Prophylactic Gene Therapy With Human Tissue Kallikrein Ameliorates Limb Ischemia Recovery in Type 1 Diabetic Mice

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Diabetes macro- and microvascular disease causes tissue hypoperfusion. This deficit, together with a failure to mount an adequate angiogenic response, might explain why vascular occlusion evolves more severely among diabetic patients. The present study investigated whether prophylactic gene therapy with human tissue kallikrein (hTK) may protect diabetic limbs from the consequences of supervening ischemia. Vehicle (saline) or an adenovirus carrying the gene for either hTK (Ad.hTK) or luciferase (Ad.Luc) was injected into left adductor muscles of streptozotocin-induced type 1 diabetic mice 2 weeks before operative occlusion of the ipsilateral femoral artery. Saline-injected nondiabetic mice served as controls. Hindlimb blood flow recovery was analyzed sequentially over the 2 weeks after ischemia induction. At necropsy, microvessel density and endothelial cell proliferation and apoptosis were quantified in skeletal muscles. We found that limb perfusion recovery of saline-injected type 1 diabetic mice is delayed because of insufficient reparative neovascularization and excessive activation of endothelial cell apoptosis. By contrast, prophylactic Ad.hTK renewed the ability to mount an appropriate neovascularization response to ischemia, suppressed apoptosis, and upregulated endothelial nitric oxide synthase expression. Ultimately, correction of diabetic endotheliopathy by Ad.hTK allowed proper perfusion recovery as seen in nondiabetic mice. These discoveries disclose new therapeutic options for the treatment of diabetic complications. Diabetes 53:1096–1103, 2004

Accelerated atherosclerosis makes peripheral ischemia dramatically common and severe in diabetic patients (1,2). In addition, clinical outcome after vascular occlusion is uniquely poor because of the failure to mount an adequate collateralization. An insufficient surge of vascular growth factors might contribute to the deficit. Therefore, supplementation with vascular endothelial growth factors (VEGFs) or hepatocyte growth factors has been proposed as a possible method to correct endogenous liabilities and rescue defective posts ischemic healing (3,4). However, master angiogenic factors may fail at advanced stages of the disease (5) as a consequence of endothelial cell unresponsiveness and reduced availability of the downstream mediator nitric oxide (NO). Thus, the main limitation of supply-side approach is that it provides the fuel but no steering power to guide the reparative process.

We reasoned that prevention of diabetic endotheliopathy might be more effective than rescue interventions. The assumption is based on preliminary studies showing that a single, local injection of an adenovirus carrying the angiogenic human tissue kallikrein (tk) gene (Ad.hTK) exerts long-lasting protection against skeletal muscle microangiopathy by restoring endothelium-dependent vasodilation, promoting vascular regeneration, and attenuating endothelial cell apoptosis, without the need of insulin supplementation (6). We have previously shown that adenovirus-mediated hTK gene delivery to skeletal muscle increases the local levels of kinins (7), which in turn mediate hTK-induced angiogenesis (7). In vitro, activation of kinin receptors causes endothelial NO synthase (eNOS) phosphorylation and NO release through the calcineurin and phosphatidylinositol (PI) 3-kinase–Akt pathways (8). In addition, we recently found that Akt-B mediates hTK-induced endothelial cell proliferation in mouse skeletal muscle (C.E., unpublished data). Interestingly, PI 3-kinase–NO–Akt signaling via inhibition of proteins Bad or caspase-9 seemingly protects endothelial cells from apoptosis, and this mechanism is relevant to insulin pro-survival effects (9).

The aim of the present study was to clarify the events responsible for impaired reparative angiogenesis in type 1 diabetes. In addition, we challenged the hypothesis that prophylactic local gene therapy with Ad.hTK may improve collateral circulation of type 1 diabetic limb muscles,
thereby restoring proper microvascular response after arterial occlusion.

We found that in type 1 diabetic mice, capillary and arteriole growth response to ischemia is profoundly impaired. Furthermore, excessive activation of endothelial cell apoptosis might contribute to microvascular destabilization. We also documented that prophylactic application of Ad.hTK potentiates collateral circulation, increases eNOS expression, and confers resistance to apoptosis, thus facilitating postischemic healing of type 1 diabetic skeletal muscles.

**RESEARCH DESIGN AND METHODS**

**Type 1 diabetes assessment.** Procedures complied with the Guide for the Care and Use of Laboratory Animals (Bethesda, MD, Institute of Laboratory Animal Resources, National Academy of Sciences, 1996) standards. Type 1 diabetes was induced by streptozotocin (STZ; 40 mg/kg body wt i.p. daily for 5 consecutive days) (6) in male 2-month-old CD1 mice (Charles River, Comerio, Italy). Mice were considered diabetic only if they had fasting glycemia >250 mg/dl and overt glycosuria at 14 days from the first STZ injection. Persistence of diabetes was determined throughout the study. Body weight was recorded before STZ and every 14 days thereafter.

**Experimental protocols.** At 1 month after the first documentation of glycosuria, mice were anesthetized (2,2,2-trichloroethanol anesthesia, 880 mmol/kg i.p.; Sigma) to receive saline or adenoviral vectors carrying hTK gene (Ad.hTK; kindly provided by Dr. Julie Chao, Medical University of South Carolina, Charleston, SC) (7) or luciferase gene (Ad.Luc; each at 1 × 10⁹ plaque-forming units) into left adductor muscles. After 14 days, mice were killed for determination of adductor microvascular density (n = 7, each group) or submitted to left hindlimb ischemia by femoral artery electrocoagulation (Ad.hTK, n = 10; Ad.Luc, n = 10; saline, n = 6). Age-matched nondiabetic mice injected with saline were studied for reference (n = 10).

Hindlimb blood flow was measured at 30 min and 4, 7, and 14 days after ischemia with a perfusion imager system (Lisca) (7). The ischemic-to-nonischemic foot blood flow ratio was calculated as an index of blood flow recovery.

After killing, the adductors of anesthetized mice were perfusion fixed and processed for evaluation of capillary density (6). For identification of arterioles, sections were stained with a mouse monoclonal antibody targeted to α-smooth muscle actin, as previously described (6). Arterioles were recognized as those vessels positive for α-smooth muscle actin and with one or more continuous layers of smooth muscle cells in their wall. Arteriole density per square millimeter of section was calculated as described previously (6). Proliferation and apoptosis were assessed in muscles harvested at 0, 3, and 14 days from ischemia (n = 5 per group). Proliferating cells were immunohistochemically identified by the use of a monoclonal antibody (Dako) against proliferating cell nuclear antigen (PCNA). The transferase-mediated dUTP nick-end labeling (TUNEL) assay revealed apoptosis as previously described (6). PCNA- or TUNEL-positive cell number was counted as an index of positive cells per square millimeter of section.

Additional experiments were performed to evaluate the protective potential of the type 1 diabetes reference drug insulin. To this aim, type 1 diabetes was induced in 2-month-old CD1 mice that were then randomly allocated into two groups receiving insulin (3 units daily Humulin U; Lilly) (6) or vehicle (n = 8 per group). Another set of nondiabetic mice was used as controls (n = 8). After 14 days, ischemia was induced in all three groups. Blood flow was

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**FIG. 1.** Type 1 diabetes impairs capillarization response to ischemia in limb skeletal muscles with no compensation by insulin. A: Bar graph shows the blunted increase in capillary density of ischemic adductor muscles (I) from type 1 diabetic (IDDM; ○) as compared with healthy mice (■). Values of nonischemic contralateral muscles (C) are shown for reference (□). B: Results expressed as capillary-to-myofiber density show the same pattern as above. C: The result of another set of experiments documenting that diabetic mice failed to mount proper capillarization regardless of whether they were given insulin (◼, ▽) or no treatment (□, △). Values are means ± SE. *P < 0.05 vs. contralateral; §P < 0.01 vs. healthy mice.
measured at 30 min, 7 days, and 14 days. Mice were then killed for evaluation of adductor microvascular density.

Evaluation of eNOS and VEGF-A gene expression. Real-time quantitative PCR (ABI Prism 7000 sequence detection system software, version 1.0; Perkin Elmer) was used to determine eNOS and VEGF-A mRNA levels in muscles harvested from type 1 diabetic (untreated or preventively given Ad.hTK) or nondiabetic mice (n/H110056 per group) before or 10 days after ischemia. Total RNA was isolated using TRIzol reagent (Invitrogen) and subsequently treated with DNase (Qiagen) to avoid DNA contamination. DNase-treated RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The sequences of primers for amplification of murine eNOS (based on the cDNA sequences from the Genebank database NM-008713) were 5’/H11032CCTTCCGCTACCAGCCAGA 3’ (forward) and 5’/H11032CAGAGATCTTCACTGCATTGGCTA 3’ (reverse). VEGF-A (from Genebank sequence M95200) primers were: 5’/H11032CCA GCG AAG CTA CTG CCG TCC A 3’/H11032 (forward) and 5’/H11032ACA GCG CAT CAG CGG CAC AC 3’/H11032 (reverse). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (based on Genebank database sequence NM-008084) were 5’/H11032CGT GGG GCT GCC CAG AAC AT 3’/H11032 (forward) and 5’/H11032TCT CCA GGC GGC ACG TCA GA 3’/H11032 (reverse). ENOS or VEGF-A cDNA levels were normalized to GAPDH housekeeping gene cDNA levels.

Statistics. Results are expressed as the means ± SE. 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, 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 denoted statistical significance.

RESULTS

Diabetes impairs postischemic healing. Spontaneous capillarization response to limb ischemia was impaired in type 1 diabetic mice (Fig. 1A and B). Insulin supplementation was able to control glycosuria (data not shown), but not to restore proper capillarization (Fig. 1C). Diabetes also blunted the increase in arteriole density in response to ischemia (Fig. 2A), with no compensation by insulin (Fig. 2B).

Ischemia induces excessive endothelial cell and myocyte apoptosis in type 1 diabetic mice. Endothelial cell proliferation (as assessed by PCNA staining) was similar...
in ischemic muscles of healthy or type 1 diabetic mice at 3 or 14 days after surgery (3.25 ± 0.52 vs. 2.92 ± 0.52 PCNA-positive endothelial cells/mm² at 3 days, \( P = \text{NS} \)) (data not shown). However, in diseased animals the effort to build up new vessels was frustrated by enhanced late activation of endothelial cell apoptosis (Fig. 3A). The effect was confirmed after normalization of the number of apoptotic endothelial cells by capillary density (37 ± 20 vs. 4 ± 3 apoptotic endothelial cells/1,000 capillaries in contralateral normoperfused muscles, \( P < 0.05 \)). Myocyte apoptosis was likewise activated in ischemic muscles of type 1 diabetic mice (Fig. 3B).

Prophylactic Ad.hTK increases microvessel density in normoperfused skeletal muscles of type 1 diabetic mice. Ad.hTK did not alter body weight or glycosuria as compared with other treatment groups. We found that hTK potentiates limb microvessels of type 1 diabetic mice. In fact, in adductors not subjected to ischemia, Ad.hTK increased capillary density (883 ± 60 vs. 547 ± 19 capillaries/mm² in contralateral, \( P < 0.001 \)) and arteriole density (7.3 ± 1.4 vs. 2.8 ± 0.5 arterioles/mm² in contralateral, \( P < 0.05 \)). In contrast, Ad.Luc did not alter adductor vascularity (587 ± 45 capillaries/mm² and 2.9 ± 0.8 arterioles/mm², \( P = \text{NS} \) vs. contralateral). Saline was also ineffective (data not shown).

Furthermore, prophylactic local administration of Ad.hTK restored the ability to mount a proper reparative neoangiogenic response to ischemia. Capillarization was increased by 40% in Ad.hTK-pretreated muscles, whereas the angiogenic response to ischemia was virtually absent in controls (Fig. 4A and B). Likewise, Ad.hTK increased arteriole density (Fig. 5).

Prophylactic Ad.hTK improves endothelial cell response to ischemic insult. As shown in Fig. 6, Ad.hTK-pretreated muscles displayed an increased number of PCNA-positive endothelial cells at 3 days from ischemia, indicative of activated endothelial cell proliferation (5.27 ± 0.27 vs. 2.92 ± 0.52 PCNA-positive endothelial cells/mm² in saline-injected muscles, \( P < 0.01 \)); however, the effect was lost at 14 days (data not shown). Thus, hTK confers endothelial cells with improved proliferative potential at the early phases of the healing process, when the sprouting of new capillaries typically occurs. In addition, Ad.hTK pretreatment drastically reduced ischemia-induced endothelial cell and myocyte apoptosis (Fig. 7A and B). Results are further illustrated in Fig. 7C, showing TUNEL-positive cells in ischemic muscles of healthy (i) or type 1 diabetic mice given saline (ii) or Ad.hTK (iii).

Ad.hTK restores eNOS expression in diabetic muscles. In nonischemic muscles from type 1 diabetic mice, the expression of eNOS and VEGF-A was reduced to 10 and 28% the levels seen in healthy mice, respectively (\( P < 0.05 \) for both comparisons). ENOS and VEGF-A expression remained downregulated after induction of ischemia in type 1 diabetic mice injected with saline (data not shown). In contrast, Ad.hTK-pretreated muscles showed twofold increased eNOS mRNA levels after ischemia (\( P < 0.05 \)) but no change in VEGF-A (\( P = \text{NS} \)).

Prophylactic Ad.hTK ameliorates blood flow recovery. Ad.hTK-pretreated type 1 diabetic mice displayed significantly improved limb hemodynamics, matching the recovery rate seen in nondiabetic mice (Fig. 8A). Insulin did not ameliorate blood flow recovery (Fig. 8B).

DISCUSSION

Type 1 diabetes is associated with accelerated atherosclerosis leading to premature myocardial, cerebral, and peripheral ischemia. Moreover, type 1 diabetic patients manifest a more severe course after vascular occlusion, including more frequent postinfarction angina, heart failure, cerebrovascular insufficiency, and nonhealing limb ulcers. The present study confirms that severe impairment in collateral growth contributes to the poor clinical outcome (3,4,10).

We explored the possibility that endothelial cell biology derangement may play a role in the deficit. In vitro conditions simulating the type 1 diabetes microenvironment reportedly reduce endothelial cell proliferation rate and branching angiogenesis (11,12). Excessive activation of apoptosis has been documented for endothelial cells cultured in high glucose or glycated collagen (13,14). Furthermore, seminal in vivo studies demonstrated that type 1 diabetes is responsible for excessive microvascular cell apoptosis in limb muscles (6) and retina (15).
Progression of diabetic microangiopathy follows a slow, inexorable course (6); however, as shown here, dramatic accelerations may occur after arterial occlusion. We documented that endothelial cell apoptosis is maintained at low levels in ischemic muscles of healthy mice. In sharp contrast, the cooperation of ischemia and diabetes dramatically increased endothelial cell death. Thus, uncontrolled apoptosis may jeopardize the attempt to build up functional collateralization.

It should be pointed out that apoptosis was mainly activated during late stages of the repair process. In this critical phase, the provisional network of endothelial tubes is, in part, dismantled and replaced by new intimal and medial smooth muscle cells instrumental in creating arteriolar connections and restoring proper circulation (16). However, arteriole remodelling could be seriously compromised if angiogenesis terminators are excessively upregulated, as reportedly occurs under conditions of hyperglycemia (17,18). Consistently, we found that reparative arteriogenesis is profoundly depressed in type 1 diabetic mice.

In previous studies, we demonstrated that diabetic microangiopathy is not an incurable condition. In fact, without the need of superimposed metabolic control, gene therapy with hTK was able to prevent and rescue microvascular rarefaction (6). Here, we report the discovery that hTK gene transfer protects type 1 diabetic muscles from the consequences of supervening ischemic insult. Adenovirus-mediated gene transfer preceded the induction of ischemia by 2 weeks. We know from previous studies that this time is sufficient for transgene expression to expire (7). Therefore, the beneficial action of hTK on posts ischemic recovery can be considered truly prophylactic. Ad.hTK-injected muscles displayed improved endothelial cell proliferative potential during early phases of postischemic recovery, and they displayed reduced apoptosis at vascular and myocyte levels during later stages of healing. Improved capillarization by Ad.hTK may benefit myocyte viability by increasing oxygen and nutrient diffusion to the muscle.

VEGF-A and eNOS expression was found to be reduced in type 1 diabetic muscles and not modulated by ischemia, which is in agreement with defective VEGF-A and NO production in diabetic animal models (3,19). Impaired eNOS expression was partially restored by hTK pretreatment. This effect could be relevant to protection from ischemia-induced apoptosis. In fact, activation of eNOS, leading to enhanced synthesis of NO, reportedly promotes endothelial cell survival via inhibition of pro-apoptotic caspases and induction of anti-apoptotic protein expression (20). Similar to NO, VEGF exerts inhibitory control of apoptosis (21). However, diabetes-related VEGF-A liabilities were not corrected by hTK. These findings favor the possibility that hTK-induced microvascular effects are mediated by a PI 3-kinase–NO–Akt mechanism independently of VEGF-A, as recently documented in a separate study (C.E., unpublished data). Other unforeseen mechanisms implicated in the pro-survival activity of Ad.hTK merit further investigation.

We documented that insulin—known to activate the pro-angiogenic and anti-apoptotic PI 3-kinase–Akt pathway in endothelial cells (22)—could not restore the proper neovascularization response to muscular ischemia in type 1 diabetic mice. Although metabolic control was seemingly achieved by the use of a standardized dose (6), it might be possible that earlier treatment or species-specific
insulin should have been used to obtain significant microvascular effects.

The theory that inhibition of endothelial cell apoptosis may facilitate angiogenesis has been already introduced by others (23). Nevertheless, the present study is the first to document the feasibility of improving the phenotype of
diabetic endothelial cells to let them regain resistance to ischemic damage. Prophylactic gene therapy with Ad.hTK seems particularly attractive for therapeutic exploitation because of its ability to improve survival and further stimulate endothelial proliferation, which is also required for neovascularization. However, much more work is necessary before proposing hTK gene transfer for clinical use, which includes testing therapeutic efficacy in different diabetic models, tailoring new vector systems, and testing more in depth the absence of adverse effects.

Conclusions and perspectives. Altogether, our results support the therapeutic utility of prophylactic angiogenesis gene therapy with hTK for secondary prevention of type 1 diabetic vascular complications. In particular, pro-

phylactic hTK gene transfer might be envisaged to benefit diabetic patients at risk of occlusion of limb arteries.

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REFERENCES

1. Kannell WB, McGee DL: Diabetes and cardiovascular disease: the Framing-
ham Study. JAMA 241:2103–2108, 1979


17. Kampfer H, Pfeilschifter J, Frank S: Expressional regulation of angio-

FIG. 8. Prophylactic hTK gene therapy of type 1 diabetic adductor muscles improves postischemic recovery, whereas insulin supplementation is ineffective. A: Line graph shows average postischemic blood flow (BF) recovery (expressed as ischemic [I]-to-contralateral [C] blood flow ratio) of type 1 diabetic mice pretreated with Ad.MTK (●), Ad.Luc (○), or saline (□). The recovery of healthy nondiabetic mice is shown for reference (●). B: Results of a second set of experiments demonstrating the inefficacy of insulin (○) to revert type 1 diabetes–induced impairment in blood flow recovery. The rate of recovery was in fact superimposable to that of saline-treated type 1 diabetic mice (□), but it was significantly less than that of nondiabetic mice (●). Values are means ± SE. $P < 0.05 vs. saline; *P < 0.05 vs. Ad.Luc; #P < 0.05 vs. healthy.


