Peripheral arterial disease is a common disease found in 10% to 25% of patients over the age of 55 years, and its occurrence and severity are strongly correlated with other cardiovascular risk factors that lead to coronary artery disease and stroke, such as hyperlipidemia, smoking, and endothelial dysfunction.1-3 An animal model has been created by ischemic injury of mouse hindlimb, and studies using this mouse model reveal that a reduction in gastrocnemius blood pressure ratios and blood flow correlate with a marked decrease in the number of capillaries surrounding each muscle fiber (capillary/fiber ratio), indicative of the inability to mount an adaptive angiogenic response.4,5 The availability of genetically deficient or transgenic mice permits the testing of genes required for postnatal hindlimb angiogenesis and remodeling. Thus, the ischemia hindlimb model is a very useful tool for studying the function of genes critical for inflammatory arteriogenesis and angiogenesis.4,5 Using this model, we have recently demonstrated that several signaling molecules (tumor necrosis factor receptor 1 [TNFR1], TNFR2, and AIP1) involved in tumor necrosis factor-α (TNF)-TNF pathways are also involved in ischemia-mediated adaptive angiogenesis.6-8

The prototype inflammatory cytokine, TNF, primarily uses TNFR1 to mediate inflammatory and pathological responses, and the role of TNFR2 signaling is not well understood. TNFR1 is ubiquitously expressed, whereas TNFR2 expression is tightly regulated and found predominantly on endothelial cells (ECs), cardiac myocytes, and hematopoietic cells.9,10 Similar to TNFR1, TNFR2 contains an extracellular domain with characteristic cysteine-rich motifs. However, TNFR2 has a unique cytoplasmic domain with different primary amino acid sequences compared with TNFR1. It is believed that the 2 receptors initiate distinct signal transduction pathways by interacting with different signaling proteins.9,10 Although TNFR1-mediated signaling has been extensively studied, much less is known about TNFR2 signaling.10 We have identified bone marrow tyrosine kinase in chromosome X (Bmx) (also named Etk-endothelial/epithelial tyrosine kinase) as a TNFR2-responsive tyrosine kinase in EC.11 Furthermore, we have shown that TNFR2-Bmx could associate with and transactivate vascular endothelial growth factor receptor 2 (VEGFR2), a potent angiogenic

Endothelial-Specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis

Yan Luo, Zhe Xu, Ting Wan, Yun He, Dennis Jones, Haifeng Zhang, Wang Min

Objective—We have previously shown that the tumor necrosis factor receptor 2 (TNFR2) protein is highly upregulated in vascular endothelium in response to ischemia, and a global deletion of TNFR2 in mice blunts ischemia-induced arteriogenesis and angiogenesis. However, the role of endothelial TNFR2 is not defined. In this study, we used endothelial cell (EC)–specific transgenesis of TNFR2 (TNFR2-TG) in mice to determine the in vivo function of TNFR2 in arteriogenesis and angiogenesis.

Methods and Results—In a femoral artery ligation model, TNFR2-TG mice had enhanced limb perfusion recovery and ischemic reserve capacity. TNFR2-TG mice also exhibited significantly enhanced arteriogenesis in the upper limb, whereas capillary formation and maturation in the lower limb were associated with reduction in cellular apoptosis and increased proliferation. Consistently, ischemia-induced TNFR2-dependent bone marrow tyrosine kinase in chromosome X–vascular endothelial growth factor receptor 2 proangiogenic signaling was augmented in TNFR2-TG mice. To further determine whether EC-expressed TNFR2 is sufficient to mediate ischemia-induced angiogenesis, we crossed TNFR2-TG with TNFR2-deficient mice to generate TNFR2-knockout (KO)/TG mice, in which only vascular ECs express TNFR2. The EC-expressed TNFR2 partially rescued the defects of TNFR2-KO in ischemia-induced angiogenic signaling and flow recovery.

Conclusion—These in vivo data support a critical role for endothelial TNFR2 in ischemia-mediated adaptive angiogenesis. Therefore, specific expression and activation of TNFR2 in ECs may provide a novel strategy for the treatment of vascular diseases such as coronary artery disease and peripheral arterial disease. (Arterioscler Thromb Vasc Biol. 2010; 30:1307-1314.)

Key Words: angiogenesis ■ endothelium ■ hypoxia ■ ischemia ■ TNFR2
receptor tyrosine kinase in ECs. This association was critical for EC proliferation, migration, and tube formation. Consistent with aforementioned in vitro observations, we and others have shown that TNFR2-knockout (KO) mice exhibit reduced ischemia-initiated angiogenesis and arteriogenesis compared with wild-type (WT) mice in a femoral artery ligation model. Similar observations were obtained for Bmx-KO mice. Interestingly, the TNFR2 protein and its downstream effector, Bmx, are both highly induced in vascular ECs but not in other cell types, such as myocytes, in ischemic tissue. These data indicate that vascular TNFR2 may play an important role in ischemia-induced adaptive repair.

However, the role of endothelial TNFR2 in arteriogenesis and angiogenesis has not been directly addressed. To this end, we have created EC-specific transgenic mice expressing the human TNFR2 with Myc-tag at the C terminus driven by an EC-specific vascular endothelial (VE)-cadherin promoter (pVE-cad) (Figure 1a). Twelve founders were obtained as determined by polymerase chain reaction genotyping (not shown) and Western blot of the tail tissues with anti-TNFR2 antibody (Figure 1b). We chose 2 lines (no. 1 and no. 2 male) to backcross with C57BL/6 mice for more than 6 generations. All experiments were performed with hemizygous TNFR2-TG mice and their non-TG littermates (WT) as controls. The TNFR2 transgene was highly expressed in the aorta and bone marrow as determined by Western blot with anti-Myc (not shown) or anti-TNFR2 antibody. A nontransgenic tail was used as a control (Ctrl). Tissue distribution of the TNFR2 transgene. Expression of the TNFR2 protein in different tissues (aorta, bone marrow, and heart) was determined by Western blot with anti-Myc or anti-TNFR2 antibody. The relative levels of TNFR2 in TG lines are quantified, with WT as 1.0. TNFR1 was also detected by Western blot with anti-TNFR1. β-Tubulin was used as a protein loading control, e. TNFR2 transgene is expressed in the vascular endothelium. The TNFR2 transgene in WT and TNFR2 TG hindlimb sections was detected by immunohistochemistry with anti-Myc antibody. Large vessel and capillary are indicated by arrowhead and arrow, respectively. Scale bar: 100 µm. IB indicates immunoblotting.

Methods

Detailed materials and methods are provided in the supplement, available online at http://atvb.ahajournals.org. All the methods have been previously published, including generation of the EC-specific transgenic mice, mouse hindlimb ischemic model, postcontraction hyperemia, histology and immunohistochemistry, gene expression in ischemic muscle and image analysis, immunoblotting for Bmx-VEGFR2 signaling, bone marrow transplant (BMT), and aortic-ring assay.

Results

EC-Specific Expression of TNFR2

Previously, we have shown that TNFR2 protein is highly upregulated in vascular endothelium in response to ischemia. Therefore, we reasoned that a transgene of TNFR2 in ECs would increase ischemia-mediated vascular repair. To test this hypothesis, we generated EC-specific transgenic mice by expressing the human TNFR2 with Myc-tag at the C terminus driven by an EC-specific vascular endothelial (VE)-cadherin promoter (pVE-cad) (Figure 1a). Twelve founders were obtained as determined by polymerase chain reaction genotyping (not shown) and Western blot of the tail tissues with anti-TNFR2 antibody (Figure 1b). We chose 2 lines (no. 1 and no. 2 male) to backcross with C57BL/6 mice for more than 6 generations. All experiments were performed with hemizygous TNFR2-TG mice and their non-TG littermates (WT) as controls. The TNFR2 transgene was highly expressed in the aorta and bone marrow as determined by Western blot with anti-Myc and anti-TNFR2 (Figure 1c). A relatively low level of the TNFR2 transgene was found in the heart despite the fact that cardiac myocytes express a high basal level of endogenous TNFR2 (Figure 1c). This is consistent with the report that the VE-cadherin promoter is less active in adult cardiac vessels than in developing or newly formed vasculature. Total TNFR2 protein (both endogenous and the transgene) in muscle tissue was absent from TNFR2-KO mice, but it was 3-fold higher in TNFR2-TG mice than in WT littermates, as determined by Western blot with anti-TNFR2.
Western blotting with anti-TNFR2 antibody (Figure 1d). Furthermore, the TNFR2 transgene was detected in vascular endothelium of both large and small vessels, as determined by immunohistochemistry with anti-Myc antibody (Figure 1e). The TNFR2 transgene in ECs had no effects on the basal capillary densities in a variety of tissues examined (Supplemental Figure I, hindlimb and heart). There were no apparent morphological abnormalities in TNFR2-TG mice. Male and female mice appeared equally fertile, and there were no significant differences in growth rate or body weight compared with WT littermates (25.2±4 g for TNFR2-TG versus 24.8±4.0 g for nontransgenic littermates, n=20).

**TNFR2-TG Augments Perfusion Recovery With Enhanced Arteriogenesis and Angiogenesis in Ischemic Hindlimbs**

To determine whether the EC-specific transgene of TNFR2 could augment ischemia-mediated angiogenesis, WT and TNFR2-TG mice were subjected to femoral artery ligation and various analyses at different time points as described previously.6,7 Blood flow in ischemic and nonischemic limbs was measured on gastrocnemius muscle at the indicated times postsurgery. The ratios of perfusion unit from nonischemic (left) to ischemic limbs (right) are shown. Data are mean±SEM, n=10 for each group. *P<0.05. b and c, TNFR2-TG mice show enhanced postcontraction hyperemia. Adductor muscle groups of mice from presurgery (b) and 2 weeks postsurgery (c) were electrostimulated, and gastrocnemius blood flow was recorded. Both baseline and stimulated lower leg perfusion in legs were measured as an index of the maximal vasodilatory capacity. Pre-sti indicates prestimulation. Data are mean±SEM, n=5 for each group. *P<0.05 comparing TNFR2-TG to WT. d through f, 4 weeks after femoral ligation, gastrocnemius muscles were harvested. Capillary density was immunostained with CD31 (an EC marker) and anti-smooth muscle actin (α-SMA). Representative images of CD31 (d) and SMA (e) staining are shown. Arterioles and capillaries are indicated by arrowheads and arrows, respectively. Scale bar: 100 μm. f, Quantification of capillaries (number/mm² muscle area), ratios of CD31/myocyte, and SMA-positive staining (number/mm² muscle area). Data from 3 sections of each mouse muscle tissue are shown in graphics. n=4 for each strain (total, 12 sections). *P<0.05.
WT and TNFR2-TG mice before ligation. Next, we measured the same physiological response after limb ischemia in WT and TNFR2-TG mice at 2 weeks postsurgery. Electric stimulation of the adductor muscle of WT mice 2 weeks surgery showed only 65% of the peak response measured in the gastrocnemius muscle group compared with preischemia. However, TNFR2-TG mice had a nearly complete recovery in peak blood flow (Figure 2b, preischemia; Figure 2c, postischemia, 95±5%). These data show that TNFR2-TG mice have augmented perfusion recovery in ischemic hindlimbs.

As demonstrated previously, enhanced clinical recovery and limb perfusion could be due to increased arteriogenesis from existing vessels of the upper limb or increased neovascularization/vessel maturation in the lower limb. Consistently, ischemia-induced arteriogenesis in TNFR2-TG mice was significantly increased, as measured by Microfil casting (Supplemental Figure II). Similarly, angiogenesis and vessel maturation in TNFR2-TG mice were increased in gastrocnemius muscles, as measured by immunostaining with anti-CD31 and anti–smooth muscle α-actin (anti-SMA). Four weeks postischemia, CD31-positive capillaries surrounding the skeletal muscle fibers in WT mice were significantly increased (Figure 2d, with quantification of capillary number/mm² and ratio of CD31/myocyte in Figure 2f). Consistent with increased functionally perfused vessels in TNFR2-TG mice, ischemia-induced vessel maturation (SMA-positive vessels, arrowheads) was also increased in TNFR2-TG (Figure 2e, with quantification of SMA-positive cells in Figure 2f).

Ischemia-Induced Cellular Survival and Proliferation and TNFR2-Bmx-VEGFR2 Angiogenic Signaling Are Enhanced in TNFR2-TG Mice

TNFR2 mediates cellular survival and proliferation responses in vitro and in vivo. To determine the effects of TNFR2-TG on ischemia-induced cellular proliferative/apoptotic responses, we measured tissue apoptosis by TUNEL assay and cellular proliferation by proliferating cell nuclear antigen (PCNA) staining. Kinetics studies suggested that apoptosis peaked at day 3 postsurgery, and ischemia-induced apoptosis is an early event in the adaptive response. Results showed that ischemia-induced tissue apoptosis was dramatically decreased in TNFR2-TG mice (Figure 3a, with quantification in Figure 3c). Cellular proliferation started at day 7 and continued until week 4 in the ischemia hindlimb models. The TNFR2 transgene increased basal PCNA-positive staining in capillaries (Figure 3b, arrows), consistent with TNFR2-dependent induction of cellular proliferation in ECs. Both capillaries and myocytes showed PCNA-positive staining on ischemia (Figure 3b, arrow and arrowhead, respectively), consistent with the increased total numbers of capillaries and muscle fibers at 4 weeks postsurgery (Figure 2f). Myocytes in ischemic tissue showed central nuclei location (Figure 3b, arrowhead), likely an indicative of regenerative response. No significant differences between WT and TNFR2-TG mice in percentage of PCNA-positive myocytes were found. However, the number of PCNA-positive ECs was significantly increased in TNFR2-TG mice compared with WT mice (Figure 3b, with quantification in Figure 3c).

We have previously shown that ischemia induces activation of TNFR2-dependent gene expression and signaling complex (TNFR2-Bmx-VEGFR2), which are defective in TNFR2-KO mice. To determine whether the TNFR2-Bmx-VEGFR2 pathway is upregulated in TNFR2-TG mice, we examined gene expression and activation of TNFR2-dependent signaling molecules. Gene expression of angiogenic factors and their cognate receptors was determined by quantitative RT-PCR. The gene expression in both nons ischemia and ischemic muscles was analyzed, and relative abundance (fold of induction) of each gene is shown by taking nons ischemic WT as 1.0. Ischemia rapidly induces the expression of inflammatory molecules (TNF and vascular cell adhesion molecule-1 [VCAM-1]) and angiogenic molecules (Ang-2 and vascular endothelial growth factor [VEGF]; VEGFR2, TNFR2, and Bmx) on day 3 posts ischemia. These molecules are known to play important roles in inflammatory angiogenesis. Gene expression of VEGFR2 and Bmx was significantly augmented in TNFR2-TG mice (Figure 3d). Interestingly, inflammatory molecules (TNF and VCAM-1) were reduced, whereas TNFR1 and TRAF2 were not significantly altered in TNFR2-TG mice (Figure 3d). Consistently, a reduced infiltration of leukocytes to ischemic hindlimb was observed, as determined by immunostaining with the anti-CD45 antibody (Supplemental Figure III). Activation of TNFR2-dependent signaling pathways was also determined by Western blot with phospho-specific antibodies. Ischemia induced activation of Bmx-VEGFR2 to a greater extent in TNFR2-TG mice compared with WT mice (Figure 3e). The TNFR2-TG mice also showed a greater induction of the total Bmx protein. Notably, phosphorylation of c-Jun N-terminal kinase (JNK), a TNFR1-dependent apoptotic signaling kinase, and the JNK-responsive gene VEGF were slightly decreased in TNFR2-TG mice (Figure 3e).

The TNFR2 Transgene in ECs Is Sufficient to Mediate Ischemia-Induced Responses

To further determine whether the TNFR2 transgene in ECs is sufficient to mediate ischemia-induced angiogenesis, we crossed TNFR2-TG with TNFR2-deficient mice to generate TNFR2-KO/TG mice (KO/TG). In these mice, only the TNFR2 transgene is expressed in ECs, as determined by genotyping and immunostaining with anti-TNFR2 (Supplemental Figure IV). Similar to TNFR2-TG, TNFR2-KO/TG had no apparent morphological abnormalities compared with WT and TNFR2-KO mice. Male and female mice appeared equally fertile, and there were no significant differences in growth rate or body weight compared with WT littermates (24.5±3 g for TNFR2-KO versus 24.2±3.0 g for TNFR2-KO/TG, n=15). However, we did observe that the TNFR2 transgene increased basal endothelium-dependent vascular reactivity as determined by aortic ring assay in response to the vasoconstrictor phenylephrine (PE) and the vasodilator acetylcholine. Aortas from TNFR2-KO mice displayed enhanced constriction in response to PE compared with WT. However, reexpression of TNFR2-TG to KO mice completely restored the normal PE response (Supplemental Figure V). To determine whether the TNFR2 transgene restores the basal release of endothelial nitric oxide synthase–derived
nitric oxide (NO), aortic rings from WT, TNFR2-KO, and TNFR2-KO/TG mice were preconstricted with a submaximal dose of PE, and the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (100 μmol/L) was added at the peak of the constriction to remove endogenous NO tone. N\textsuperscript{G}-Nitro-L-arginine methyl ester caused a further constriction in all vessels, resulting in an increase in isometric tension reflecting the removal of basal NO. The ratio of EC\textsubscript{50} by PE in the presence versus absence of N\textsuperscript{G}-nitro-L-arginine methyl ester indicates a basal NO activity.\textsuperscript{14} The ratio for TNFR2-KO vessels was half that of WT vessels, suggesting a reduction in a basal NO release. However, the TNFR2 transgene restored the basal NO activity (Figure Vb). The TNFR2 transgene in TNFR2-KO mice also restored endothelium-dependent vasodilation in response to acetylcholine (Figure Vc). These effects of TNFR2-transgene on vasomotion occurred selectively in the endothelium because the vasoconstrictive responses to KCl and relaxation in response to the NO donor drug sodium nitroprusside (SNP) were similar to those of TNFR2-KO (Supplemental Figure V). The exact mechanism by which TNFR2 regulates NO is unclear. The TNFR2-dependent NO activity and endothelium function could be attributed to the increased VEGFR2 activity in TNFR2-TG mice (see below), an upstream activator of endothelial nitric oxide synthase–NO.

We next examined whether the TNFR2 transgene in ECs could restore the defects in ischemic responses observed in
TNFR2-KO. Consistent with previous findings,7 TNFR2 deletion caused severe impairment in ischemia-induced gene expression of VEGFR2 and Bmx, as well as TNFR2-dependent Bmx-VEGFR2 signaling. Indeed, the basal level of TNFR2 in TNFR2-TG driven by the VE-cadherin promoter is similar to that induced by ischemia in WT muscle, and the TNFR2 transgene could restore ischemia-induced gene expression of VEGFR2 and Bmx, as determined by RT-PCR (Figure 4a), and activation of Bmx-VEGFR2 signaling in hindlimb, as determined by Western blot with phospho-specific antibodies (Figure 4b). The increased Bmx-VEGFR2 signaling in TNFR2-KO/TG correlated with reduced infiltration (by CD45 staining) and increased proliferation (by PCNA staining) compared with TNFR2-KO mice (Figure 4c through 4e).

The TNFR2 Transgene in Both Intrinsic Vasculature and Bone Marrow Contributes to Ischemia-Induced Angiogenesis and Flow Recovery Finally, we examined whether the TNFR2 transgene in ECs could restore angiogenesis and functional flow recovery. WT, TNFR2-KO, and KO/TG mice were subjected to femoral artery ligation followed by morphological and functional blood flow analyses as described for WT and TNFR2-TG mice. Results show that EC-expressed TNFR2 rescued the defects in ischemia-induced neovascularization and flow recovery observed in TNFR2-KO (Figure 5a through 5c). This increased angiogenesis observed in vivo seems to be EC autonomous because isolated mouse ECs from TNFR2-KO/TG also showed increased migration in response to TNF in vitro compared with TNFR2-KO ECs (Supplemental Figure VI), likely attributable to TNF-induced TNFR2-Bmx-VEGFR2 activation.7,12 In contrast, TNFR1-dependent JNK signaling was reduced by the TNFR2 transgene, as observed in vivo (Supplemental Figure VI).

The TNFR2 transgene is expressed at a very high level in bone marrow (see Figure 1). Moreover, we have previously shown that Bmx in bone marrow–derived cells contributes to ischemia-induced arteriogenesis/angiogenesis.6 This led us to determine the role of the TNFR2 transgene in bone marrow in ischemia-induced responses. To this end, we performed BMT experiments using TNFR2-KO as recipients. First, TNFR2-KO mice received lethal irradiation followed by transplantation of bone marrow cells from TNFR2-KO or TNFR2-KO/TG to TNFR2-KO mice (KO BMT and KO/TG BMT). Six weeks after BMT, the mice were then subjected to femoral artery ligation. Success of BMT was verified by genotyping of blood samples with TNFR2-KO or TNFR2-TG-specific primers (not shown) and by Western blot of bone marrow samples with anti-TNFR2 antibody (Figure 5d). KO and KO/TG mice without BMT were used as controls. Both control KO mice and KO BMT mice showed a similar impairment of blood flow recovery, and no significant differences were observed between the 2 groups. Blood flow recovery was dramatically increased in the KO/TG BMT group compared with KO on days 14 and 28 (Figure 5e). Consistently, KO/TG BMT mice had enhanced ischemia-mediated arteriogenesis and angiogenesis (not shown). However, blood flow recovery on day 28 in the KO/TG BMT groups was less compared with control KO/TG (Figure 5e). These data suggest that TNFR2-expressing cells in both intrinsic vasculature and bone marrow contribute to ischemia-mediated vascular repair and functional flow recovery.

Discussion The rationale for generating TNFR2-TG is that TNFR2 is highly induced in vascular ECs in response to ischemia.7 The
The important finding of this study is that the EC-specific TNFR2 plays a critical role in ischemia-mediated arteriogenesis and angiogenesis. Specifically, EC-specific TNFR2 transgene enhances limb perfusion and ischemic reserve capacity compared with the nontransgenic WT mice. Immunohistochemical analyses indicate that TNFR2-TG mice had increased ischemia-initiated EC proliferation, neovascularization, and vessel maturation. These functional changes in TNFR2-TG mice correlate with augmented Bmx-VEGFR2 proangiogenic activities, which have been previously shown to be TNFR2-dependent in vascular ECs. Moreover, the EC-expressed TNFR2 rescues the defects of TNFR2-KO in ischemia-induced angiogenic signaling and flow recovery. Moreover, BMT suggests that TNFR2-expressing cells in both intrinsic vasculature and bone marrow contribute to ischemia-mediated angiogenesis and flow recovery. Our data support a critical role of endothelial TNFR2 in ischemia-mediated adaptive angiogenesis.

The EC-expressed TNFR2 transgene did not significantly induce inflammation and basal capillary density in a variety of tissues that we examined. There were no apparent morphological abnormalities in TNFR2-TG or TNFR2-KO/TG mice. This is in contrast to TNF-transgene mice. Previous data have shown that transgenic mice expressing low levels of soluble TNF cause chronic inflammation and arthritis. This is likely due to systemic activation of TNFR1-dependent signaling. EC-specific transgenic mice expressing a membrane-bound form of TNF also induced constitutive EC activation and inflammatory angiogenesis. It is not known whether these responses are TNFR1 or TNFR2-dependent, despite the proposal of the membrane-bound form of TNF as a ligand for endogenous TNFR2 in vivo. Interestingly, ischemia-induced inflammatory responses (expression of TNF, TNF-induced JNK mitogen-activated protein kinase signaling, and its responsive genes VCAM-1 and VEGF, as well as infiltration of immune cells) are actually reduced in the TNFR2-TG mice. Because TNF-induced inflammatory events are primarily mediated by TNFR1, the current study suggests that TNFR1 signaling in ECs may be suppressed by the TNFR2 transgene. The mechanism by which TNFR2 suppresses TNFR1-dependent signaling in ECs needs to be further defined. In contrast to the TNFR1-dependent inflammatory signaling, TNFR2-dependent Bmx-VEGFR2 proangiogenic signaling pathways are upregulated. This is consistent with our previous findings that TNFR2 in ECs uses Bmx to transactivate VEGFR2, a pathway critical for in vitro EC angiogenesis. Moreover, we have demonstrated that TNFR2 contains a Bmx-binding domain at the C-terminal 59 amino acid residues, and a mutant with a deletion of those 59 amino acid residues (TNFR2-59) failed...
to activate VEGFR2 and EC angiogenesis; it induces apoptosis in cultured ECs.7 We consistently failed to obtain an EC-specific transgenic mouse line expressing TNFR2-59. It is conceivable that the TNFR2-59 transgene in ECs leads to embryonic lethality by inducing vascular EC apoptosis.

Our data clearly demonstrate that the TNFR2 transgene in bone marrow–derived cells plays a role in ischemia-induced vascular remodeling. This is supported by the results that bone marrow–derived cells expressing the TNFR2 transgene implanted into TNFR2-KO mice augmented ischemia-induced blood flow recovery. However, blood flow recovery on day 28 in KO/TG BMT groups was less compared with control KO/QTG. These data suggest that TNFR2-expressing cells in both intrinsic vasculature and bone marrow contribute to ischemia-mediated vascular repair and functional flow recovery. These results are similar to our previous findings that Bmx in bone marrow–derived cells is critical for the early phase of ischemia-induced tissue repair, whereas Bmx in intrinsic vasculature may contribute to the later phase of arteriogenesis/angiogenesis.6 Given that the TNFR2 transgene is expressed in both bone marrow and muscle tissues, it is conceivable that the TNFR2-Bmx-VEGFR2-dependent signaling may enhance EC survival and mobilization in bone marrow and promote EC migration and tube formation in local muscle tissue, contributing to ischemia-induced arteriogenesis and angiogenesis.

ECs are a primary target of TNF, and TNF elicits a broad spectrum of biological effects, including proliferation, differentiation, and apoptosis. It has been shown that anti-TNF therapy targeting both TNFR1 and TNFR2 has been successful in treating inflammatory rheumatoid arthritis but has failed in the treatment of cardiovascular diseases, although the TNF protein is increased in heart disease.23 Our previous and current studies strongly suggest that TNFR2 signaling plays beneficial roles in the cardiovascular system, including ischemia-initiated arteriogenesis (growth of existing collaterals) and angiogenesis (formation of new blood vessels). Therefore, specific expression and activation of TNFR2 in ECs may provide a novel strategy for the treatment of vascular diseases, such as coronary artery disease and peripheral arterial disease.

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Disclosures

None.

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SUPPLEMENTAL MATERIALS

Endothelial-specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis

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MATERIALS AND METHODS

**Animal protocol.** Mice were housed in specific pathogen-free animal facilities according to guidelines from the *Office of Laboratory Animal Welfare at the National Institute of Health*, and all experimental procedures were approved by the *institutional animal care and use committees* at Yale University.

**Creation of EC-specific transgenic mice expressing TNFR2 (TNFR2-TG).** The human TNFR2 cDNA with a Myc-tag sequence at 3’-end was inserted into the *EcoRI* and *XbaI* sites of pVE-pA vector \(^1\) between the VE-cadherin promoter and bGH pA to obtain pVE-TNFR2 plasmid. The plasmid was linearized with *XhoI* digestion and the pronuclear injection was performed at Yale Transgenic Core. The founder were identified by PCR of tail DNA with a 5’ primer of TNFR2 and 3’ primer of Myc. TNFR2-TG mice were backcrossed with C57BL/6 mice for more than 6 generations before experiments. All experiments were performed with heterozygous TNFR2-TG mice and their non-TG littermates as controls.

TNFR2-KO (strain name B6.129-tnfrsf1b) were initially purchased from Jackson Laboratory (Bar Harbor, Maine) and subsequently bred with C57BL/6 for at least six generations prior to experiments. Mice were confirmed by genotyping with specific primers suggested by the Vendor. TNFR2-KO/TG mice were obtained by mating of TNFR2-TG with TNFR2-KO and the KO/TG mice express TNFR2 only in vascular EC.

**Immunohistochemistry for transgene expression.** 8 to 10-week-old TNFR2-TG or WT littermates were perfused with PBS for 5 min and then with 4% paraformaldehyde for 5 min at physiological temperature and pressure. Blood vessels were then harvested and diffusion-fixed overnight at 4°C followed by dehydration in 30% sucrose overnight at 4°C. The vessels were then embedded, and 5-µm sections were stained for anti-Myc antibody (Roche) or anti-TNFR2 (rabbit polyclonal, Abcam). Endothelium was visualized by goat anti-CD31 antibody (Santa Cruz).
In vivo Angiogenesis Models. All animal studies were approved by the institutional animal care and use committees of Yale University. Wild type C57BL/6 mice were mated with TNFR2-TG to obtain the transgene-negative (WT) and transgene-positive (TG) littermates. Similarly, TNFR2-KO and TNFR2-KO/TG were mated to obtain littermates of KO and KO/TG. Age and sex-matched 8-12 week old male mice of WT, TNFR2-TG, TNFR2-KO and TNFR2-KO/TG mice were used for all experiments.

Mouse hindlimb ischemia model was performed as described recently. Briefly, following anesthesia (79.5 mg/kg ketamine, 9.1 mg/kg; xylazine), the left femoral artery was exposed under a dissection microscope. The proximal of femoral artery and the distal portion of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arteriectomy was performed. Sham operation is without femoral artery ligation but skin incision. Blood flow was measured by PeriFlux system with Laser Doppler Perfusion Module (LDPU) unit (Perimed, Inc. North Royalton, OH). Deep measurement probe was placed directly on gastrocnemius muscle to ensure a deep muscle flow measurement. Ischemic and non-ischemic limb perfusion was measured pre-, post-surgery, 3 days, 2 weeks and 4 weeks after surgery. The final blood flow values were expressed as the ratio of ischemic to non-ischemic hind limb perfusion.

Post-contraction hyperemia pre- and post- ischemia. After anesthesia, mice were placed on a heated pad. The adductor muscle group and gastrocnemius muscle were exposed by a middle line incision of the limb. After baseline gastrocnemius blood flow was measured, adductor muscles were stimulated with 2 electrodes at 2Hz, 5mA by using electrostimulator for 2 min. Blood flow was taken and recorded by MacLab Chart software (ADIstruments, Grand Junction, CO) during stimulation and for 10 minutes post-stimulation.

Microfil Perfusion. 4 weeks after femoral ligation, mice were anesthetized and perfused with 20 ml of 37°C PBS plus 10 units/ml heparin at a flow rate of 10–15 ml/min through the left ventricle. To analyze
the vasculature after onset of ischemia, we employed a silicone-based contrast formulation (Microfil, FlowTech Inc., USA) to optimize visualization of the vascular structures in mice. Arterial growth was quantitated as ratio of diameters (left/right).

**Histology and Immunohistochemistry.** Mice were sacrificed at indicated times and tissues were perfused in situ with PBS and then fixed with 10% buffered formalin. The sections were paraffin embedded, sectioned in 5 µM, and stained with hematoxylin & eosin. Tissue sections were also stained with certain antibodies (e.g., anti-CD31, anti-α-SMA, anti-TNFR2 and anti-CD45) as described previously ², ³. Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed™ peroxidase substrate kit, Vector Laboratories, Burlingame, CA). Pictures from 4 random areas of each section, and 5 sections per mice were taken using a Kodak digital camera mounted on a light microscope (40x objective). Images were quantified using the Matlab software (The Math Works, Inc. Natick, MA) as described recently ², ³. TUNEL assay was performed according to the instruction provided by the Manufacturer’s protocol (Roche) ³.

**Gene expression in ischemic muscle.** Total RNA of lower limb muscles was isolated by using phenol/chloroform and isolated using RNeasy kit with DNase I digestion (Qiagen, Valecia, CA). Reverse transcription was done by standard procedure (Super Script First-Strand Synthesis System, Qiagen) using 1µg total RNA. Quantitative real-time PCR was performed by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). Specific primers for mouse TNFR2, VEGFR2, Bmx, TNF, TNFR1, TRAF2 and 18S ribosomal RNA were described previously ²-⁴. Relative amount of mRNA in mouse lower limb muscle 3 days and 2 week post-ischemia was quantified.

**Aortic-Ring Assay.** The thoracic aorta was dissected from mice (8–10 weeks old) and cut into cylindrical, 3-mm-long segments. The rings were suspended by two tungsten wires mounted in a vessel
myograph system (Danish Myotechnologies, Aarhus, Denmark). The aortas were bathed in oxygenated Krebs buffer and submitted to a resting tension of 9.8 mN. After 60 min of equilibration with frequent washings, concentration response curves for phenylephrine (PE) were generated to determine vasoconstrictor responses. To study vasodilator and L-nitro arginine methyl ester (L-NAME) responses, the rings were preconstricted with a submaximal concentration of PE, and acetylcholine (ACh) (10^{-9} to 10^{-5} M), sodium nitroprusside (SNP) (10^{-9} to 3x10^{-7} M), or L-NAME (100 µM) was injected at the plateau of the PE-induced contraction.

**Bone marrow transplantation (BMT).** Bone marrow transplantation (BMT) was performed as described previously. Briefly, lethally irradiated mice (irradiation 2x 5.5 Gy within 3h) were transplanted with cells derived from donor mice, and bone marrow cells from TNFR2-KO or TNFR2-KO/TG mice were harvested by flushing femur. Red blood cells were removed by lysis using ammonium chloride and subsequent washing with PBS. Cells were counted and 1x10^{5} cells were injected into the tail vein of the TNFR2-KO recipient. Successful BMT was controlled by genotyping of TNFR2 gene six weeks after BMT. The mice were then subjected to femoral artery ligation as described in the methods.

**Mouse EC culture.** Mouse EC isolation from lung tissues was performed as we described previously. For immuno-selection, 10 µl beads (per T-75 of mouse lung cells) were washed with 1 ml of buffer A (PBS +2% FBS) for 3 times and resuspended in 100 µl of buffer A. 10 µl (10 µg) of anti-mouse ICAM-2 or 10 µl (10 µg) of PECAM-1 were added and rocked at 4°C for 2 hrs. Beads were washed for 3 times and resuspended in 160 µl of buffer A. Confluent mouse lung cells cultured in a T-75 flask were placed at 4°C for 5 min and incubated with the beads at 4°C for 1 hr. Cells were then washed with warm PBS and treated with 3 ml of warm Trypsin/EDTA. When cells were detached, 7 ml of growth media were added. An empty 15-ml tube in the magnetic holder was placed on the holder and the cell suspension
(~10 ml) was added slowly by placing the pipette on the wall of the tube so that the cells pass through the magnetic field. Cells were incubated for 5 min, and the media was carefully aspirated. The 15-ml tube was removed from the magnetic holder and the beads/cells were resuspended in 10 ml of media. The selected cells were plated on 0.2% gelatin-coated flasks and cultured for 3-7 days. When the cells were confluent, another round of immunoselection was repeated.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM). Statistical differences were measured by either Student’s T-test, one or two-way analysis of variance followed by Bonferonni post-hoc test. A value of $p < 0.05$ was considered as statistically significant.
SUPPLEMENTAL FIGURE AND FIGURE LEGENDS

Endothelial-specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis

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Supplemental Fig. I

a. Isotype control

Ctrl IgG  aCD31

b. Hindlimb muscle

WT  TG

Fig. I. TNFR2 transgene expressed in vascular endothelium has no effect on the basal capillary density of tissues. a. The specificity of anti-CD31. Hindlimb tissue sections were immunostained with a goat IgG isotype control or anti-CD31. Arterioles are indicated by arrowheads and capillaries are indicated by arrows. b-c. Basal capillary density in hindlimb muscle and heart was immunostained with CD31. Representative images of CD31 staining in muscle and heart. c. Quantification of capillaries (number/mm² muscle area. Data from 3 sections of each mouse muscle tissue are shown in graphics and n=3 for each strain (total 9 sections). *, p<0.05.
Fig.II. TNFR2 transgene increases ischemia-induced arteriogenesis. a. Diagram of blood flow changes after occlusion of the femoral artery in the hind limb. After occlusion of the femoral artery (X, left panel), blood flow (arrows) follows the gradient between high pressures proximal to the occlusion site and very low distal pressures. Preexisting collateral arterioles are recruited. b-c. Arteriogenesis was determined by Microfil casting analyses. 4 weeks after femoral ligation, mice were anesthetized and subjected to microfil perfusion. A low and high power are shown in a and b, respectively. Collateral artery growth is indicated by arrows. d. Arterial growth was quantified as a ratio of diameters (left/right) and n=5 for each strain. *, p<0.05.
**Supplemental Fig.III**

a. Infiltration (CD45 staining)

Fig.III. Effects of TNFR2 transgene on ischemia-induced infiltration. WT and TNFR2-TG mice were subjected to ischemia ligation, hindlimb tissues were harvested on day 7. Recruitment of leukocytes in response to ischemia was determined by anti-CD45. a. Representative images of CD45 staining in ischemic and non-ischemic hindlimbs of WT and TNFR2-TG mice are shown. b. CD45-positive cells were quantified as number/mm² muscle area. *, p<0.05.
**Fig.IV. Characterization of TNFR2-KO/TNFR2-TG mice.**

*a.* Genotyping of TNFR2-KO/TNFR2-TG mice. TNFR2-TG mice were mated with TNFR2-KO mice (both are C57BL/6 background) to obtain TNFR2-KO/TNFR2-TG (KO/TG) mice. Genomic DNA of mouse tails was genotyped by PCR with specific primers for the human TNFR2 transgenic fragment and the endogenous mouse TNFR2. *a.* A representative genotyping of WT, TG, KO and KO/TG is shown.

*b.* Immunostaining of TNFR2. Muscle hindlimb tissues from TNFR2-KO and TNFR2-KO/TNFR2-TG mice were immunostained with anti-TNFR2. A rabbit IgG isotype was used as a control. Positive staining of TNFR2 in capillary is detected in KO/TG but not in KO mice.
Supplemental Fig. V

Fig. V. TNFR2-KO mice show reduction of basal NO release in aortic endothelium but TNFR2-TG restored the basal NO activity. Aortas from WT, KO and KO/TG (n=3 for each group) were used for aortic ring assays. a. PE responses. Aortas were contracted with PE at a full range of doses (10^{-9}-10^{-4} M). b. Basal NO activity. Aortic rings were incubated with a NOS inhibitor L-NAME to remove basal NO synthesis and then contracted with PE as in a. Ratio of EC{50} in response to PE in the presence of L-NAME to PE in the absence of L-NAME. c. Ach responses. Aortic rings were precontracted with PE and then relaxed with Ach at a full range of doses (10^{-9}-10^{-4} M). % of relaxation is shown. d. Vessel constriction in response to KCl. Aortic rings were contracted with 50 mM of KCl. e. TNFR2 expression had no effects on vessel relaxation in response to the NO donor drug SNP. Aortic rings were incubated with L-NAME to remove basal NO synthesis followed by a precontraction with PE, and were then relaxed with SNP at a full range of doses (10^{-9}-10^{-6} M). All data are mean±SEM, with n=3 animals and eight aortic rings per animal, *, p<0.05.
Supplemental Fig.VI

Fig.VI. TNFR2 expression increases EC migration with no effects on TNFR1-dependent JNK activation. a-b. Effects of TNFR2 transgene on EC migration. Mouse microvessel EC (MEC) from lung were isolated from KO and TNFR2-KO/TG mice, and TNF-induced EC migration was determined in a monolayer injury model by treating cells with TNF (1 ng/ml) for 0, 12 and 24 h. The wound areas were marked by white lines (a). Data are mean ±SEM of the two triplicates from two independent experiments. *, p<0.05 (b). c. Effects of TNFR2 transgene on TNFR1-dependent JNK activation. MEC were treated with TNF (10 ng/ml) for indicated time. JNK1 activation was determined by Western blot with a phospho-JNK1 specific antibody. Total JNK1 as well as expression of TNFR2, TNFR1 and β-tubulin were determined by Western blot with respective antibodies. The relative levels of phospho-JNK are quantified, with untreated TNFR2-KO EC as 1.0 (e). Similar results were obtained in two additional experiments.
References


