Tumor Necrosis Factor-α Receptor p75 Is Required in Ischemia-Induced Neovascularization

David A. Goukassian, MD, PhD; Gangjian Qin, MD; Christine Dolan, MS; Toshinori Murayama, MD, PhD; Marcy Silver, BS; Cynthia Curry, BS; Elizabeth Eaton, BS; Corinne Luedemann, BS; Hong Ma, BS; Takayuki Asahara, MD, PhD; Victor Zak, PhD; Shanu Mehta, MS; Aaron Burg, BS; Tina Thorne, MS; Raj Kishore, PhD; Douglas W. Losordo, MD

Background—Aging is a risk factor for coronary and peripheral artery disease. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is expressed in ischemic tissue and is known to modulate angiogenesis. Little is known about the role of TNF-α receptors (TNFR1/p55 and TNFR2/p75) in angiogenic signaling.

Methods and Results—We studied neovascularization in the hindlimb ischemia model in young and old TNFR2/p75 knockout (p75KO) and wild-type age-matched controls. Between days 7 to 10 after hindlimb surgery, 100% of old p75KOs experienced autoamputation of the operated limbs, whereas none of the age-matched wild-type mice exhibited hindlimb necrosis. Poor blood flow recovery in p75KO mice was associated with increased endothelial cell apoptosis, decreased capillary density, and significant reductions in the expression of vascular endothelial growth factor and basic fibroblast growth factor-2 mRNA transcripts in ischemic tissue and in circulating endothelial progenitor cells. The number of circulating bone marrow–derived endothelial progenitor cells was significantly reduced in p75KO mice. Transplantation of wild-type bone marrow mononuclear cells into irradiated old p75KO mice 1 month before hindlimb surgery prevented limb loss.

Conclusions—Our present study suggests that ischemia-induced endothelial progenitor cell–mediated neovascularization is dependent, at least in part, on p75 TNF receptor expressed in bone marrow–derived cells. Specifically, endothelial cell/endothelial progenitor cell survival, vascular endothelial growth factor expression, endothelial progenitor cell mobilization from bone marrow, endothelial progenitor cell differentiation, and ultimately ischemia-induced collateral vessel development are dependent on signaling through TNFR2/p75. Furthermore, because TNFR2/p75 becomes an age-related limiting factor in postischemic recovery, it may be a potential gene target for therapeutic interventions in adult vascular diseases. (Circulation. 2007;115:752-762.)

Key Words: angiogenesis ■ cytokines ■ endothelium ■ ischemia ■ collateral circulation

Aging is associated with an increased risk for development of coronary and peripheral artery diseases. The extent of ischemic damage and functional recovery in case of extensive impairment of perfusion due to arterial obliteration is largely dependent on the ability to develop new collateral blood vessels. Previous reports have indicated that angiogenesis responsible for collateral development in limb ischemia in murine and rabbit models is impaired with aging.

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Angiogenesis is accompanied by perivascular inflammation and monocyte/macrophage accumulation. Tumor necrosis factor-α (TNF-α), a macrophage/macrophage-derived pluripotent mediator, can function as both a proangiogenic and antiangiogenic factor. These divergent TNF-α effects have been attributed to TNF-α concentration and duration of the exposure. TNF-α induces the expression of many angiogenesis-related genes through 2 different TNF-α receptors, TNF-αR1 (p55) and TNF-αR2 (p75). For example, in vascular endothelial cells (ECs), TNF-α increases the expression of angiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin-8. Recent reports suggest that aging is associated with increased expression of p55 and decreased expression of p75 in human lymphocytes.
Accordingly, on the basis of the constellation of preexisting data outlined above, we hypothesized that p75 receptor expression may be important in angiogenic signaling in aging adults. To test our hypothesis, we studied neovascularization in the hindlimb ischemia model in young and old p75KO mice and age-matched wild-type (WT) counterparts. In the present report, we show that with advanced age, signaling through the p75 receptor (TNFR2) is required for collateral vessel development in ischemia-induced neovascularization.

Methods

Endothelial Progenitor Cell Culture and In Vitro Functional Assay
Bone marrow (BM)-derived endothelial progenitor cells (EPCs) from young WT and TNFR2 knockout (KO) mice were isolated and expanded ex vivo and used between days 6 and 8 after initial plating as described.

Chemotaxis and chemokinesis of WT and TNFR2 KO EPCs in response to TNF-α (1 and 10 ng/mL), recombinant mouse VEGF (20 ng/mL), and granulocyte/macrophage colony-stimulating factor (50 ng/mL) were evaluated with the use of a modified checkerboard assay with Coster Transwell chambers (6.5-mm diameter, 5-μm pore) as described previously.

Circulating peripheral blood (PB) EPCs in WT and TNFR2 KO mice were evaluated with the use of EPC culture assay and by fluorescence-activated cell sorter (FACS) analysis, as described previously. (For additional EPC characterization, please refer to Figure IV in the online-only Data Supplement.)

To examine the formation of tubulike structures, ex vivo expanded WT and p75KO EPCs were seeded at 5 × 10^4 cells per well on 4-well chamber slides coated with Matrigel (Collaborative Biomedical Products, Mass) and incubated for 12 hours in medium containing 5% fetal bovine serum and supplemented with medium alone or 1 and/or 10 ng/mL of recombinant mouse TNF-α (BD PharMingen, San Diego, Calif). Cells in the chambers were examined and photographs were taken 12 hours after stimulation.

Quantitative mRNA expression of angiogenic factors in vivo was determined with the use of RNA obtained from the tissues of WT and p75KO mice, at days 3 and 10 after hindlimb surgery, by Taqman real-time polymerase chain reaction, as described previously.

mRNA expression of angiogenic factors in vitro in ex vivo expanded WT and p75KO EPCs treated with 10 ng/mL of mrTNF-α was determined by ribonuclease protection assay with the use of a custom-made multiprobe DNA template (PharMingen) and RPA III TM kit (Ambion, Tex) following manufacturers’ instructions.

To examine the effect of TNF on nuclear factor-kB (NF-kB) nuclear translocation in WT and p75 EPCs, immunostaining with NF-kB p65 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was performed. Electrophoretic mobility-shift assay with NF-κB consensus sequence was performed to evaluate NF-κB DNA binding activity as described.

To examine the role of p75 in TNF-induced, NF-kB-mediated VEGF promoter activity, EPCs from WT and p75KO mice were transiently transfected with VEGF promoter/luciferase reporter constructs. The constructs, including full-length (2.6-kb) VEGF promoter, a deletion construct (0.35 kb) containing 2 putative NF-κB sites and 1 Sp1 cluster, and an inactive deletion construct spanning up to −70 from the transcription origin site and containing an incomplete Sp1 cluster, have been described before. Twenty-four hours after transfection, cells were treated with TNF (1 ng/mL), and reporter activity was measured by luciferase activity as described.

Hindlimb Ischemia Studies
Unilateral hindlimb ischemia in male young and old WT C57BL/6J and p75KO mice was established by ligation and excision of femoral artery as previously described. Serial assessments of HL blood flow were performed with a PIM 2.0 laser Doppler perfusion imager (Lisca) as previously described. Calculated perfusion was expressed as a ratio of left (ischemic) to right (control) limb. Results represent the mean ± SEM of at least 10 to 15 mice per group.

Capillary density in nonischemic and ischemic limb tissue was determined in at least 5 mice from each group on day 28, and capillary networks were evaluated as described previously.

To evaluate the kinetics of VEGF expression in operated limbs of WT and p75KO mice after hindlimb surgery, tissue from 5 mice from each group was collected before and 1, 3, 7, and 10 days after surgery, immediately fixed in methanol overnight, and then processed for immunofluorescent staining with VEGF (Santa Cruz Biotechnology). In addition, continuous sections of hindlimb tissue from same animals were immunostained with terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) to assess viability of the hindlimb tissue in operated limbs in WT versus p75KO mice. Both VEGF- and TUNEL-stained slides were evaluated by laser scanning confocal microscopy (Axiovert 100, Zeiss, NY).

Murine BM Transplantation Studies
All protocols were approved by St Elizabeth’s Medical Center Institutional Animal Care and Use Committee. BM transplantation models were established wherein BM mononuclear cells (MNCs) from young (4-week-old) WT and p75KO mice were injected into old p75KO animals. Inversely, to evaluate the effect of BM-derived p75KO MNCs in the ischemic recovery in the WT tissue setting, we transplanted BM-derived MNCs from young WT and p75KO mice into old WT mice. BM cells were obtained by flushing the tibias and femurs of young (3- to 4-week-old) donor WT (C57BL/10j/d) and p75KO (on C57BL/6j background) mice (both, The Jackson Laboratory, Bar Harbor, Me). Low-density BM MNCs were isolated by density centrifugation over Histopaque-1083 (Sigma, St Louis, Mo). To evaluate the impact of BM MNC transplantation on therapeutic neovascularization, old (12- to 14-month-old) TNFR2/p75KO mice were lethally irradiated with 9 to 11 Gy and received tail-vein injections of 3 × 10^7 of donor (WT/GFP or TNFR2 labelling) with before injection with 1.1′-diocadecyl-3,3′,3′-tetramethylindocarbocyanine [DiI]; Molecular Probes, Ore) BM MNCs. At 4 weeks after BM transplantation, by which time the BM of the recipient mice usually regenerates by donor BM cells, hindlimb surgery was performed, and animals were evaluated at different times after hindlimb surgery for physiological recovery with the use of the laser Doppler perfusion imager, histological assessment of capillary network (CD31 and/or isoclin B4 staining), evaluation of total muscle loss by the ratio of operated versus unoperated limbs on day 28, and homing of WT/GFP and TNFR2/DiI-labeled BM-derived MNCs in the areas of ischemia by confocal microscopy.

Statistical Analysis
Results are expressed as mean ± SEM. To determine differences in postschemic recovery in vivo (laser Doppler perfusion imager, capillary density, EPC mobilization, EC apoptosis), we used repeated-measures ANOVA (StatView software, SAS Institute Inc, Cary, NC) and the general linear model with post hoc analysis (statistical package, SPSS Inc). Differences among treatment group/time (tissue and EPC mRNA expression, electrophoretic mobility-shift assay, VEGF promoter studies) were evaluated by ANOVA f test and 2- and 3-way ANOVA by the Fisher protected least significant difference test and corrected by Scheffé and Bonferroni/ Dunn test with the use of StatView software (SAS Institute Inc). Differences were considered significant at P < 0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Ischemia-Induced Angiogenesis Is Impaired in Old TNFR2 KO Mice
To examine whether age-associated decreases in p75 TNFR2 expression may contribute to the failure of post-
ischemic recovery in adults, we subjected 4- to 8-week-old (young) and 10- to 12-month-old (old) WT, p55KO (Figure Ia to Ie in the online-only Data Supplement) and p75KO mice to hindlimb surgery.22 Mean blood flow in young WT mice 28 days after hindlimb surgery reached 80% of the preischemic flow (Figure 1A, black bars). In contrast, recovery of blood flow was delayed up to 14 days in old WT (gray bars) and young p75KO (clear bars) mice (40% of preischemic value versus 80% in young WT mice; \( P < 0.03 \)) but was similar to the recovery in young WT mice thereafter (Figure 1A, days 21 and 28). These results suggest that old WT and young p75KO mice exhibit a partial and temporal insufficiency of postischemic recovery compared with young WT mice. All of the old p75KO mice (n=12; Figure 1B, black bars and inset) experienced autoamputation of the operated limb between days 7 to 10 after hindlimb surgery, suggesting an absolute requirement of TNFR2 p75 for postischemic blood flow recovery in adult mice.

As a measure of collateral blood flow recovery after ischemia, we evaluated capillary density in hindlimb muscles of young and old mice of all genotypes 28 days after surgery (Figure 1C and Figure Ie in the online-only Data Supplement). Compared with young WT mice, there was a 50% decrease in capillary density in young p75KO mice (1.83 ± 0.3 versus 0.99 ± 0.14; \( P < 0.05 \)) (Figure 1C).

Mobilization of BM-derived EPCs into PB with subsequent homing into ischemic areas has been shown to contribute to postischemic recovery in animal models and in humans.23,24 To assess the number of circulating BM-derived EPCs in PB after hindlimb surgery in WT versus p75KO, MNCs isolated from PB were evaluated for the expression of Flk1 and Sca1 by FACS analysis.15 Only Flk1/Sca1 double-positive cells were considered EPC and were included in our calculation (Figure II in the online-only Data Supplement). FACS analysis revealed a gradual increase in circulating BM-derived EPCs in WT compared with p75KO mice between days 1 and 3, with a maximal 4-fold increase by day 3 (11.1 ± 3.1 versus 2.6 ± 1.8; \( P < 0.05 \)) (Figure 1D). Circulating EPCs remain 2-fold higher (7.6 ± 0.5 versus 3.2 ± 0.7; \( P < 0.01 \)) in WT versus p75KO mice up to 7 days after hindlimb surgery, suggesting that ischemia-induced mobilization of EPCs from BM at least in part depends on TNFR2 p75 signaling. By day 10, the EPCs were similar in mice of both genotypes (4.75 ± 3.3 versus 4.5 ± 0.9; \( P = \text{NS} \)) (Figure 1D).

Figure 1. Graphic representation of blood flow recovery presented as the laser Doppler perfusion ratio up to 28 days after hindlimb surgery in WT young (black bars, n=15), WT old (gray bars, n=12), and p75KO young (clear bars, n=15) mice (A); and p75KO old mice (black bars, n=12) and (inset) a representative image of 5 old p75KO mice showing autoamputation of operated limb (B). C, Representative images of hindlimb muscles immunostained with isolecitn B4 to identify capillaries and relative capillary density at postoperative day 28 in young WT, p55KO, p75KO, and old WT mice. Capillary density is expressed as number of capillaries per muscle fiber. Results represent the mean±SEM of 12 randomly chosen areas (0.06 mm²) of muscle fiber from 3 mice per genotype. D, Kinetics of mobilization of BM-derived EPCs into PB after hindlimb (HL) surgery in WT and p75KO mice, measured by staining the mononuclear fraction of PB with antibodies against VEGF receptors Flk1 and Sca1.
Ischemia-Induced VEGF Expression Is Lower in the Limbs of p75KO Mice

Real-time PCR analysis of homogenized hindlimb muscle on days 3 and 10 after hindlimb surgery revealed that, compared with WT tissue, VEGF expression was decreased 40% to 50% (P<0.05) in p75KO mice between days 3 and 10 after hindlimb surgery (Figure 2A). The mRNA expression of bFGF was also decreased by 15% and 36% (day 3 and 10, respectively) in p75KO versus WT tissue, but differences were not significant statistically (data not shown).

Interestingly, mRNA levels of angiopoietin-1 in p75KO were comparable to those in WT mice up to day 10 after surgery (data not shown), suggesting that ischemia-induced angiopoietin-1 gene expression does not require signaling through p75 TNFR2.

VEGF was highly expressed in the muscles of operated limbs of WT mice between days 3 and 10 after surgery (Figure 2B, top panel). In contrast, VEGF expression was decreased in the tissue of p75KO mice between days 3 and 10 (Figure 2B, bottom panel), suggesting that ischemia-induced VEGF expression is impaired in p75KO mice.

Ischemia-Induced EC Apoptosis Is Greater in the Limbs of p75KO Mice

To evaluate the viability of ECs, we triple stained the sections of operated hindlimb muscle for TUNEL, a marker of apoptosis, for isoelectin B4, a marker of endothelial cells, and for TopRo3 to visualize nuclei (Figure III in the online-only Data Supplement). No EC apoptosis was detectable in WT mice before hindlimb surgery, a few TUNEL/EC-positive cells were detected between days 1 to 3, and no TUNEL/EC-positive cells were detectable up to day 10 after surgery (Figure 3A, top panel and Figure 3B, solid line). In contrast, p75KO mice revealed extensive EC apoptosis as early as day 1 after hindlimb surgery that decreased gradually and was comparable to the number of TUNEL/EC-positive cells in WT control muscles up to day 7 after surgery (Figure 3A, bottom panel and Figure 3B, dotted line), suggesting that ischemia-induced apoptosis was augmented in p75KO mice.

Interestingly, by day 10 in p75KO mice, there was a second significant increase in the number of TUNEL/EC-positive cells (Figure 3A, bottom panel and 3b, dotted line), indicating continued impaired postischemic EC survival mechanism(s) in p75KO mice after hindlimb surgery. It is also possible that this second increase in EC apoptosis may represent an inability of recruited BM-derived EPCs to survive in the ischemic tissue of p75KO mice.

Loss of p75 Impairs the Function of Cultured EPCs

Our in vivo studies showed that loss of p75 TNFR2 impairs postischemic recovery by affecting angiogenesis (Figure 1A and 1B) and that ischemia-induced mobilization of EPCs from BM is decreased in p75 KO mice (Figure 1D). To examine whether EC functions may be altered in p75KO cells, we used ex vivo expanded cultures of BM-derived EPCs from WT and p75KO mice.13 EPC identity of ex vivo expanded cells was confirmed before functional experiments (Figure IVa to IVe in the online-only Data Supplement). In addition, we also confirmed by real-time polymerase chain reaction expression of TNF receptors p55R1 and p75R2 in ex vivo expanded EPCs (Figure V in the online-only Data Supplement).

One of the important functional features of ECs is their ability to migrate toward chemotactic stimuli.14 We found no difference in the chemotactic activity between WT versus p75KO EPCs in migration toward TNF, VEGF, and granulocyte/macrophage colony-stimulating factor (Figure 4A).

The ability to form tubelike structures on VEGF-enriched Matrigel is another important EC functional characteristic. EPCs from WT mice formed tubelike structures in control chambers and those treated with TNF, whereas EPCs from
p75KO mice failed to form tubelike structures in either type of chamber, indicating a functional loss in the BM-derived EPCs of p75KO mice (Figure 4B).

Because induction of endothelial growth factors and cytokines (ie, VEGF, bFGF, platelet-derived growth factor, interleukin-8, TNF-α) is required for initiation of angiogenesis and neovascularization,9,22,25 we evaluated the effect of TNF treatment on the mRNA expression of bFGF, VEGF, and angiopoietin-1 by ribonuclease protection assay (Figure 4C). Densitometric analysis revealed that TNF-induced expression of bFGF mRNA was undetectable in p75KO mice up to 16 hours after treatment and was only a third of the bFGF mRNA level of WT cells (Figure 4D). The difference in VEGF expression in p75KO versus WT mice was marked. VEGF expression was nearly undetectable in p75KO cells up to 8 hours after TNF treatment, and it was still 8-fold lower by 24 hours in p75KO versus WT EPCs (Figure 4D). Interestingly, TNF-induced mRNA levels of angiopoietin-1 were comparable at 8 and 16 hours in EPCs from p75KO versus WT cells; by 24 hours, they were reduced to half of the levels in WT cells (Figure 4D), corroborating our in vivo findings of expression of these angiogenic factors in tissue homogenates of WT and p75KO mice after hindlimb surgery (Figure 2B) and confirming loss of several functions characteristic of ECs in p75KO cells.

Figure 3. A, Representative merged images of triple-stained hindlimb muscle of young (8- to 10-week-old) WT (top) and p75KO (bottom) mice up to 10 days after hindlimb ischemia (HLI) surgery showing a significant increase in the number of TUNEL/EC-positive cells in p75KO mice 1 and 10 days after hindlimb surgery. B, We analyzed immunofluorescence (number of double TUNEL/EC-positive cells) in at least 8 to 10 randomly selected areas of ~10 600 μm² (measured by computer-assisted software of the confocal microscope) of hindlimb muscle from at least 3 animals per treatment group. All samples were coded and then evaluated by a single blinded observer to eliminate the bias and interobserver variability. Compared with WT, by days 1 and 10 there was a statistically significant increase in the number of TUNEL/EC-positive cells in p75KO mice after hindlimb (HL) surgery (day 1 to 26±5 vs 7±5, and day 10 to 37±8 vs 3±0.5; P<0.0001, p75KO vs WT mice), representing a 67% and 93% increase in EC apoptosis in p75KO mice on days 1 and 10, respectively. HPF indicates high-power field.

Signaling Through TNFR2 p75 Is Required for NF-κB–Mediated VEGF Gene Expression

Because treatment with TNF activates transcription factor NF-κB and NF-κB is known to regulate VEGF expression,9 we examined NF-κB nuclear translocation and DNA binding activity in WT versus p75KO cells. Thirty minutes after TNF treatment, NF-κB nuclear translocation was completely abrogated in EPCs from p75KO mice, whereas NF-κB was translocated to the nucleus in 100% of EPCs from WT mice (Figure 5A). Electrophoretic mobility-shift assay with NF-κB consensus sequence probe showed that constitutive NF-κB DNA binding was higher (lane 1 versus 3) in p75KO EPCs (Figure 5B). However, TNF treatment failed to activate NF-κB DNA binding activities in p75KO EPCs (lane 3 versus 4), whereas in WT cells TNF treatment increased (3- to 4-fold) (lane 1 versus 2) the NF-κB DNA binding activity in WT cells at 30 minutes after stimulation (P<0.002). This finding suggests that under unstimulated conditions, signaling through the p55 receptor alone maintains a slightly higher NF-κB DNA binding activity in p75KO cells than in WT cells. However, in the setting of TNF stimulation, NF-κB signaling in p75KO EPCs is impaired.

To further investigate the molecular mechanisms of TNF signaling in the regulation of VEGF expression, serial VEGF promoter/luciferase reporter constructs were transfected into EPCs from WT and p75KO mice (Figure VIa and VIb in the
online-only Data Supplement). TNF exposure resulted in a 2- to 3-fold increase in the activity of full-length (2.6 kb) VEGF promoter in p75KO EPCs compared with WT EPCs (Figure 5C). Interestingly, in WT cells transfected with NF-κB construct (0.35 kb), there was a similar 3-fold increase in VEGF promoter activity compared with WT cells transfected with full-length construct, suggesting that in WT cells under similar TNF treatment conditions, NF-κB alone can activate VEGF promoter to the same degree as full-length promoter (Figure 5C). In contrast, VEGF promoter activity was completely counteracted in p75KO cells transfected with NF-κB construct, indicating that signaling through p75 receptor is required for TNF-induced activation of VEGF promoter (Figure 5C) and that NF-κB may mediate the induction of VEGF expression through the TNF-p75 pathway. We observed no difference between WT and p75KO EPCs transfected with inactive (0.07 kb) construct in VEGF promoter activity.

Transplantation of BM Cells From Young WT Mice Rescues Limbs of Old Mice From Ischemia-Induced Autoamputation
Because BM-derived MNCs contribute to postnatal neovascularization and because our present study showed that postischemic recovery is substantially impaired in p75KO mice, we examined whether restoration of p75 receptor expression in BM of old p75KO mice would augment ischemic recovery. To test this hypothesis, we transplanted lethally irradiated old p75KO mice with BM MNCs isolated from WT GFP(+) and control p75KO Dil-labeled cells from young (4-week-old) mice (for detailed BMT protocol, see the diagram in Figure VII in the online-only Data Supplement). Four weeks after BMT, to allow for complete engraftment of transplanted BM (by day 28, recipient BM was completely reconstituted with donor marrow, and no difference between WT or p75KO engraftment was observed; the number of BM-derived PB EPCs

Figure 4. A. No difference was found in migration of WT and p75KO BM-derived EPCs toward specific chemotactic agent. Graphs represent data pooled from independent experiments. GM-CSF indicates granulocyte/macrophage colony-stimulating factor. B, Baseline and TNF-induced tubulogenesis was inhibited in BM-derived p75KO EPCs. C, A representative radiogram of multiprobe ribonuclease protection assay after TNF stimulation using 5 μg total RNA per lane. Ang-1 indicates angiopoietin-1. D, Graphic representation of bFGF, VEGF, and angiopoietin-1 mRNA expression after densitometric analysis. Black bar=p75KO; clear bar=WT. All values are adjusted relative to actin mRNA expression.
were also similar in recipient mice of both genotypes, as evaluated by FACS analysis; Figure VIII in the online-only Data Supplement), hindlimb surgery was performed. Twenty-eight days after hindlimb surgery, 100% of old p75KO mice transplanted with WT BM had a preserved ischemic limb, whereas only half of the old p75KO mice transplanted with BM from young p75KO mice had a preserved ischemic limb (Figure 6A and Figure IXa in the online-only Data Supplement), suggesting that BM-derived EPCs play an important role in postischemic recovery.

**Functional p75 Receptor Is Required for Efficient Contribution of BM-Derived EPCs in Postischemic Recovery**

To explore further the contribution of BM-derived EPCs, we transplanted old WT mice with BM MNCs from young WT or p75KO mice. Although old WT mice that received either WT or p75KO BM avoided postischemic limb loss at 28 days after hindlimb surgery, the mice that received p75KO BM experienced a greater loss of total muscle tissue in the ischemic limb (50% versus 23%; Figure 6B and Figure IXb in the online-only Data Supplement). These data suggest that...
even in WT ischemic tissue, BM-derived EPCs with functional p75 TNFR2 are required for efficient postischemic recovery.

We also examined homing of BM-derived EPCs to the ischemic tissue. Confocal microscopy of hindlimb tissues from the operated limbs of GFP-labeled BM transplanted mice showed that BM-derived cells homed only into ischemic areas of operated limbs (Figure 7A to 7C, green GFP-positive cells).

In addition, we examined homing of endothelial lineage cells into the areas of ischemia. Twenty-eight days after hindlimb surgery, 60% to 70% of GFP-positive cells in the ischemic limbs were identified as BM-derived endothelial lineage cells (Figure 8A and 8B, yellow double-positive cells), strongly suggesting a substantial contribution of BM-derived EPCs to postischemic recovery.

**Discussion**

Age-related impairment of angiogenesis has been documented previously. Specifically, investigators have delineated deficiencies in several components of ischemia-induced neovascularization, including inhibition of EC proliferation and function22,29,30 and impaired expression of angiogenic growth factors, such as VEGF, bFGF, and transforming growth factor-$

Impaired signaling by TNF-$\alpha$ and other cytokines in ECs has been correlated with enhanced apoptotic responses in the cutaneous microvasculature in adult tissue. Moreover, it is well known that TNF-$\alpha$ can induce the expression of many important immune- and angiogenesis-related genes through 2 different TNF-$\alpha$ receptors: p55 and p75. However, the individual role of the 2 TNF-$\alpha$ receptors in mediating these responses remains incompletely characterized.
Our results demonstrate that deficiency of p75 TNFR2 expression led to the failure of postischemic recovery in adult mice, manifested by 100% limb loss in old p75KO. The negative effect of p75 receptor deficiency on postischemic recovery was also evident in young animals. Likewise, the numbers of capillaries per muscle fiber in young p75KO and old WT mice were significantly lower than in young WT mice. These data strongly suggest that age-associated decrease in angiogenesis is, at least in part, a result of impaired signaling through TNFR2/p75. Aging is associated with increased expression of p55 and decreased expression of p75 in human cells and a decrease in the expression of p75 receptor in PB EPCs from adult donors.

Numerous reports suggest that VEGF is a critical growth factor in therapeutic angiogenesis. Our data indicate that VEGF expression in muscle tissue from ischemic limbs of p75KO mice was lower in both mRNA and protein levels than in WT mice. Our data indicate that VEGF expression in muscle tissue from ischemic limbs of p75KO mice was lower in both mRNA and protein levels than in WT mice. VEGF, which is present early in the response to ischemia, has been shown to mobilize BM-derived EPCs in murine models and in humans. It is conceivable that significantly lower mobilization of BM-derived EPCs into PB that we observed in p75KO mice was a direct result of decreased VEGF expression. Aging is associated with alterations in cytokine signaling pathways that result in enhanced apoptosis. The reduced expression of TNFR2/p75 associated with increased age, coupled with postischemic increases in the systemic levels of TNF-α, favors apoptosis in adult ECs, which could subsequently lead to inhibition of angiogenesis. Suppression of TNF with soluble TNFR1/p55 was reported to accelerate angiogenesis via upregulation of VEGF receptor KDR/Fk-1. Although in this experiment TNFR1/p55 was used, the improved angiogenesis was achieved not through preferential signaling via TNF receptors but rather through inhibition of TNF bioavailability, which correlates well with previous in vitro findings that low doses of TNF are angiogenic and high doses are antiangiogenic. In our study, we observed an exaggerated apoptotic response in the hindlimb of p75KO mice. It is possible that unopposed signaling through the p55 receptor in p75KO mice, and conceivably in human adult tissue with decreased p75 receptor expression, increases predominantly proapoptotic cascade via increases in Fas-associating protein with death domain (FADD), TNFR1-associated death domain protein (TRADD), and Fas death domain (FASDD), whereas impaired p75-mediated antia apoptotic signaling via NF-κB potentiates further apoptotic responses in the same tissue.

Previous studies have demonstrated that aged ECs show impaired proliferation and migration in response to various cytokines. In our study of BM-derived EPCs from WT and p75KO mice, we were not able to show impaired in vitro proliferation or migration in response to several cytokine and growth factor stimuli, suggesting that these EC functions may not depend on TNFR2 p75 signaling. However, we did find substantial decrease in VEGF mRNA expression after TNF-α stimulation in vitro of BM-derived EPCs from WT and p75KO mice. Similar to previous findings in adult tissue, we found bifactorial reduction in VEGF expression in our p75KO mouse model. First, we documented lower VEGF protein expression by immunostaining in the ischemic hindlimb in p75KO compared with WT mice. Second, we found that VEGF promoter activity was lower in BM-derived EPCs from p75KO than in WT EPCs, strongly suggesting that previously reported decreases in VEGF reporter activity in adult cells may be a direct consequence of age-associated decreases in the expression/signaling via TNFR2/p75. Impairment of TNF-induced NF-κB activity coupled with repressed VEGF promoter activity in TNFR2/p75 KO cells further corroborated the role of TNFR2/p75 in the regulation of VEGF expression and resultant VEGF-mediated neovascularization in the ischemic tissue.

Previous studies in animal models of ischemia and limited human clinical trials have documented that transplantation of BM cells or BM-derived EPCs significantly augmented ischemia-induced neovascularization by recruitment and retention of these cells to the areas of ischemia. Furthermore, animal studies have shown that transplanted BM cells or BM-derived EPCs contribute to the processes of neovascularization and the development of collateral vessels that contribute to more rapid recovery of blood flow in the ischemic areas. Our BM transplantation data showing that transplantation of young WT but not p75KO BM MNCs into old p75KO mice rescued ischemic limbs from autoamputation suggest that ischemia-induced neovascularization and mobilization of EPCs from BM are mediated, at least in part, by TNFR2/p75 signaling. Our BM transplantation studies further delineated that BM-derived EPCs with functional TNFR2/p75 are required even in WT ischemic tissue for efficient postischemic recovery. In addition to substantiating the importance of BM-derived EPCs in ischemia-induced neovascularization, our observations suggest that the recapitulation of TNFR2/p75 expression and signaling in autologous BM-derived cells from aged patients may augment their functional efficacy during cell therapy.

In summary, our present study led to the following important conclusions regarding the role of TNF receptors in neovascularization after ischemic injury: (1) Loss of p75 TNFR2 expression impairs postischemic recovery; (2) TNFR2 p75 is important for ischemic recovery in part via upregulation of VEGF gene expression; (3) postischemic apoptotic responses are exaggerated in the absence of TNFR2/p75; (4) ischemia-induced neovascularization and mobilization of EPCs from bone marrow are mediated, at least in part, via TNFR2/p75; (5) TNFR2/p75 expression by BM-derived MNCs is important for ischemia-induced neovascularization; and (6) with advanced age, signaling through TNFR2/p75 is required for collateral vessel development. Moreover, our results suggest that augmentation of TNFR2/p75 in older patients may represent a gene target with therapeutic potential, one that could be used to improve recovery after ischemia and prevent the development of severe ischemia-induced damage in adult coronary and peripheral vascular disease.
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References


### CLINICAL PERSPECTIVE

The findings of the present study indicate that loss of tumor necrosis factor (TNF) receptor function mimics the effects of aging on the response to tissue ischemia, thus suggesting that the worsening of vascular disease that occurs with aging may be the result of a TNF receptor defect. This suggestion is supported by the observation that the expression of the TNF receptor 2 (TNFR2) is decreased in older versus younger individuals. The hypothesis generated by the present study is supported by in vivo findings in the mouse hindlimb ischemia model, demonstrating a deficit in functional recovery and an increase in limb loss when the p75 receptor (TNFR2) is absent. Moreover, the replacement of the bone marrow (in an otherwise normal mouse) with marrow that lacks TNFR2 also significantly impairs the response to ischemia, implying that TNF receptor expression by circulating cells plays a critical role in ischemic recovery. From these findings one might extrapolate that increasing TNFR2 expression in elderly patients could enhance ischemic recovery; a better understanding of the pathways triggered by TNF receptor activation, however, will likely lead to more precise therapeutic targets.
Tumor Necrosis Factor-α Receptor p75 Is Required in Ischemia-Induced Neovascularization

David A. Goukassian, Gangjian Qin, Christine Dolan, Toshinori Murayama, Marcy Silver, Cynthia Curry, Elizabeth Eaton, Corinne Luedemann, Hong Ma, Takayuki Asahara, Victor Zak, Shanu Mehta, Aaron Burg, Tina Thorne, Raj Kishore and Douglas W. Losordo

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