Prevention of Diabetes-Induced Microangiopathy by Human Tissue Kallikrein Gene Transfer

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Background—Microvascular insufficiency represents a major cause of end-organ failure among diabetics.

Methods and Results—In streptozotocin-induced diabetic mice, we evaluated the potential of human tissue kallikrein (hTK) gene as a sole therapy against peripheral microangiopathy. Local delivery of hTK gene halted the progression of microvascular rarefaction in hindlimb skeletal muscle by inhibiting apoptosis, thus ensuring an improved hemodynamic recovery in case of supervening vascular occlusion. The curative action of hTK did not necessitate insulin supplementation. Application of gene therapy at a stage of established microangiopathy stimulated vascular regeneration.

Conclusions—Our studies indicate that hTK may represent a useful tool for the treatment of microvascular complications in diabetics. (Circulation. 2002;106:993-999.)

Key Words: diabetes mellitus ■ microangiopathy ■ gene therapy ■ angiogenesis ■ apoptosis

Periperal ischemia is uniquely common among diabetic patients because of the concomitance of accelerated atherosclerosis and microvascular insufficiency. Early in the course of diabetes, intracellular hyperglycemia causes endothelial dysfunction and hemodynamic abnormalities. With time, endothelial cell (EC) loss by apoptosis and arteriole and capillary occlusion lead to microvascular rarefaction, which favors the formation of nonhealing limb ulcers and limits the benefit of revascularization. The disease follows an inexorable course, so that limb amputation is often the ultimate remedy for unbearable symptoms. Recently, supply-side approaches with angiogenic substances or EC precursors have been proposed as a new remedy for rescuing diabetic hindlimb ischemia. Application of therapeutic angiogenesis for prevention of microangiopathy has been disregarded on the basis of current opinion that the strategy would fail in the absence of an ischemic milieu.

We documented that delivery of human tissue kallikrein (hTK) can induce angiogenesis in normoperfused skeletal muscle and in a hindlimb ischemic model. Several clues suggest that potentiation of the kallikrein-kinin system (KKS) might also benefit peripheral diabetic complications. Kinins generated by tissue kallikrein (TK) from kininogen stimulate EC proliferation and survival through the release of the vasodilator autacoids NO and prostacyclin. In skeletal muscle, kinin binding to the B2-receptor promotes phosphorylation of insulin receptor and activation of the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB pathway, thus favoring translocation of glucose transporter to plasma membrane. Furthermore, experimental and clinical evidence suggests involvement of the KKS in the pathogenesis of diabetic complications. The B2-knockout phenotype encompasses characteristics of syndrome X, namely, arterial hypertension, myocardial hypertrophy, and damage due to microvascular rarefaction, as well as impaired insulin-dependent glucose transport. Among patients with peripheral vascular disease, the diabetics show reduced collateralization and lower circulating TK levels than nondiabetics.

The present study focuses on local hTK gene delivery as sole therapy for the treatment of peripheral microangiopathy in the streptozotocin (STZ) diabetic mouse model.

Methods

Induction of Diabetes

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, 1996). Eight-week-old male CD1 mice (Charles River, Comerio,
Italy) received STZ 40 mg/kg IP (Sigma Chemical Co) in 0.05 mol/L citrate buffer, pH 4.5, daily for 5 days. Only mice showing consistently elevated fasting glucose levels (>250 mg/dL) and overt glycosuria were included in the study.

**Time Course of Microangiopathy**

Capillary density was measured in adductor muscles harvested at selected time points from first assessment of glycosuria. Arteriolar profile and density were evaluated at 60 days. Age-matched nondiabetic mice were used for reference.

**Gene Delivery**

Adenovirus Ad.CMV-hTK consisted of shuttle vector pAdLink.1 inserted with plasmid CMV-hTK at the EcorV site. In the transcription unit, the hTK sequence is followed by the bovine growth hormone gene polyadenylation signal sequence, and expression is controlled by the cytomegalovirus enhancer/promoter. Two doses of adenovirus (3 × 10^8 and 1 × 10^9 plaque-forming units [pfu] in 10 μL) were tested in separate experiments. Adenovirus harboring the luciferase gene (Ad.CMV-Luc) and sterile saline served as controls. Each agent was administered into 3 sites in the left adductors of mice anesthetized with 2.2,2-tribromoethanol (880 mmol/kg IP, Sigma). In prevention experiments, gene therapy was given 14 days after diabetes induction, whereas in rescue experiments, it was given 60 days after diabetes induction. To determine whether imposed metabolic control may exert additional protection, a subgroup was treated once per day with 3 IU of insulin (Humulin U, Lilly) beginning on the first assessment of glycosuria. Fourteen days later, Ad.CMV-hTK, Ad.CMV-Luc, or saline was injected intramuscularly. Adductors were harvested 2 weeks later for histological evaluation.

**Histological Assessment of Micrcirculation**

Anesthetized mice were perfusion fixed, and limb muscles were processed for histological analyses, as described previously. Vasculcular ECs were identified by immunostaining for factor VIII–related antigen with a rabbit polyclonal antibody (Dako). For identification of arterioles, sections were stained with a mouse monoclonal anti-a-smooth muscle actin (Sigma).

Histological analysis was performed in a blinded fashion. Capillaries and myofibers were counted with an ocular reticle (9604-H11003) distributed among vessels of various luminal sizes. Morphometric analysis of arterioles and myofiber profiles was performed at 400× magnification.

**Detection of Apoptosis**

DNA fragmentation was determined through a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Deraffinnized 4-μm-thick sections were stained with streptavidin-conjugated peroxidase (with DAB as a chromogen) or FITC-conjugated extravadin and then counterstained with methyl green or Hoechst 33342. Positive controls were pretreated with 1 μg/mL DNase I, whereas negative controls were incubated in the absence of the terminal deoxynucleotidyl transferase. Sections were examined in a blinded fashion at 200× magnification. TUNEL-positive cell density was calculated as number of apoptotic cells per square millimeter of section.

**Hemodynamic Measurements**

Systolic blood pressure and heart rate were measured in unanesthetized mice by tail-cuff plethysmography (Visitec Systems) according to a procedure validated previously. In addition, the acute blood pressure–lowering effect of intravenous acetylcholine (Ach, 0.1 to 100 ng) was evaluated in unrestrained mice instrumented 1 day in advance with jugular and carotid catheters.

The impact of gene therapy on local hemodynamics was evaluated by determining the rate of blood flow recovery after transient interruption of left femoral artery (LFA) flow. To this aim, mice made diabetic 14 days in advance received local Ad.CMV-hTK or Ad.CMV-Luc (both 3 × 10^7 pfu) and 14 days later underwent 1 minute of LFA occlusion. Limb blood flow of anesthetized mice was measured before LFA occlusion and then sequentially every 30 seconds for the first 5 minutes of reperfusion with a perfusion imager system (Lisca Inc.). Untreated nondiabetic mice undergoing the same procedure were used as reference.

**Muscular cAMP and cGMP Levels**

Mice were killed 28 days from the onset of overt glycosuria (14 days after gene transfer). Untreated nondiabetic mice served as controls. Adductors were frozen by immersion in liquid nitrogen. After homogenization, the supernatants were used for determination of cAMP and cGMP by enzyme immunoassay (Amersham).

**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 in which ANOVA indicated significant differences, the statistical value was determined according to the Bonferroni method. Differences within and between groups were determined with paired or unpaired Student’s t test, respectively. A probability value <0.05 was interpreted to denote statistical significance.

**Results**

**Time Course of Microangiopathy**

In hindlimb muscles of STZ-induced diabetic mice, capillary density was initially augmented (P<0.03) and then progressively decreased, reaching at 120 days a value 2-fold less (P<0.01) than that of nondiabetic mice (Figure 1a). By that time, the myocyte component underwent a 36% overall decrease (P<0.01), attributable equally to fiber rarefaction (Figure 1b) and shrinkage (data not shown). However, vascular pathology prevailed on muscular damage, as reflected by a 43% reduction in capillary/myofiber ratio (Figure 1c).

Arteriole rarefaction was documented at 60 days from diabetes onset (2.55±0.29 versus 4.90±0.60 arterioles/mm² in nondiabetic mice; P<0.001), the reduction being equally distributed among vessels of various luminal sizes. Morphometric analysis revealed a heterogeneous remodeling, with small arterioles (5 to 10 μm in diameter) showing a 41% increase in wall thickness and larger arterioles (25 to 55 μm in diameter) showing a 31% decrease in the same linear measure compared with nondiabetic mice (P<0.01 for both comparisons).

Microvascular rarefaction was associated with enhanced apoptosis. At 28 days, TUNEL-positive ECs averaged 4.0±0.6/mm², whereas apoptosis was virtually absent in nondiabetic mice.

**Prevention of Microangiopathy by hTK**

As shown in Figure 2a, 3×10^7 pfu of Ad.CMV-hTK completely prevented capillary rarefaction over 120 days, a result confirmed after normalization of capillary density by the number of myofibers (Figure 2c). Microvascular improvement was associated with preserved myofiber density (Figure
2b), whereas myofiber atrophy was not halted by gene therapy (data not shown).

The therapeutic action was also evident at the arteriole level. In fact, the arteriole density of hTK-treated muscles (7.7 ± 0.7 arterioles/mm²) exceeded by 196% that of saline-treated muscles (2.6 ± 0.3 arterioles/mm², P < 0.001) and by 57% that of muscles in nondiabetic mice (4.9 ± 0.6 arterioles/mm², P < 0.01), with the increase encompassing vessels of all sizes of luminal diameter (Figure 3). In Luc-treated muscles, arterioles were augmented (5.1 ± 0.4 arterioles/mm²), although less than with hTK (P < 0.01) and limited to those <15 μm in luminal diameter. Enlargement of arteriole mean luminal area by hTK (390 ± 43 versus 276 ± 33 μm² in saline and 227 ± 23 μm² in Luc, P < 0.05 for both comparisons), combined with increased vessel density, accounted for the larger section fraction occupied by arteriole lumens (0.34 ± 0.06%) compared with other diabetic groups (saline

Figure 1. Time course of changes in capillary density (a), myofiber density (b), and capillary to myofiber ratio (c) in adductor muscles from STZ-induced diabetic mice. Adductors from nondiabetic mice are shown for reference. Values are mean ± SEM, and number within each column represents sample size. *P < 0.05 and **P < 0.01 vs nondiabetic mice. cap indicates capillaries.

Figure 2. Effects of gene therapy on capillary density (a), myofiber density (b), and capillary to myofiber ratio (c) in adductor muscles from STZ-induced diabetic mice. At 14 days from first appearance of glycosuria, mice were randomly allocated into 3 different groups receiving single intramuscular injection of saline (hatched columns), Ad.CMV-Luc (dotted columns), or Ad.CMV-hTK (solid columns). Values are mean ± SEM, and number within each column represents sample size. *P < 0.05 and **P < 0.01 vs nondiabetic, $P < 0.05 and $$$P < 0.01 vs saline, #P < 0.05 and ###P < 0.01 vs Ad.CMV-Luc. cap indicates capillaries.
Apoptosis was drastically reduced by hTK (Figure 4). In fact, the number of TUNEL-positive ECs (0.7 ± 0.3 cells/mm², n = 6) was 84% and 87% less than with untreated (4.0 ± 0.6 cells/mm², n = 6, P < 0.01) or Luc-treated (5.0 ± 0.6 cells/mm², n = 6, P < 0.01) adductors, respectively.

We found that 10⁵ pfu of Ad.CMV-hTK was sufficient to retard microvascular rarefaction. For instance, arteriole density averaged 3.1 ± 0.4 arterioles/mm² in hTK-injected muscles, a value 40% higher (P < 0.05) than with saline-treated (2.2 ± 0.2 arterioles/mm²) or Luc-treated (2.1 ± 0.2 arterioles/mm²) muscles. Neither the high nor the low dose produced changes in the microvasculature of contralateral hindlimb muscles (data not shown).

**Effect of Insulin Alone or in Combination With hTK Gene Transfer**

Insulin supplementation led to reduced fasting blood glucose levels (saline 109 ± 28, hTK 101 ± 24, and Luc 110 ± 14 mg/dL versus 380 ± 20 mg/dL in untreated diabetic mice; P < 0.05 for all comparisons) and higher body weight (saline 35.2 ± 0.7, hTK 33.2 ± 1.0, and Luc 32.2 ± 0.7 g versus 30.6 ± 0.6 g in untreated diabetic mice; P < 0.05 for all comparisons). No treatment-related effect on systolic blood pressure or heart rate was observed among groups given saline, hTK, or Luc, with or without superimposed insulin (data not shown). Systemic vasodilator response to Ach was significantly depressed in untreated diabetic mice, and the defect was partially prevented by insulin (data not shown).

Histological analysis revealed that insulin per se was not effective in preventing diabetic microangiopathy (Figure 5). Furthermore, no additive effect on vascular pathology was observed when hTK and insulin were combined. Nevertheless, a synergistic protection was documented with regard to myofiber rarefaction (Figure 5b) and atrophy (data not shown).

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**hTK Gene Therapy Accelerates Hindlimb Postischemic Recovery**

Next, we tested whether microvascular potentiation by hTK would be functionally relevant to induction of transient hindlimb ischemia. In saline- or Luc-treated diabetic mice, blood flow regained baseline levels 3 minutes after the release of LFA occlusion, whereas 1 minute was sufficient for hTK-treated animals. At this time point, no significant difference in limb blood flow was observed between hTK-treated diabetic mice and nondiabetic controls. Thus, diabetes-induced vascular rarefaction is responsible for reper-
fusion delay. hTK gene therapy potentiates collateral circulation, thus preventing the hemodynamic deficit.

Cyclic Nucleotide Levels in Diabetic Muscles
At 28 days from diabetes onset (14 days from gene therapy), cGMP averaged 153 ± 37 pmol/mg protein in hTK-injected muscles (n = 6), a value higher than that of saline- or Luc-treated muscles (55 ± 5 and 56 ± 18 pmol/mg protein, respectively, n = 6 each group, P < 0.01 for both comparisons) although not high enough to match nondiabetic values (444 ± 70 pmol/mg protein, n = 6). Similarly, cAMP levels were higher in hTK-treated muscles (2592 ± 202 fmol/mg protein) than in untreated (391 ± 45 fmol/mg protein, P < 0.01) or Luc-treated (909 ± 120 fmol/mg protein, P < 0.05) muscles.

Rescue of Microangiopathy by hTK
To test the therapeutic potential for established microangiopathy, gene transfer was applied 60 days after diabetes onset, and capillary density was measured 14 days later. We documented that hTK promotes vascular regeneration in diabetic muscles (Figure 6).

Discussion
The balance between mechanisms that favor EC growth and vascular stabilization and those that promote EC death and vascular regression is deregulated in diabetes. In limb skeletal muscles of STZ-induced diabetic mice, vascular rarefaction is indeed the result of an abnormally activated program that commits ECs to premature death. During the first 28 days, capillary loss occurred at the rate of 1% per day without any reduction in myofiber density. In the long term, this mismatch significantly contributes to altering the path length for oxygen transport to myocytes, thus ultimately leading to ischemia and additional activation of cell-death mechanisms. Accordingly, from 28 to 120 days, a supplemental 35% decrease in capillarity occurred in association with myofiber rarefaction and atrophy. Detrimental hemodynamic effects and tissue hypoxia derive especially from the drastic rarefaction of arterioles, which normally provide the largest part of hindlimb blood flow. Arteriole remodeling in response to altered
shear stress may ultimately lead to vascular collapse and regression.\textsuperscript{21}

We documented that hTK is able to prevent diabetic microangiopathy by attenuating EC apoptosis and promoting capillarization. A similar therapeutic profile has been reported in a rat model of myocardial infarction.\textsuperscript{22} Putative mechanisms may include kinin-mediated stimulation of pro-survival and proliferative pathways. For instance, in cultured myocardial ECs, bradykinin reportedly promotes rapid phosphorylation of the vascular endothelial growth factor receptor KDR/Flik-1, resulting in endothelial NO synthase activation.\textsuperscript{23} Similar to vascular endothelial growth factor, bradykinin activates endothelial NO synthase via the PI3K-Akt/PKB and calcineurin pathways.\textsuperscript{24} NO is fundamental for hTK-induced angiogenesis\textsuperscript{7} and functions as the final effector of various antiapoptotic pathways, including Akt/PKB.\textsuperscript{25,26} Besides inhibiting EC apoptosis, Akt may contribute in EC proliferation instrumental to hTK-induced angiogenesis. hTK-induced capillarization was consistently prevented in part by cotransfection with the 308/547 dominant negative mutant of Akt (Emanuelli et al, unpublished data, 2002). Another piece of information comes from in vitro studies showing that activation of the kinin B\textsubscript{1} subtype receptor exerts pro-survival effects on ECs via stimulation of a prostacyclin-cAMP-mediated mechanism.\textsuperscript{10,11} Finally, hTK-generated kinins may retard capillary thrombosis and occlusion by stimulating tissue plasminogen activator release.\textsuperscript{27}

Another novel finding is that hTK supplementation promotes arteriole regeneration in diabetic hindlimb muscles and accelerates reperfusion after transient LFA occlusion. The newly generated arterioles may derive from pericyte encapsulation and muscularization of nascent endothelial channels. Furthermore, hTK might stimulate the growth and remodeling of preexisting collateral arteries, which is supported by concomitant arteriole luminal enlargement. In line with this theory, clinical studies documented the importance of hTK as a biomarker of collateral growth in ischemic patients.\textsuperscript{18}

A single injection of the hTK gene produces long-term protection that largely exceeds the duration of transgene expression. Shortly after gene therapy, muscular vasculature surpassed the figure normally found in nondiabetic muscles but was then subject to progressive regression. This phenomenon may represent a physiological response aimed at clearing unnecessary capillaries. Accordingly, at 106 days from gene delivery, the capillary/myofiber ratio exactly matched the value of nondiabetic animals. Given the mouse life span, this undeniably represents a significant therapeutic result. We cannot, however, rule out the possibility that uncontrolled hyperglycemia and continued oxidative stress may decrease capillarity over a longer period of time. Furthermore, the intrinsic limitations of an adenovirus would make necessary the use of alternative, longer-expressing vectors for the treatment of diabetic microangiopathy in humans. On the other hand, the demonstration that hTK can rescue established diabetic microangiopathy is of importance. Thus, at late stages of the disease, potentiation of endogenous KKS could provide a remedy for peripheral vascular complications and improve limb salvage. In addition, hTK, as a sole therapeutic agent, exerted an appreciable benefit for myofiber viability. It was necessary to combine gene therapy and insulin to prevent late-stage muscular atrophy, a condition that usually improves when correcting hyperglycemia.\textsuperscript{28}

It might be argued that vector proangiogenic activity contributes to the therapeutic effects of hTK. This argument was circumvented by delivery of the therapeutic gene at a low infecting dose of 10\textsuperscript{5} pfu. Ad.CMV-hTK retained curative properties against arteriole rarefaction, whereas the reporter gene was devoid of angiogenic effects.

Although systemic hTK gene delivery to nondiabetic animals has already been proven to benefit myocardial ischemia and stroke,\textsuperscript{22,29} local angiogenesis gene therapy was intentionally chosen for the present study to avoid endangering distant tissues, especially the retina. The safety of the approach is shown by the absence of obvious side effects or angiogenesis in contralateral muscles. On the other hand, the therapeutic potential of hTK in other organs affected by microangiopathy requires additional, specifically designed experiments.

Given that kinins are responsible for the therapeutic action of hTK, one might hypothesize about the application of ACE inhibitors to combat diabetic microangiopathy. However, the ability of ACE inhibitors to promote reparative angiogenesis by a kinin-mediated mechanism is not universally accepted.\textsuperscript{30,31} The argument was raised that kinins are not able to balance the negative impact of reduced proangiogenic angiotensin II formation by ACE inhibition.\textsuperscript{31} Thus, experimental evidence points to hTK as a more direct approach to exploit the curative properties of kinins in ischemia.

In conclusion, our findings documenting a therapeutic benefit of hTK for the treatment of diabetic microangiopathy represent solid progress toward the potential clinical application of this gene therapy approach.

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