Rescue of Impaired Angiogenesis in Spontaneously Hypertensive Rats by Intramuscular Human Tissue Kallikrein Gene Transfer

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Abstract—Angiogenesis represents a compensatory response targeted to preserve the integrity of tissues subjected to ischemia. The aim of the present study was to examine whether reparative angiogenesis is impaired in spontaneously hypertensive rats (SHR), as a function of progression of hypertension. In addition, the potential of gene therapy with human tissue kallikrein (HK) in revascularization was challenged in SHR and normotensive Wistar-Kyoto rats (WKY) that underwent excision of the left femoral artery. Expression of vascular endothelial growth factor and HK was upregulated in ischemic hindlimb of WKY but not of SHR. Capillary density was increased in ischemic adductor muscle of WKY (from 266 ± 20 to 633 ± 73 capillaries/mm² at 28 days, P < 0.001), whereas it remained unchanged in SHR (from 276 ± 20 to 354 ± 48 capillaries/mm², P = NS), thus compromising perfusion recovery as indicated by reduced plantar blood flow ratio (0.61 ± 0.08 versus 0.92 ± 0.07 in WKY at 28 days, P < 0.05). In separate experiments, saline or 5 × 10⁹ pfu adenovirus containing the HK gene (Ad.CMV-cHK) or the β-galactosidase gene (Ad.CMV-LacZ) was injected intramuscularly at 7 days after the induction of ischemia. Ad.CMV-cHK augmented capillary density and accelerated hemodynamic recovery in both strains, but these effects were more pronounced in SHR (P < 0.01). Our results indicate that native angiogenic response to ischemia is impaired in SHR, possibly as a result of defective modulation of endothelial cell mitogens. Supplementation with kallikrein, one of the growth factors found to be deficient in SHR, restores physiological angiogenic response utilitarian for tissue healing. Our discoveries may have important implications in vascular medicine for therapeutic benefit. (Hypertension. 2001;38:136-141.)

Key Words: angiogenesis ■ ischemia ■ genes ■ muscles ■ kallikrein

Peripheral vascular disease represents a major health problem, particularly in elderly persons. It is becoming clear that endothelial dysfunction, in association with defective expression of endothelial cell mitogens, compromises reparative processes consequent to vascular injury and ischemia. This concept was clearly documented in aged animals,1 as well as in models of atherosclerosis2,3 and diabetes.4 Hypertension, a major risk factor for cardiovascular disease, is characterized by endothelial dysfunction2 and an altered control of vascular cell growth and death.6 Cardiovascular cells from hypertensive animals are subjected to an enhanced turnover since the early phases of development, leading to accelerated aging of these cells and progressive microvascular rarefaction.7-9 The mechanisms responsible for these functional and structural alterations have not been completely elucidated. Expression of the kallikrein-kinin system (KKS), a mechanism relevant to vascular protection and repair,10-12 is reduced in hypertension, and this defect may contribute to endothelial dysfunction and hypertensive vascular remodeling.13,14 However, it is not known whether the same concept can be extended to other endothelial cell-specific mitogens, such as vascular endothelial growth factor (VEGF). Most importantly, the impact of arterial hypertension on native compensatory response to ischemia has not yet been explored.

The present study was designed to investigate the hypothesis that spontaneous angiogenic response to ischemia is impaired in spontaneously hypertensive rats (SHR) as a function of the progression of hypertension and, if this hypothesis is confirmed, to identify potentially implicated mechanisms. Moreover, the recent discovery that human tissue kallikrein (HK) gene transfer promotes collateral vessel growth15 and accelerates postischemic recovery of otherwise normal animals12 prompted us to challenge this approach of angiogenesis gene therapy in the context of a hypertensive background. This issue may have important implications for
the use of such a strategy in hypertensive patients who are at risk for vascular complications.

**Methods**

**Animals**

All procedures complied with the standards for the care and the use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985).

Eight- or 20-week-old male SHR and age-matched normotensive Wistar-Kyoto rats (WKY) (Charles River, Italy) were used.

**Surgical Induction of Limb Ischemia**

With rats anesthetized (20 mg/kg ketamine and 0.4 mg/kg medetomidine chlorhydrate, both IM) and maintained at 37°C on a heating pad, the left femoral artery was exposed through an incision of the skin, dissected free from its proximal origin to the bifurcation into the saphenous and popliteal arteries, closed with a microtip electrocaugulator, and excised.

**Kallikrein and VEGF Gene Expression**

Expression of rat tissue kallikrein (tK) was evaluated in the gastrocnemius muscle of 20-week-old WKY and SHR (n=3 per group) at 0, 1, 3, and 7 days after induction of ischemia by RT-PCR analysis, as described previously.16 VEGF mRNA levels were determined on the same samples with the following RT-PCR primers: 5′-GAT TCT TGT ATA CCA GCC GTC TCC G-3′, and 3′-ACA AAT GCT TTT TTC GCT C-3′. Briefly, total RNA was isolated from frozen skeletal muscles according to the RNAzol B method, in the presence of DNase. The reaction mixture for RT contained 1 μg total RNA, 10 pmol random hexamer, 10 mmol dNTP, 0.2 μmol dTT, 4 μL of 5X reverse transcription buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L MgCl₂), and 200 U Moloney murine leukemia virus reverse transcriptase (BRL), in a total volume of 20 μL. The RT reaction mixture was incubated at 23°C for 10 minutes followed by 37°C for 50 minutes to synthesize the first strand of cDNA. Then, 10 pmol 5′ primer, 10 pmol 3′ primer, 5 μL 10X PCR buffer, 40 nmol dNTP, and 2.5 U of Taq DNA polymerase were added to the 20 μL of RT mixture to a total volume of 50 μL followed by 30 cycles of hot-start PCR (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute) in a thermal cycler. In previous experiments, the specificity of PCR products obtained for VEGF was confirmed by Southern blot analysis (J.C., unpublished data). In negative control experiments, RT was omitted. GAPDH was used for normalization in densitometric analysis.

**Adenovirus Delivery**

Seven days after the induction of ischemia, 150 μL sterile saline or 5x10⁉ pfu (in 150 μL) adenovirus containing the HK gene (Ad.CMV-cHK) or the β-galactosidase gene (Ad.CMV-LacZ)16 was injected into 3 different sites of the left adductor muscle of anesthetized rats. Animals that had been allocated at random to each treatment were tagged, and the code of randomization was broken at the end of the experiments.

**Hemodynamic Measurements**

Systolic blood pressure (SBP) and heart rate were measured in unanesthetized rats by tail-cuff plethysmography before adenovirus injection and sequentially after gene transfer. The superficial blood flow of the plantar skin was measured by laser Doppler flowmetry (Transonic) (1) before femoral artery removal, (2) at 1 day after surgery, (3) on virus or saline injection (ie, 7 days after surgery), and (4) at weekly thereafter until the rats were killed at 28 days. Measurements were performed in anesthetized rats (n=3 per group) maintained on a heating pad at 37°C, and results were expressed as the ratio of ischemic to nonperfused limb blood flow.

**Histology and Morphometric Analysis**

Capillary density of ischemic and contralateral adductors was measured at 14 and 28 days after removal of the left femoral artery by a single observer who was blinded to treatment regimen. Hindlimbs of anesthetized 20-week-old rats were perfused at physiological pressure with PBS (1 minute), followed by 10% formalin (10 minutes) via a cannula inserted into the abdominal aorta. After paraffin embedding, 3-μm-thick sections were cut from each adductor muscle with muscle fibers oriented in a transverse direction, stained with hematoxylin-eosin, and examined at 200X magnification. For each area of tissue section, 75 fields were randomly examined. The number of capillary profiles was counted to compute the capillary numerical density per mm² of muscle. The number of myofibers per mm² of muscle was also evaluated, and then the ratio of capillary to myofiber number was calculated.

**Statistical Analysis**

All results were 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 F test indicated significant differences, the statistical value was determined according to Bonferroni’s method. Differences within and between groups were determined using paired or unpaired Student’s t test, respectively. P<0.05 was considered statistically significant.

**Results**

**Effects of Ischemia on Gene Expression**

As shown in Figure 1, tK and VEGF gene expression in the skeletal muscle of WKY were markedly increased at 1 day after the induction of ischemia (P<0.05 for both comparisons) and then returned to baseline at 7 days. Ischemia failed to modulate gene expression in SHR.

**Hindlimb Blood Flow Recovery**

At all ages examined, the SBP of SHR was higher than that of WKY (157±8 versus 122±10 mmHg at 8 weeks, P<0.05; 186±12 versus 131±6 mmHg at 20 weeks, P<0.001), whereas heart rate did not differ (data not shown).

The induction of ischemia was followed by a dramatic drop in plantar blood flow in both WKY and SHR. During the next days, perfusion of the ischemic limb increased at the same rate in 8-week-old SHR and age-matched WKY (data not...
shown), thus indicating that arterial hypertension per se does not alter postischemic hemodynamic recovery.

As shown in Figure 2, perfusion recovery was instead reduced and delayed in 20-week-old SHR. In fact, at 7 days after surgery, plantar blood flow ratio averaged $0.26 \pm 0.03$, a value 2.3- and 1.7-fold lower than that observed in age-matched WKY ($0.61 \pm 0.07$) or 8-week-old SHR ($0.44 \pm 0.04$, $P < 0.01$ for both comparisons). In 20-week-old SHR, the perfusion ratio was still reduced 28 days after the induction of ischemia ($0.61 \pm 0.08$ versus $0.92 \pm 0.07$ in age-matched WKY, $P < 0.01$). Therefore, SHR showed impaired hemodynamic recovery as a function of age.

**Ischemia-Induced Native Angiogenesis in WKY and SHR**

As shown in Figure 3, the capillary density of normoperfused adductors was similar in 20-week-old WKY and SHR ($266 \pm 20$ versus $276 \pm 30$ capillaries/mm$^2$, respectively, $P = \text{NS}$). Furthermore, no strain difference was detected when considering capillarity normalized by myofiber number ($0.86 \pm 0.07$ versus $0.74 \pm 0.04$ capillaries per fiber in WKY and SHR, respectively, $P = \text{NS}$).

In 20-week-old WKY, at 28 days after femoral artery excision, capillary density of ischemic adductor was found to be 2.4-fold higher than that of contralateral normoperfused muscle ($P < 0.01$), with this result being indicative of compensatory neovascularization. In contrast, no angiogenic effect was manifested at the capillary level in the ischemic adductor of 20-week-old SHR ($354 \pm 48$ versus $276 \pm 30$ capillaries/mm$^2$ in contralateral muscle, $P = \text{NS}$).

**Effect of Kallikrein Gene Transfer on Perfusion Recovery and Muscular Capillary Density**

Local HK gene transfer did not affect SBP of 20-week-old rats (SHR from $186 \pm 12$ to $188 \pm 11$ mm Hg, WKY from $131 \pm 6$ to $121 \pm 5$ mm Hg at 7 days postinjection, $P = \text{NS}$ for both comparisons).

As shown in Figure 4, perfusion recovery was accelerated by Ad.CMV-cHK in both strains, but the beneficial effect exerted by HK was significantly greater in SHR ($P < 0.001$). Therefore, this angiogenesis gene therapy approach was of benefit for the treatment of ischemia with particular regard to hypertensive animals, inasmuch it rescued impaired postischemic recovery typical of long-term hypertension.

As shown in Figure 5A and 5B, ischemic adductor muscles of 20-week-old WKY given Ad.CMV-chk showed increased capillarity at 14 days from surgery ($797 \pm 59$ versus $208 \pm 16$ capillaries/mm$^2$ in LacZ and $1.94 \pm 0.18$ versus $0.87 \pm 0.11$ capillaries per fiber in LacZ, $P < 0.001$ for both comparisons), anticipating by 2 weeks the spontaneous angiogenic response...
observed in untreated animals. At 28 days, no difference in muscular capillary density was seen among normotensive rats given HK, Lac-Z, or saline (Figure 4C and 4D).

In 20-week-old SHR, HK increased the capillary density of ischemic muscle by 3.4-fold at 14 days (605 ± 60 capillaries/mm² and 1.87 ± 0.16 capillaries per fiber, P < 0.05 versus LacZ for both comparisons) and by 2.2-fold at 28 days (625 ± 18 capillaries/mm² and 2.35 ± 0.44 capillaries per fiber at 28 days, P < 0.01 versus LacZ for both comparisons). Therefore, HK gene transfer was able to accelerate spontaneous reparative angiogenesis in normotensive animals and, most importantly, to correct defective neovascularization typical of SHR.

Discussion

Survival of endothelial cells is critical for the maintenance of blood vessel integrity. Consistently, cardiovascular diseases characterized by accelerated cell turnover and death are generally associated to progressive reduction in vascularity. Microvascular rarefaction has been reported in hypertensive humans, but in SHR this finding is not universal. Under basal conditions, we did not find appreciable differences in capillary density and capillary-to-myofiber ratio between SHR and WKY at 20 weeks of age. It is conceivable that hypertensive remodeling may require a longer period to occur at the level of muscular microvasculature.

The present study also documents that reparative angiogenesis is impaired in SHR as a function of progression of the hypertensive disease. In fact, no abnormality was observed at 8 weeks (an age at which SHR blood pressure was already 35 mm Hg above that of normotensive controls), whereas failure of muscular capillary density to increase after induction of limb ischemia was documented in 20-week-old SHR. This suggests that hypertension per se is not sufficient to determine the defective phenotype. It appears more likely that the development of endothelial dysfunction related to the duration of the hypertensive disease might have contributed to the impaired response to ischemia.

Recent studies have demonstrated the relevance of aging for collateral development in limb ischemia. Impaired angiogenesis has been observed in mice and rabbits age ≥2 years compared with controls of a younger age. Our results showing defective angiogenesis in 20-week-old SHR support the hypothesis that cardiovascular cells from hypertensive animals are subjected to an accelerated aging. This microvascular deficit severely compromised posts ischemic perfusion recovery to the distal part of the limb, without evidence of resolution up to 28 days. It should be noted that in age-matched WKY, hemodynamic recovery anticipated the increase in capillary density, probably due to the activation of preexisting quiescent collaterals on the induction of ischemia. This mechanism may also be operative in SHR, inasmuch plantar blood flow recovered over time, although at a slower rate compared with WKY, despite no evidence of neoangiogenesis at the capillary level.

It should be pointed out that we did not intend ascertain possible inheritance of the angiogenic defect or its linkage with the genetics of hypertension. The use of nongenetic hypertensive models would be necessary to address this intriguing question, although we may expect that results would not be univocal depending on the particular mechanism that underlies each specific animal model.

Angiogenesis is a complex process modulated by a number of factors, the activity of which is under the control of genetic and environmental determinants. The formation of new vessels involves endothelial cell activation, migration, and proliferation. From this concept, the hypothesis was advanced that disease states characterized by endothelial dysfunction may be unable to mount an efficient response to vascular injury or tissue ischemia. Furthermore, a deficit in endogenous growth factors may contribute to reduce angiogenic potential. Experimental evidence for these concepts has been provided in genetic models of atherosclerosis and diabetes. Aged animals also combine an inability to mount an efficient neovascularization to impairment in the ischemia-induced activation of endogenous angiogenic cytokines. Similarly,
we found that ischemia-induced upregulation of tK and VEGF expression is lacking in 20-week-old SHR, reinforcing the view of an early impairment of mechanisms that control vascular cell growth and repair in SHR.

Angiogenesis gene therapy, which is based on the concept that a continuous supply of growth factors can rescue impaired collateral growth, proved to be effective in peripheral vascular disease. In consideration of the reduced expression of KKS in hypertension and of the evidence that ACE inhibitors exert angiogenic effect in the rabbit skeletal muscle and rat hearts, possibly via the stimulation of kinin B2 receptor, we thought it would be worthwhile to test whether potentiation of kinin signaling could be of benefit to postischemic recovery in the context of a hypertensive background. Two important arguments favored our hypothesis: (1) adenovirus-mediated HK gene transfer is able to induce vascular healing after mechanical injury, and (2) HK gene delivery significantly enhances native angiogenic response to hindlimb ischemia in the mouse, via generation of kinins, and consequent release of angiogenic NO and prostacyclin.

This approach was also of benefit in hypertension, with the important addition that the effect was achieved in the context of an impaired angiogenic potential. Rescue of defective angiogenesis by HK was associated with amelioration of blood flow recovery and occurred without any change in systemic hemodynamics. It should be noted that the dose used here was less than that able to decrease blood pressure in SHR.

In summary, our results show that native angiogenic response to ischemia is impaired in SHR, possibly because of a deficit in compensatory mechanisms relevant to the promotion and regulation of endothelial growth and repair. Supplementation with kallikrein, one of the angiogenic factors found to be defective in SHR, was able to correct impaired angiogenesis and allowed a complete hemodynamic recovery. Our

Figure 5. Capillary density per mm² or per myofiber in ischemic adductors of 20-week-old WKY and SHR at 14 (A and B) and 28 (C and D) days after induction of hindlimb ischemia. Sterile saline (open columns), Ad.CMV-LacZ (hatched columns), or Ad.CMV-cHK (full columns) was injected 7 days after induction of ischemia. Values are mean ± SEM. §P < 0.05 vs saline, #P < 0.05 vs Ad.CMV-LacZ, *P < 0.05 vs WKY.
discoveries not only shed light on the importance of endogenous KKS in reparative angiogenesis but also support the hypothesis that interventions able to enhance local kinin levels may deserve consideration as therapeutic strategies for hypertensive patients with critical limb ischemia.

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