Intramuscular Gene Transfer of Soluble Tumor Necrosis Factor-α Receptor 1 Activates Vascular Endothelial Growth Factor Receptor and Accelerates Angiogenesis in a Rat Model of Hindlimb Ischemia

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Background—In a pathological setting, tumor necrosis factor (TNF)-α inhibits the proliferative response of endothelial cells through inactivation of receptors for vascular endothelial growth factor (VEGF). Soluble TNF-α receptor 1 (sTNFR1) is an extracellular domain of TNFR1 and an antagonist to TNF-α. In the present study, we examined the effect of sTNFR1 expression plasmid on receptor for VEGF (KDR/flk-1) and angiogenesis in a rat model of hindlimb ischemia.

Methods and Results—The left femoral artery was exposed and excised to induce limb ischemia. A total of 400 μg of sTNFR1 or LacZ plasmid was injected into 3 different sites of the adductor muscle immediately after the induction of ischemia. TNF-α bioactivity in ischemic adductors increased in rats receiving LacZ plasmid compared with sham-operated rats. However, sTNFR1 plasmid significantly suppressed the increase in TNF-α bioactivity. KDR/flk-1 mRNA and tyrosine phosphorylation of KDR/flk-1 were significantly increased in the muscles injected with sTNFR1 plasmid compared with those injected with LacZ plasmid. VEGF increased both in muscles injected with sTNFR1 plasmid and in muscles injected with LacZ plasmid but did not differ significantly between them. At 21 days after the induction of ischemia, the sTNFR1 plasmid–transfected muscles showed significantly increased capillary density compared with LacZ plasmid–transfected muscles.

Conclusions—In a rat model of hindlimb ischemia, VEGF increased but activation of KDR/flk-1 was suppressed, possibly by TNF-α, which might impair angiogenesis. Suppression of TNF-α with sTNFR1 plasmid upregulated KDR/flk-1 and accelerated angiogenesis. Local transfection of the sTNFR1 gene can be a new strategy for therapeutic angiogenesis in peripheral ischemic diseases. (Circulation. 2004;109:797-802.)

Key Words: tumor necrosis factor ■ angiogenesis ■ ischemia ■ gene therapy

Lower-limb ischemia is a major health problem. Because of the absence of effective pharmacological treatment, amputation is undertaken at the end stage as a unique solution to unbearable symptoms. Delivery of angiogenic factors via recombinant protein or by gene transfer proved to be effective in animal models of ischemia and is now emerging as a new therapeutic strategy in peripheral vascular disease.1,2 These studies using animal models of peripheral ischemia have demonstrated that vascular endothelial growth factor (VEGF), which is known as a vascular permeability factor as well as a secreted endothelial mitogen, can stimulate the development of collateral arteries.1,2 The high efficiency of therapeutic angiogenesis with VEGF gene transfer has also been reported in human patients with critical limb ischemia.3-4 VEGF promotes the growth of vascular endothelial cells and the development of new blood vessels through interaction with its specific receptors, KDR/flk-1 and flt-1.5,6

In a pathological setting, conversely, TNF-α inhibits endothelial cell proliferative response and modulates angiogenesis through the inactivation of KDR/flk-1; these are mediated by a protein tyrosine phosphatase.7 A direct antiangiogenic effect of TNF-α on endothelial cells has also been demonstrated in association with the downregulated expression of KDR/flk-1.8 We thus hypothesized that blocking TNF-α might stimulate angiogenesis in vivo via KDR/flk-1 activation. Soluble TNF-α receptor 1 (sTNFR1) is an extracellular domain of TNF-α receptor 1 and an antagonist to TNF-α.9 Accordingly, in the present study, we examined whether sTNFR1 stimulates angiogenesis in vivo.

Methods

sTNFR1 Expression Vector

Total RNA (1 μg) isolated from the rat muscle was reverse transcribed into cDNA by use of an antisense strand primer (5'-
CAGAGTGGGGTTGAAGCC-3'). The cDNA produced was amplified by use of the primers designed to amplify the sequences encoding the extracellular domain of the rat type 1 TNF receptor.10 The sense primer was 5'-ATGGTCTCCCATCTGTCCT-3', and the antisense primer was 5'-ATTTGCAACTGGAGGAGGCA-3' or 5'-AGTCTCATGTTACATTTTGACCTAGTAG-3'. The polymerase chain reaction (PCR) fragment was gel-purified by use of a QIAEX II gel extraction kit (Qiagen) and cloned into the TA expression vector by use of a pCDNA3.1/V5-His TOPO TA expression kit (Invitrogen). The latter antisense primer was designed to contain 2 in-frame stop codons, but the former antisense primer was not. Thus, the TA expression vector containing the PCR fragment produced by the latter antisense primer expresses only sTNFR1 (sTNFR1 plasmid), but the TA expression vector containing the PCR fragment produced by the former antisense primer expresses not only sTNFR1 but also V5 epitope and His6 (sTNFR1-flag plasmid).

Animal Treatment
Male Wistar rats (250 to 300 g; bred in our lab) were anesthetized, and the left femoral artery was exposed, dissected free, and excised. Immediately after ischemia was induced, a total of 400 ng sTNFR1 plasmid, sTNFR1-flag plasmid, LacZ plasmid, or saline was injected into 3 different sites of the adductor muscle of each anesthetized rat.

Reverse Transcription–PCR Analysis
Soluble TNFR1 mRNA was measured by reverse transcription (RT)–PCR. The sense primer was 5'-TAATACGACTCACTATAGGG-3', the antisense primer was 5'-ATTGCCAACTGGAGGAGGCA-3', and the PCR product size was 693 bp. The sTNFR1 primer set amplified an sTNFR1 product from cDNA derived from sTNFR1 plasmid but not from endogenous sTNFR1 cDNA.

Transfection Assay
A successful transfection resulted in V5 epitope expression in a sTNFR1-flag plasmid. Briefly, the tissues injected with plasmids were homogenated with 5 volumes of homogenization buffer, then supernatants were fractionated on 12.5% SDS-PAGE and blotted to a Hybond-P membrane (Amersham). The membranes were blocked and incubated with the antibody (horseradish peroxidase [HRP]-conjugated antiphosphotyrosine) for 2 hours at room temperature. Detection was performed with the ECL detection system (Amersham). After incubation with the primary antibody, the blotted membranes were treated with the WEHI cell line as described previously.12 Recombinant TNF-α was also incubated with the WEHI cell line and used as a standard. Then, the TNF-α bioactivity was expressed as pg/mg protein. TNF-α mRNA and β-actin mRNA levels were measured by RT-PCR using a modification of the method described by Iversen et al.13 except that in the present study, fluorescein 11-dUTP (Boehringer) was used to label the PCR product as described previously.14 After we examined the relation between the amount of RT-PCR products and the PCR cycles in each mRNA, the PCR cycles for determining the amount of each mRNA were determined (24 cycles for TNF-α and 18 cycles for β-actin). The profile for each mRNA involved denaturation at 95°C for 50 seconds, annealing at 55°C for 50 seconds, and extension at 72°C for 1 minute.

Histology and Morphometric Analysis
Twenty-one days after the gene transfer, anesthetized rats were perfused with PBS via the abdominal aorta. The tissue samples were fixed in formalin and embedded in paraffin. Sections 3 μm thick were cut and placed onto silane-coated slides with muscle fibers oriented transversely. Vascular endothelial cells were identified by immunohistochemical staining for factor VIII–related antigen (von Willebrand Biologicals). In control sections, the primary antibody was replaced with nonimmune sheep immunoglobulin G. Ten different fields from 1 muscle section located relative to the injection sites were randomly selected, and the number of the capillaries was counted. The same procedure was followed in 2 additional muscle sections from different paraffin blocks of the same limb. Capillaries present in 5 mm² were counted in each limb. Arterioles were not included in the study. Most of them were located at the periphery of the muscle bundle, and their number was not adequate. The number of capillary profiles was used to compute capillary numerical density per square millimeter of muscle according to the method described by Emanuelli et al.15 To determine the infiltrates, T lymphocytes were identified with anti-CD-3 antibody (Sigma Chemical Co). T cells were counted manually in 80 randomly chosen high-power fields (20 in each rat) of light microscopic sections harvested from 4 rats in each group at each time.

Thermography
Twenty-one days after the gene transfer, each rat was reanesthetized, and the lower-body coats were shaved. The skin temperature of the rat hindlimb was measured with infrared thermography (TH3107ME, NEC San-ei Instruments).

Statistical Analysis
All values are presented as the mean ± SEM. Statistical significance was evaluated by unpaired Student’s t test for comparison between 2 means. Comparisons among 3 or more groups were made by 1-way ANOVA followed by Dunnett’s modified test. Differences were considered statistically significant at a probability value of P<0.05.

Results
The vector-derived sTNFR1 transcripts were present as early as 5 hours until 21 days after intramuscular injection of the sTNFR1 plasmid (Figure 1A). These results confirm the successful transfection of the sTNFR1 gene into the ischemic muscle, given that the sTNFR1 primer set amplified an sTNFR1 product from cDNA derived from the vector but not from endogenous sTNFR1 cDNA. In addition, plasmid-
derived protein was expressed in muscle injected with the sTNFR1-flag plasmid. V5 epitope with sTNFR1 expression (~55 kDa) was detected from 1 to 21 days after the plasmid injection (Figure 1B). The vector-derived sTNFR1 transcripts and sTNFR1 expressions were absent in the LacZ plasmid-treated rats and in the saline-treated rats (data not shown). In addition, the plasmid-derived protein was not detected in circulating plasma throughout the experiment. The TNF-α bioactivity in the muscle injected with sTNFR1 plasmid was significantly lower throughout the experiment than in the muscle injected with LacZ plasmid (Figure 1C). In contrast, the mRNA for TNF-α was not significantly different throughout the experiment between the rats injected with the sTNFR1 plasmid and LacZ plasmid (Figure 2). The mRNA for KDR/flk-1 increased both in muscles injected with the sTNFR1 plasmid and in those injected with the LacZ plasmid compared with the sham-operated rats (Figure 3). At 7 and 14 days after the injection, the sTNFR1 plasmid treatment significantly upregulated the mRNA for KDR/flk-1 (Figure 3B). Because KDR/flk-1 activation by the tyrosine phosphorylations mediates endothelial cell proliferation, we examined whether the sTNFR1 plasmid might upregulate KDR/flk-1 activation (Figure 4A). Throughout the experiment, the sTNFR1 plasmid significantly stimulated tyrosine phosphorylation of KDR/flk-1 in the muscle compared with the LacZ plasmid (Figure 4B). Western blot analysis was performed to detect the VEGF protein in muscle injected with either the sTNFR1 plasmid or the LacZ plasmid (Figure 5). Throughout the experiment, the intensity of the bands did not differ significantly between the muscle injected with sTNFR1 plasmid and that injected with the LacZ plasmid (data not shown). In the sham-operated rats, VEGF was not detected throughout the experiment (data not shown). Immunohistochemical studies demonstrated that sTNFR1 plasmid accelerated angiogenesis compared with LacZ plasmid (Figure 6A). As indicated in Figure 6B, muscle transfected with sTNFR1 plasmid showed significantly increased capillary density compared with the LacZ plasmid. The ischemic limb developed infiltrates until 2 weeks after induction of ischemia, and no infiltrates were observed at 3 weeks. T cells were significantly reduced in the limb injected with sTNFR1 plasmid until 7 days (1 day, 0.49±0.06 versus 0.79±0.07; 7 days, 0.64±0.07 versus 1.03±0.11; 14 days, 0.19±0.04 versus 0.20±0.05, sTNFR1 versus LacZ plasmid, respectively; P<0.05 at 1 and 7 days). To assess the improved perfusion of ischemic limbs, we measured the skin temperature of the rat hindlimbs with infrared thermography. The skin temperature of the LacZ-transfected ischemic limb was ~3°C lower than that of the contralateral limb. Conversely, sTNFR1 gene transfer nearly restored the skin temperature in the ischemic limb (Figure 7).
TNF-α inhibits the proliferative response of endothelial cells and modulates angiogenesis, both of which are a result of the inactivation of KDR/flk-1 and the downregulation of expression of KDR/flk-1. Moreover, TNF-α inhibits angiogenesis by reducing integrin (β3, an adhesion receptor that plays a key role in tumor angiogenesis). We thus hypothesized that blocking TNF-α might stimulate angiogenesis in vivo. In the present study, muscle injected with the sTNFR1 plasmid revealed the presence of vector-derived sTNFR1 transcripts as early as 5 hours after injection until 21 days. A successful transfection also resulted in V5 epitope expression in a sTNFR1-flag plasmid. Except that the sTNFR1-flag plasmid expresses not only sTNFR1 but also V5 epitope and His6, both the sTNFR1-flag plasmid and the sTNFR1 plasmid are made with the same vectors. The success of the transfection may be partly a result of the plasmid used in the present study. In addition, in skeletal muscle, the transfection efficiency of intramuscular gene transfer is augmented from 5- to 7-fold when the injected muscle is ischemic. The direct injection of nonviral plasmid DNA into skeletal muscle resulted in expression of the transgene in skeletal myocytes. In the present study, VEGF expression in the muscle increased after either plasmid was injected, with no differences appearing between them until 21 days, whereas the increased TNF-α bioactivity was significantly suppressed in the muscle injected with the sTNFR1 plasmid. The TNF mRNA increased in both muscles injected with sTNFR1 plasmid and with LacZ plasmid but did not differ significantly between them. Thus, the suppression of TNF-α bioactivity with the sTNFR1 plasmid was thought to be primarily a result of the neutralization of TNF-α. The increased expression of the TNF mRNA was thought to be derived from myocytes and in part from infiltrates of ischemic muscles, because the TNF-α bioactivity and the infiltrates were not reduced completely to the levels of sham-operated ones even in the muscle treated with sTNFR1 plasmid. In hindlimb ischemia, it is thought that TNF-α increases primarily in myocytes of the ischemic muscle and that it affects KDR/flk-1 in endothelial cells. In the present study, it is thus hypothesized that the sTNFR1 plasmid reduced the increased TNF-α bioactivity in myocytes of the ischemic muscle and then upregulated KDR/flk-1 mRNA and tyrosine phosphorylation of KDR/flk-1 in endothelial cells. In KDR/flk-1–null mutant mice, endothelial and hematopoietic cell development is impaired, whereas in flt-1–null mutant mice, endothelial and hematopoietic cell development is impaired.

Figure 3. A, Northern blot analysis for KDR/flk-1 mRNA (top). Amount of total RNA loaded in each lane (10 µg) is presented after ethidium bromide staining (bottom). Lanes 1 and 5, 1 day; lanes 2 and 6, 7 days; lanes 3 and 7, 14 days; lanes 4 and 8, 21 days; lane 9, sham-operated rats (1 day). B, Values are ratios of band density to mean band density of sham-operated rats at each time and are expressed as mean±SEM (n=6 in LacZ- and sTNFR1-treated rats at each time and n=3 in sham-operated rats at each time). a, P<0.01 and b, P<0.001 vs rats receiving LacZ plasmid.

Figure 4. A, KDR/flk-1 was immunoprecipitated by use of anti–KDR/flk-1 antibody from an equal amount of tissue homogenates. Western blot was probed with antibody to phosphotyrosine. Lanes 1 and 5, 1 day; lanes 2 and 6, 7 days; lanes 3 and 7; 14 days; lanes 4 and 8, 21 days; lane 9, sham-operated rats (1 day). B, Values were ratios of band density to mean band density of sham-operated rats at each time and are expressed as mean±SEM (n=6 in LacZ- and sTNFR1-treated rats at each time and n=3 in sham-operated rats at each time). a, P<0.05; b, P<0.001; and c, P<0.0001 vs rats receiving LacZ plasmid.

Figure 5. Western blot analysis of VEGF in muscles from an equal amount of tissue homogenates. Lanes 1 and 5, 1 day; lanes 2 and 6, 7 days; lanes 3 and 7, 14 days; lanes 4 and 8, 21 days; lane 9, sham-operated rats (1 day).
mice, endothelial cells overgrow and blood vessels are disorganized. In addition, flt-1 tyrosine kinase–deficient homozygous mice develop normal blood vessels and survive, indicating that flt-1 lacking the tyrosine kinase domain is sufficient for normal angiogenesis and development. The kinase activity of KDR/flk-1 by tyrosine phosphorylation is essential to activation of cytoplasmic signaling proteins that contain Src homology 2 domains, and this process is associated with endothelial cell proliferation. In the present study, sTNFR1 is thought to activate KDR/flk-1 and stimulate angiogenesis by blocking the effect of TNF-α. The clinical relevance of therapeutic angiogenesis induced by sTNFR1 compared with that induced by VEGF lies in the fact that sTNFR1 not only augments angiogenesis, as shown in the present study, but also antagonizes TNF-α. It is thought that VEGF therapy activates KDR/flk-1 by increasing VEGF without reducing the TNF-α bioactivity, whereas the anti-TNF therapy presented in the present study activates KDR/flk-1 by reducing the TNF-α bioactivity (Figure 8). In fact, in hindlimb ischemia, as seen in the muscle injected with the LacZ plasmid, VEGF increased, whereas KDR/flk-1 was not fully activated compared with that injected with sTNFR1 plasmid, possibly because of an increase in TNF-α bioactivity (Figure 8). To activate KDR/flk-1 in a pathological setting such as hindlimb ischemia, we think reducing TNF-α is a better therapeutic strategy. TNF-α is thought to play an important role in the progression of atherosclerosis and vascular injury. In the present study, sTNFR1 not only stimulated angiogenesis but also reduced TNF-α bioactivity, which is thought to be antiatherogenic. In contrast, the recent studies suggest that VEGF may be atherogenic even though it stimulates angiogenesis. Therefore, in vascular diseases, in which TNF-α is usually highly expressed, local transfection of the sTNFR1 gene can be a better tool for therapeutic angiogenesis than that of the VEGF gene. Etanercept, which is a fusion protein of soluble TNF-α receptor 2, has been used in patients with advanced heart failure. It might be possible that repeated administration of etanercept or a fusion protein of sTNFR1 can induce angiogenesis in hindlimb ischemia, although there are no studies that have examined the effect of the protein on angiogenesis. In the present study, however, the sTNFR1 was not detected in the circulating plasma. Intramuscular injection of the sTNFR1 gene, in lieu of the protein, provided a local depot effect of protein secretion over a period of 3 weeks. This may provide an advantage relative to systemic treatment with purified TNF antagonist proteins such as etanercept. In conclusion, the present study provides new insights into the role of sTNFR1 in vascular diseases and may have significant implications for gene therapy in the treatment of peripheral ischemic diseases.

Figure 6. A, Immunohistochemical identification of vascular endothelial cells using antibodies against von Willebrand factor. Representative pictures show higher capillary density of adductor muscles injected with sTNFR1 plasmid (left) vs LacZ plasmid (right). B, Values show capillary numerical density per square millimeter of muscle and are expressed as mean±SEM (n=6 in each group). a, P<0.05 vs rats receiving LacZ plasmid.

Figure 7. Thermography of rat hindlimbs was performed at 3 weeks after gene transfer. Representative color images of infrared thermograph. L, ischemic limbs; R, contralateral limbs.

Figure 8. Schematic of hypothesized sTNFR1 therapy in angiogenesis.
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References
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