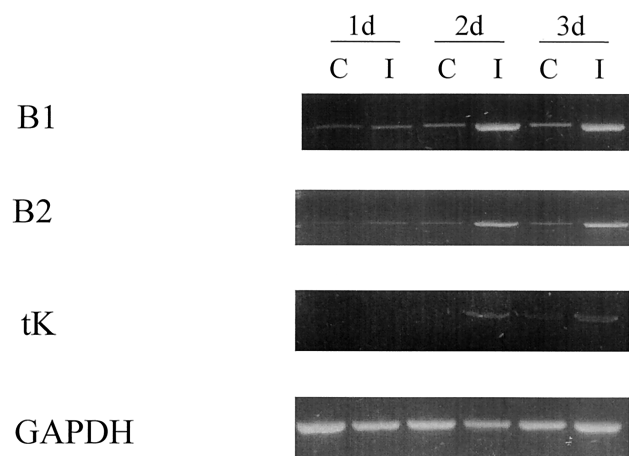
Figure 1. RT-PCR analysis showing time course of mouse B_1 receptor (B1), B_2 receptor (B2), and tissue kallikrein (tK) gene expression in ischemic (I) and contralateral (C) adductors from day 1 through day 3 after removal of femoral artery. GADPH was used for normalization.
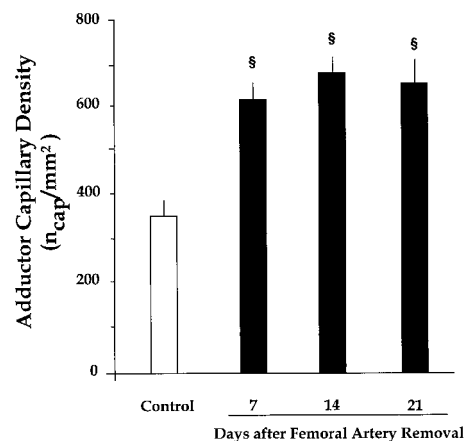
Figure 2. Time course of capillary density after femoral artery excision (filled bars). Capillary density in the adductor muscle of control nonoperated mice (open bar) is shown for reference. Values are mean±SEM. §$P < 0.001$ vs control.

previously.[8,15] Although the partial agonist effect of DALBK was recognized in the isolated mouse stomach,[21] this compound is devoid of residual agonistic activity in vivo (P.M., unpublished data, 1999).
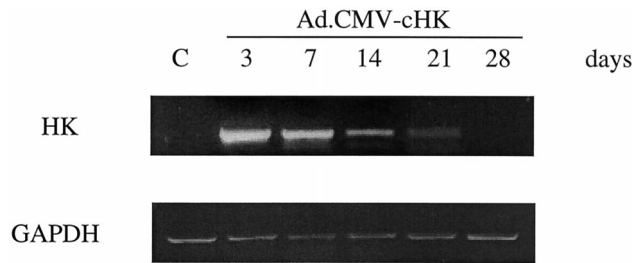
HK Expression After Ad.CMV-cHK Injection

The expression level of HK transgene in hindlimb muscles and liver was determined at 0, 3, 7, 14, 21, and 28 days after intramuscular adenovirus (n=3 for each time point). Immunoreactive HK was measured in muscle homogenates and plasma at the same time points (n=3 for each group) by use of an ELISA specific for the active form of the enzyme.[18] Kinin, cAMP, and cGMP levels in homogenates (at least 6 in each group) were determined by radioimmunoassay (Phoenix). Protein concentration was determined by the Lowry assay.

Effect of HK on Angiogenic Response to Ischemia

Seven days after femoral artery excision, Ad.CMV-cHK, Ad.CMV-LacZ, or vehicle was injected in ischemic adductors (n=6 per group). Fourteen days later, muscular capillary density was measured.
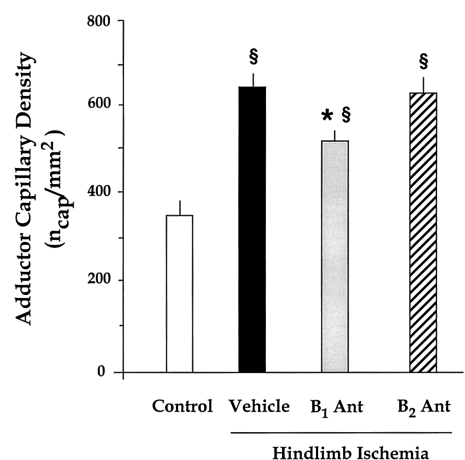
Figure 3. Effect of chronic blockade of B_1 or B_2 receptors on spontaneous angiogenic response to ischemia. Capillary density was increased in ischemic adductor muscle at day 21 after surgery in mice given vehicle (filled bar) compared with control nonoperated mice (open bar). Angiogenic response to ischemia was blunted by B_1 antagonist DALBK (B_1 Ant, stippled bar) but not by B_2 antagonist Icatibant (B_2 Ant, hatched bar). Values are mean±SEM. §$P < 0.001$ vs control; *$P < 0.01$ vs vehicle.

Figure 4. RT-PCR analysis showing time course of HK mRNA expression in adductor muscle from day 3 through day 28 after Ad.CMV-cHK. Expression was not detected in muscle injected with Ad.CMV-LacZ (lane C, 3 days after injection). GADPH was used for normalization.

In additional experiments, ischemic mice were injected with Ad.CMV-cHK or Ad.CMV-LacZ and were infused with DALBK or Icatibant (same doses as above, n=6 per group). Capillary density was measured 14 days later.

Effect of HK on Perfusion Recovery

Hemodynamic parameters were evaluated before and sequentially after gene delivery (Ad.CMV-cHK, n=13; Ad.CMV-LacZ, n=10). Systolic blood pressure was measured by tail-cuff plethysmography (Visitech Systems).[22] Hindlimb and plantar perfusion was then measured in anesthetized mice by laser Doppler flowmetry with use of a perfusion imager system (Lisca Inc)[23] and a Transonic flowmeter, respectively. In separate experiments, muscular blood flow was measured by radioactive microspheres (n=6 per group for each time point). Microspheres (10 μm in diameter), labeled with [141]Ce (Dupont-NEN), were injected into the left ventricle of anesthetized mice, and reference arterial blood was withdrawn through 10 seconds before injection and continuing for 60 seconds at a rate of 0.2 mL/min. Tissue specimens were weighed and counted for radioactive emission by a γ counter. Blood flow was calculated with the following equation: blood flow=(withdrawal rate/tissue weight)$\times$(counts of the tissue/counts of blood sample), expressed as mL $\cdot$ min^{-1} $\cdot$ 100 g tissue^{-1}. The ratio of ischemic to nonischemic blood flow was then calculated.

Effect of HK on Muscular Energetic Charge

Three days after the induction of unilateral ischemia, Ad.CMV-cHK, Ad.CMV-LacZ, or saline was injected in both adductors (n=8 each group). Four days later, hindlimb muscles were harvested for measurement of adenylate concentration by capillary electrophoresis (P/ACE 2100 System, Beckman Instruments).[22] Energetic charge was calculated according to the following formula: (ATP+1/2 ADP)$\times$100/(ATP+ADP+AMP).

RT-PCR Analysis

Total RNA was isolated from frozen skeletal muscles and liver by use of the RNAzol B method, in the presence of DNAase. The Table shows primers used for RT-PCR.[18] Primers for mouse tissue kallikrein gene were kindly provided by Dr Pierre Meneton (INSERM, Paris, France). In negative control experiments, RT was omitted. GADPH was used for normalization in densitometric analysis.

Histology and Morphometric Analysis

Anesthetized mice were perfused with PBS (1 minute) and then with 10% buffered formalin (10 minutes) at 100 mm Hg via the abdominal aorta. After paraffin embedding, 3-μm-thick sections were cut from each sample with muscle fibers oriented in the transverse direction, stained with hematoxylin and eosin, and examined at $\times$200 magnification.

Vascular endothelial cells were identified by immunohistochemical staining for factor VIII–related antigen (von Willebrand factor) with a rabbit polyclonal antibody (Dako). In control sections, the primary antibody was replaced with nonimmune rabbit immunoglobulin G. Sections (n=3 per animal) were analyzed in a blinded fashion

by use of an ocular reticle (area 9604 μm^2) at $\times$1000 magnification. The number of capillary profiles (n$_{cap}$) was used to compute capillary numerical density per square millimeter of muscle, according to the following formula: n$_{cap}$/mm^2=n$_{cap}$ in total fields/total field area.[24]

Statistical Analysis

All results are expressed as mean$\pm$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 F test indicated significant differences, the statistical value was determined according to the Bonferroni method. Differences within and between groups were determined by the paired or unpaired Student t test, respectively. A value of $P<0.05$ was interpreted to denote statistical significance.

Results

Effects of Ischemia on Muscular Capillary Density and Gene Expression

As shown in Figure 1, B$_1$ receptor gene expression in skeletal muscle was increased by 7.5$\pm$1.2-fold ($P<0.05$) at 2 days after the induction of ischemia. At the same time point, B$_2$ receptor and tissue kallikrein mRNA levels were increased by
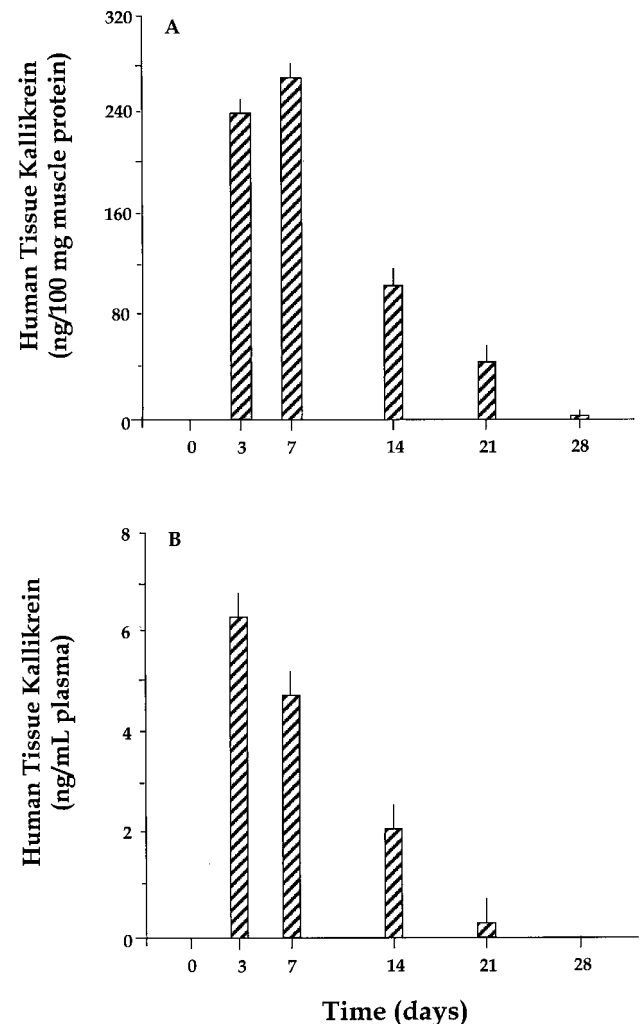
Figure 5. Immunoreactive HK levels in adductor muscles (top) and plasma (bottom) after intramuscular Ad.CMV-cHK. Expression of HK peaked between 3 and 7 days and then declined to become undetectable at 28 days. Values are mean$\pm$SEM.
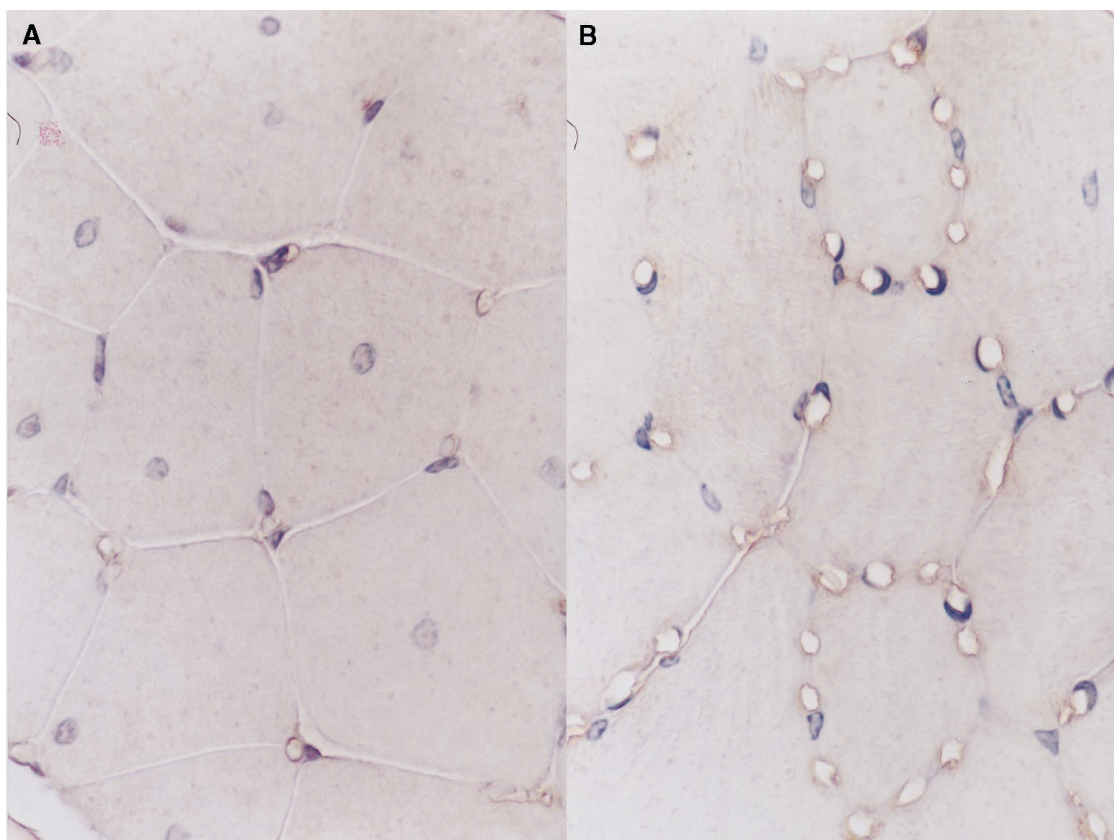
Figure 6. Immunohistochemical identification of vascular endothelial cells using antibodies against von Willebrand factor. Skeletal muscle sections were harvested from ischemic hindlimbs 21 days after surgery. Representative pictures showing higher capillary density of adductor muscles injected with Ad.CMV-cHK (B) compared with Ad.CMV-LacZ–injected controls (A) are presented.

3.5 ± 0.7- and 3.3 ± 0.8-fold, respectively ($P<0.05$). Seven days after surgery, B_1 and B_2 receptor and tissue kallikrein mRNA levels returned to baseline (data not shown).

As shown in Figure 2, muscular capillary density was significantly increased by ischemia ($P<0.001$). This response was blunted by chronic blockade of B_1 receptors ($P<0.01$) but not by B_2 receptor antagonism (Figure 3). Neither the B_1 nor B_2 receptor antagonist affected capillary density in nonischemic muscle (data not shown).

Histological examination of skeletal muscle at day 3 after induction of ischemia revealed scattered foci of inflammation, consisting in interstitial edema and perivascular infiltrates of lymphocytes, monocytes, and granulocytes. The cellular response was not altered by B_1 receptor blockade. Furthermore, no inflammation was observed at 21 days after surgery.

HK Transgene Expression
HK mRNA was not detected in muscles injected with Ad.CMV-LacZ (Figure 4, lane C). In Ad.CMV-cHK–injected muscles, HK expression peaked between 3 and 7 days and then declined to undetectable levels at day 28 (Figures 4 and 5). Secretion of recombinant protein into the circulation was documented by recognition of immunoreactive HK in plasma (Figure 5, bottom panel). HK expression was not detected in the liver or in the adductor contralateral to the site of Ad.CMV-cHK injection (data not shown).

Seven days after Ad.CMV-cHK injection, muscular immunoreactive kinins were doubled (0.59 ± 0.10 versus 0.30 ± 0.05 pg/mg protein in controls, $P<0.05$). cAMP and cGMP levels after injection were also increased (1.02 ± 0.06 and 99 ± 5 fmol/mg protein, respectively) compared with control levels (0.48 ± 0.04 and 68 ± 5 fmol/mg protein, respectively; $P<0.01$).

### Effect of HK on Angiogenic Response to Ischemia
Immunohistochemical studies demonstrated that HK potentiates the spontaneous angiogenic response to ischemia (Figure 6).

As indicated in Figure 7, Ad.CMV-cHK–infected muscles showed increased capillary density (969 ± 32 versus 541 ± 18 capillaries/mm^2 in controls, $P<0.001$); this effect was prevented by chronic blockade of B_1 or B_2 receptors.

Neither induction of ischemia nor injection of Ad.CMV-cHK in the left adductor altered capillary density in the contralateral nonischemic muscle (data not shown).

Effect of HK on Perfusion Recovery
Systolic blood pressure was not affected by femoral artery removal or adenovirus injection (data not shown). As shown in Figures 8 and 9, a dramatic drop of perfusion ratio was observed on removal of the femoral artery. In mice given saline or Ad.CMV-LacZ, this effect was followed by a gradual recovery, except for the most distal part of the
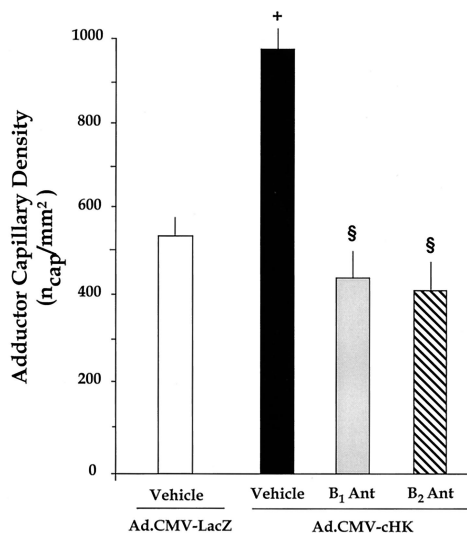
Figure 7. Effect of chronic blockade of B_1 or B_2 receptors on HK-induced angiogenesis. Ad.CMV-LacZ (open bar) or Ad.CMV-cHK was injected in left adductor muscle 7 days after induction of ischemia. Vehicle (filled column) or B_1 (stippled bar) or B_2 (hatched bar) receptor antagonist was infused, starting on day of Ad.CMV-cHK injection. Capillary density in ischemic adductor muscles was counted 21 days after surgery. Values are mean±SEM. +$P<0.01$ vs Ad.CMV-LacZ; §$P<0.01$ vs Ad.CMV-cHK+vehicle.

ischemic hindlimb. Gene therapy with HK accelerated hemodynamic recovery of the whole limb ($P<0.01$). Energetic charge was reduced in ischemic muscles injected with saline (71±3% versus 85±3% in contralateral muscles, $P<0.01$) or Ad.CMV-LacZ (66±4% versus 84±2%, $P<0.01$). In contrast, energetic charge was preserved in ischemic muscles of Ad.CMV-cHK–injected mice (84±1% versus 86±2%, $P=0.66$ [not significant]).

Discussion

Increased kinin levels in the coronary sinus blood of dogs and humans have been reported after myocardial ischemia.[25,26] The compensatory relevance of this phenomenon is intrinsic to the ability of kinin to cause vasodilation and preserve muscular energy content.[16,27] Yet the possibility that kinins exert long-term protection on ischemic skeletal muscles by potentiation of angiogenesis has not been explored. We have shown that B_1 receptor gene expression is activated in ischemic skeletal muscles. In the ischemic milieu, cells expressing this receptor may act as magnets for kinin peptides generated by kallikrein. Although identification of B_1 receptor–positive cells was not addressed in the present study, the functional relevance of B_1 receptors in postischemic angiogenesis was documented by antagonism with DALBK. The same antagonist did not alter the inflammatory response to ischemia, thus suggesting that different mechanisms modulate leukocyte recruitment after ischemia. However, B_1 receptors might be implicated in other cellular events responsible for ischemia-induced angiogenesis. In vitro, BK induces proliferation of vascular endothelial cells, one of the initial events in angiogenesis, via activation of the B_1 receptor–cAMP pathway.[8] Furthermore, BK in synergism with

interleukin-1α enhances the angiogenic response to the subcutaneous implantation of a polyether sponge in rats; this potentiation is abolished by B_1 antagonism, whereas B_2 blockade is not effective.[7] In line with the above reports, native angiogenesis in the ischemic hindlimb is partially prevented by DALBK but not by Icatibant.

The model used in the present study simulates the ischemia typical of patients with lower-extremity arterial occlusive disease. After an initial profound drop of blood flow, hindlimb perfusion progressively recovers, with morphometric evidence of muscular neovascularization. In the present study, a 7-day interval was incorporated between surgery and gene transfer to allow for the complete development of spontaneous angiogenesis. Successful infection by Ad.CMV-cHK was documented at mRNA and protein levels. The expression of HK transgene was limited to the site of injection and vanished within 28 days. Detection of recombinant protein in plasma demonstrates the secreted nature of the gene product, a property recognized to be relevant for therapeutic angiogenesis.[4] Contrary to the possibility that secreted HK may trigger an immune response, antibodies against HK or kallikrein DNA were not developed up to 7 weeks after delivery of HK gene constructs.[28]

The success of HK gene therapy is documented by anatomic and functional evidence. Immunohistochemical identification of vascular endothelial cells demonstrated that HK gene transfer potentiates the angiogenic response to ischemia. This result was associated with improved perfusion recovery and preserved energetic charge of ischemic muscle, consistent with amelioration of blood supply and/or with a direct favorable metabolic effect of kinins.[27]

The increase in kinin levels caused by HK was insufficient to evoke hypotension in normal rats[19] and mice, whereas systemic vasodilatory activity was documented in hypertensive animals.[18,19] Furthermore, dissociation between the duration of HK transgene expression and improvement in perfusion suggests that the latter effect was due to augmented vascularity rather than to kinin-induced muscular vasodilation.

Various mechanisms may be implicated in HK-induced angiogenesis. Kallikrein, acting as a proteinase[9] and activating the metalloproteinase type IV collagenase,[29] might favor the degradation of vascular basal membrane and extracellular matrix protein, thus leading to endothelial cell invasion and migration. Generated kinins may stimulate vascular endothelial cell to proliferate.[8] Experiments using kinin antagonists indicate that activation of B_1 or B_2 receptor signaling is implicated in the angiogenic process. Additional growth factors[4] plus tissue kallikrein and kininogen substrate[30,31] may be released from migrated leukocytes, thus amplifying the initial angiogenic response.

The possibility that NO, a well-known angiogenic factor,[32] represents the final mediator of the vascular effects of HK is supported by previous studies showing that infection with Ad.CMV-cHK leads to activation of the NO-cGMP pathway.[20] Consistently, we report in the present study increased cGMP levels in Ad.CMV-cHK–injected skeletal muscles. Furthermore, preliminary experiments showed that pharma-
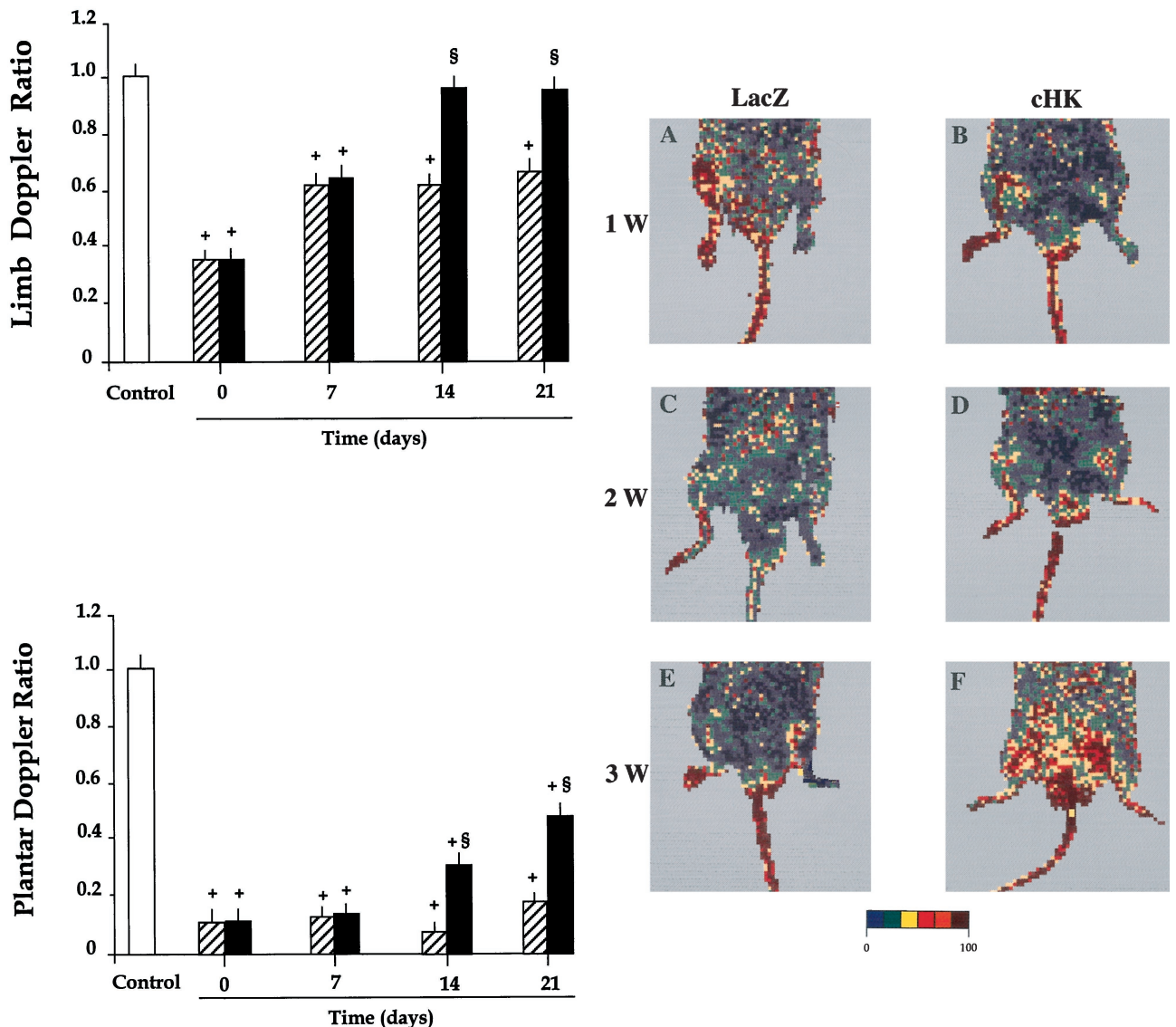
Figure 8. Time course of perfusion recovery after induction of ischemia evaluated by laser Doppler flowmetry. Left femoral artery was excised at time 0. Seven days later, mice received intramuscular adenovirus. Bar graphs on left show ratio of ischemic to nonischemic perfusion in whole limb (top graph) or plantar region (bottom graph) of mice injected with Ad.CMV-LacZ (hatched bars) or Ad.CMV-cHK (filled bars). Perfusion ratio before ischemia (control, open bar) is shown as reference. Values are mean±SEM. +$P<0.01$ vs control; §$P<0.01$ vs Ad.CMV-LacZ. Representative images of laser Doppler perfusion measurements are shown on right. Abdominal area and ventral parts of limbs and tail are shown. Colors displayed in scale correspond to 6 intervals of perfusion from 0% (dark blue) to 100% (red). Images A and B were recorded on the day of adenovirus delivery at 1 week (1W) after induction of ischemia. Perfusion recovery during following weeks (2W and 3W) was accelerated in mice injected with Ad.CMV-cHK (D and F) compared with controls given Ad.CMV-LacZ (C and E).

cological inhibition of NO synthase prevents the angiogenic effect of HK in normoperfused skeletal muscle.[32a]

Prostacyclin represents an important mediator for kinin-induced vascular effects.[20] Activation of B_2 receptors stimulates phospholipase A_2 with increased prostacyclin formation. Prostacyclin activates adenylate cyclase, which results in increased cAMP levels. Enhancement of cAMP by HK in vascular tissue[20] and skeletal muscle might favor the involvement of prostaglandins as mediators of the angiogenic action of HK.[33]

Interestingly enough, kinins share important features with the potent angiogenic factor vascular endothelial growth factor (VEGF). Both induce plasma extravasation, vasodila-

tion, and endothelial cell proliferation. On a molar basis, BK proved to be more potent than VEGF in in vitro proliferation assays using human coronary endothelial cells.[8,34] However, only VEGF is able to stimulate cell migration. In vivo angiogenic activities of HK and VEGF appear to be superimposable, with both depending on the stimulation of NO release.[32,35] However, scrotal edema, a side effect of VEGF angiogenesis gene therapy,[36] was not observed in Ad.CMV-cHK–treated mice.

An increase in the capillary density of skeletal muscle and myocardium induced by ACE inhibitors has been hypothesized to be attributable in part to the prevention of kinin breakdown.[37,38] The present study is the first to demonstrate
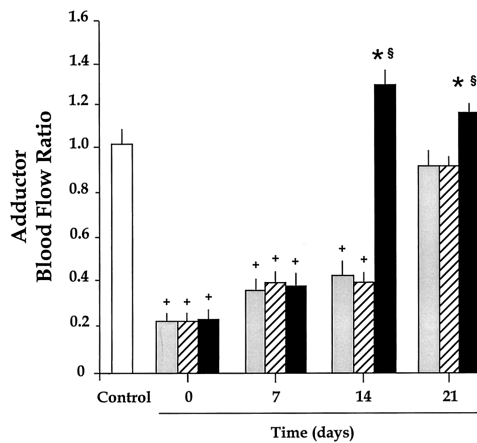
Figure 9. Perfusion recovery after induction of ischemia evaluated by radioactive microspheres and expressed as ratio of ischemic to nonischemic blood flow. Left femoral artery was excised at time 0. Seven days later, mice received intramuscular saline (stippled bars), Ad.CMV-LacZ (hatched bars), or Ad.CMV-cHK (filled bars). Perfusion ratio in control nonoperated mice (open bar) is shown as reference. Values are mean±SEM. +$P<0.01$ vs controls; §$P<0.01$ vs Ad.CMV-LacZ; and *$P<0.01$ vs saline.

that a continuous supply of HK by gene transfer potentiates angiogenesis in ischemic skeletal muscle. Altogether, these results suggest that pharmacological or genetic interventions able to enhance local kinins may deserve consideration as therapeutic strategies for patients with claudication and/or critical limb ischemia.

Angiogenesis therapy with HK is attractive for several reasons: (1) HK proved to be protective against neointimal formation in models of mechanical carotid injury,[20,39] whereas concerns have been advanced for other angiogenic factors to accelerate atherosclerotic plaque growth.[40] (2) The secreted nature of HK allows that much less myocytes need to be infected to achieve the desired biological effect, an important feature in consideration of the low capability of mature muscle fibers to be transduced by adenovirus.[41] (3) HK-induced angiogenesis is limited to the site of injection. Thus, local delivery of HK may not augment the risk of pathological angiogenesis in distant tissues. (4) HK angiogenic activity is not restricted to the mouse, inasmuch as a favorable impact was observed in rats with limb ischemia (C.E., unpublished data, 2000). (5) Availability of potent and selective kinin receptor antagonists allows modulation of the biological effects of HK in case of an excessive or undesired angiogenic response.

In conclusion, the present study provides new insights into the role of the kallikrein-kinin system in vascular medicine and may have significant implications for gene therapy in the treatment of peripheral ischemia.

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