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Dual roles of tumor necrosis factor- α receptor-1 in a mouse model of hindlimb ischemia

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Abstract

Signals in the tumor necrosis factor α (TNF- α) pathway are upregulated after ischemia, yet its role in peripheral ischemia remains unclear. We investigated the effect of TNF- α receptor 1 (TNFR-1) in acute limb ischemia of TNFR-1 knockout (TNFR-1^{-/-}) and wild type (WT, TNFR-1^{+/+}) mice. Laser Doppler scanning showed that although pre-ischemia blood flow levels were similar in these mice, the limb reperfusion after ischemia was significantly higher in TNFR-1^{-/-} mice 1–7 days after injury. Consistently, fewer TUNEL-positive cells, less DNA fragmentation, and a lower ischemic score were detected in the TNFR-1^{-/-} group when compared to WT controls. Western blot analysis revealed less expression of pro-apoptotic markers Bax and cleaved caspase-3 in TNFR-1^{-/-} mice 1 day after ischemia, supporting the hypothesis that the absence of TNFR-1 results in a reduction of apoptosis. The rate of post-ischemia amputation was 50% in WT mice versus 0% in TNFR-1^{-/-} mice. However, immunohistochemical co-staining of microvessel marker CD31 and cellular proliferation marker BrdU 21 days after ischemia showed an impaired angiogenic activity in the TNFR-1^{-/-} mice. These data were supported by Western blot analysis, which indicated a decreased expression of angiopoietin-1 (Ang-1) and its receptor Tie-2 in TNFR-1^{-/-} mice. Our results suggest that a deficiency in TNFR-1 prevents the activation of death-related proteins downstream to TNF- α and attenuates apoptosis in acute limb ischemia, but the lack of TNFR-1 signaling hinders the belated angiogenesis mediated by the Ang-1/Tie-2 pathway.

Keywords

angiogenesis; apoptosis; peripheral ischemia; TNFR-1

Introduction

Acute limb ischemia (ALI) occurs due to a sudden decrease in the blood flow to a limb, resulting in a threat to the viability of the extremity. Lower extremity peripheral arterial disease may progress to ALI or critical limb ischemia (chronic compromise in limb perfusion, which results in rest pain and tissue loss), both of which can lead to limb loss in the absence of timely treatment.¹ The two main causes of acute peripheral artery occlusion (APAO) are embolism and thrombosis; the latter usually occurs in cases of severe atherosclerotic stenoses.^{2,3} ALI may also occur in non-atherosclerotic patients when either an embolus or tumor cells become dislodged from a proximal source and is then trapped at the site of a peripheral arterial bifurcation.³ ALI caused by graft occlusion has been observed in patients subjected to lower extremity arterial bypass graft procedures. Recent epidemiological data estimated that ALI occurs in 14 out of every 100,000 and accounts for 10–16% of the vascular pathologies.⁴ Patients with peripheral arterial disease (PAD) are more susceptible to heart attacks or strokes. For example, patients with critical limb ischemia have an elevated risk of future myocardial infarction, stroke and vascular death that is threefold higher than in patients with intermittent claudication.⁵ In contrast to extensive investigations on brain and heart ischemia, APAO and ALI have received less attention and the cellular, molecular mechanisms are poorly understood. Effective pharmacological treatment for APAO or ALI has also been limited.

Tumor necrosis factor α (TNF- α) is a multifunctional cytokine that has been implicated as a mediator of diverse physiologic and pathophysiologic events, including cell survival, growth, differentiation, apoptosis, inflammation, and angiogenesis.^{6,7} The two high-affinity receptors of TNF- α are type 1 or TNF- α receptor 1 (TNFR-1, also named p55) and type 2 or TNF- α receptor 2 (TNFR-2, p75).⁸ TNFR-1 is the main receptor subtype in most cell types including the heart, and its complex and diverse downstream signaling system has been extensively studied. TNFR-2 and its role in TNF- α signaling remains less well characterized.⁹ After activation by TNF- α , TNFR-1 can couple with the adaptor protein Fas-associated death-domain protein (FADD), which can lead to the recruitment and activation of apoptotic proteases with subsequent progression to programmed cell death.^{10,11} However, TNFR-1 activation is not always associated with apoptosis; activation of downstream NF κ B-dependent and -independent transcriptional events can induce the expression of protective genes involved in cellular growth, survival and proliferation.^{10,11} Signals in the TNF- α /TNFR-1 pathway can be upregulated after human acute hindlimb ischemia.¹² It is unclear, however, whether this upregulation is a pathophysiological consequence or a compensatory response. For instance, TNF- α activity may either increase or inhibit angiogenesis^{13–16}; the mechanism by which the TNF- α /TNFR-1 pathway affects post-ischemic angiogenesis remains unclear. In the present study, we investigated the role of TNFR-1 in acute limb ischemia and post-ischemia angiogenesis in a hindlimb ischemia model in TNFR-1^{-/-} and wild-type (WT) mice.

Materials and methods

Animal model

All animal experiments and surgical procedures were approved by the University Animal Research Committee and met the NIH standard. C57BL/6 (Harlan, Indianapolis, IN, USA) and C57BL/6 TNFR-1^{-/-} mice (Jackson Laboratory, Bar Harbor, MN, USA) weighing 18–22 g (female, 8–10 weeks old) were anesthetized with intraperitoneal (i.p.) injections of 4% chloral hydrate. Under sterile conditions, the proximal portion of the left femoral artery and vein including the superficial and the deep branch as well as the distal portion of the saphenous artery were exposed and ligated with 7-0 silk sutures. All arterial branches between the ligation were ligated. The overlying skin was closed with 5-0 silk suture. A

sham procedure (dissection of vessels without ligation) was performed on the contralateral right leg.

Ischemic damage assessments

After surgery, a semiquantitative assessment of the ischemic damage to the limb was performed with a modification of an ischemic score (0 = no change, 1 = mild discoloration, 2 = moderate/severe discoloration, 3 = necrosis, 4 = amputation; $n = 8$ per group).¹⁷ Animals were euthanized at designated time points after surgery by cervical dislocation under deep anesthesia, and perfused with normal saline via the left ventricle. Equal amount of adductor muscles of both legs were harvested to prepare protein and DNA samples. Calf muscles were dissected and snap-frozen in O.C.T. solution (Sakura Finetek USA, Inc. Torrance, CA, USA) in dry ice. All the samples were transferred to a -80°C freezer and prepared for electrophoresis, Western blotting or immunohistochemical staining.

5-Bromo-2'-deoxyuridine (BrdU) administration

For mitotic labeling of cellular proliferation, BrdU (Sigma, St Louis, MO, USA) was i.p. injected to animals at a dosage of 50 mg/kg body weight once daily until the day of sacrifice.

Limb blood flow measurements

Before and at designated time points after surgery, animals were anesthetized with 4% chloride hydrate (i.p.) and placed in a water-warming incubator at 37°C for 30 min to minimize variations in temperature. Shortly following, blood flow in both limbs was measured using a laser Doppler blood perfusion scanner (PeriScan PIM 3 System, Perimed AB, Stockholm, Sweden). The average perfusions of the ischemic and non-ischemic limbs were calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, calculated perfusion was expressed as the ratio of ischemic to non-ischemic hindlimb perfusion ($n = 8$ per group).

DNA fragmentation

Equal amounts of adductor muscle from both groups ($n = 3$ per group) were homogenized in lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and incubated on ice for 20 min, then centrifuged for 20 min at 14,000 rpm to separate fragmented DNA from intact DNA (pellet). The supernatant was extracted twice with phenol/ chloroform/isoamyl (25:24:1) and centrifuged for 10–20 min to separate layers (10,000 rpm). The top layer was removed to a separate micro-centrifuge tube and precipitated with a half volume of 7.5 M $(\text{NH}_4)_2\text{SO}_4$ and two volumes of 100% ethanol and vortexed and incubated for 30 min on ice. After spinning at 14,000 rpm for 30 min in the micro-centrifuge tube, the supernatant was discarded. The pellets were then washed again with 1 ml 70% alcohol and dissolved in 30 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with 200 $\mu\text{g}/\text{ml}$ RNAase A and then incubated at 37°C for 1 h. The DNA samples were analyzed by electrophoresis in a 1.5% agarose gel containing 0.2 $\mu\text{g}/\text{ml}$ ethidium bromide and visualized under UV illumination.

TUNEL staining

A TUNEL staining kit (DeadEnd™ Fluorometric TUNEL system, Promega, Madison, WI, USA) was used to visualize cell death in 8- μm frozen sections of calf muscle ($n = 6$ per group). After fixing for 10 min in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA, USA) and pretreatment with a 2:1 ethanol/acetic acid mixture and 0.2% Triton X-100, the muscle sections were incubated in an equilibration buffer as instructed by the kit. The TdT enzyme and nucleotide mix were then added at proportions specified by the kit for 75 min at room temperature. The slides were washed with the provided $2 \times \text{SSC}$ wash buffer. To

identify nuclei, slides were incubated with Hoechst 33342 (1:20,000; Molecular Probes, Carlsbad, CA, USA) for 5 min before mounting with ProLong Antifade mounting medium (Molecular Probes) for observation and cell counting.

Immunofluorescence staining

Vessels were identified by immunostaining with an anti-CD31 (Cymbus Biotechnology, Eastleigh, UK) antibody. Fresh frozen sections of 8- μ m thick ($n = 6$ per group) were sliced using a cryostat vibratome (Ultapro 5000, St Louis, MO, USA). After the slides were completely air dried, slices were fixed twice in 100% methanol for 7 min, then air dried and rewet with phosphate buffered solution (PBS), and followed by treatment in 0.2% Triton X-100 for 5 min and washed in PBS three times between each step. Slides were blocked in 0.2% fish gelatin (Sigma) at room temperature for 1 h, and subsequently incubated with rat anti-CD31 (1:200) diluted in PBS overnight at 4°C. After rinsing with PBS, sections were then treated with Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To co-label vessels with BrdU, sections were post-fixed in 10% buffered formalin and subjected to our established BrdU staining procedure with mouse anti-BrdU (1:500; CHEMICON International, Temecula, CA, USA) and Alexa Fluor 488 anti-mouse IgG (1:200; Molecular Probes) as the primary and secondary antibodies.¹⁸ The slides were mounted and cover slipped for imaging and cell counting.

Cell and vessel counting

Six sections from each animal were analyzed for all staining. Sections used for comparing WT and TNFR-1^{-/-} mice were from similar levels of calf muscle. Twelve randomly chosen fields per section were analyzed at 400 $\times$ magnification under a microscope (BX51, Olympus, Japan). Stereological cell counting was used to avoid double counting; cell counting was performed on every tenth muscle section across the region of interest, with a distance of at least 80 μ m between each section. Counts are expressed as the average cell number per field $\pm$ standard error of mean.

Western blot analysis

Expression of caspase-3, Bax and angiogenic proteins was examined by Western blot. Adductor muscle samples were collected and proteins were extracted using the NE-PER Kit (Pierce Chemical Co., Rockford, IL, USA). The protein concentration was determined by the BCA protein assay kit (Pierce Chemical Co.). Proteins were separated on a 6–15% gradient SDS-PAGE gel and transferred to a PDVF membrane in a Hoefer Mini-Gel system (Amersham Biosciences, Piscataway, NJ, USA) and transferred overnight in the Hoefer Transfer Tank (Amersham Biosciences) to a PVDF membrane (BioRad, Hercules, CA, USA). Membranes were blocked with buffer (Tris-buffered saline containing 0.1% Tween-20 (TBS-T), pH 7.6, 7% milk) at room temperature for 2 h and incubated overnight at 4°C with rabbit polyclonal anti-cleaved caspase-3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Bax (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-Tie-2 (H-176) (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-angiopoietin-1 (Ang-1) (1:1000; CHEMICON International), rabbit polyclonal anti-angiopoietin-2 (Ang-2) (1:1000; CHEMICON International), or mouse anti-vascular endothelial growth factor (VEGF) (1:1000; Upstate, Charlottesville, VA, USA). Mouse anti- α -tubulin (1:1000; Santa Cruz Biotechnology) was blotted as protein loading control. The blots were washed in 0.5% TBS-T and incubated with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (Promega) for 2 h at room temperature. Finally, the membranes were washed with TBS-T followed by three washes with TBS. Experiments were repeated three times with samples from different animals. Signal was detected using the alkaline phosphatase substrate BCIP/NBT (Sigma).

Statistical analysis

Data were expressed as mean $\pm$ standard error. Student's two-tailed *t*-test was used for comparison of two experimental groups. Multiple comparisons were done using one-way ANOVA followed by a Tukey test for multiple pairwise examinations. Differences between the ratios were determined with the chi-squared test. Changes were considered as statistically significant if the *p*-value was less than 0.05.

Results

Effects of TNFR-1 on ischemia-induced local blood flow reduction and leg damage

Before surgery, laser Doppler blood flow measurement on WT and TNFR-1^{-/-} mice (*n* = 16 in each group) showed that the mean limb perfusion was similar in the left and right limbs of the same animals and between TNFR-1^{-/-} and WT mice. The blood flow to the left leg was markedly reduced by the left femoral artery occlusion to similar low levels in TNFR-1^{-/-} and WT mice. One day after ischemia, the mean limb perfusion in the left leg remained low in both groups, but it was significantly higher in TNFR-1^{-/-} mice compared to WT mice (Figure 1). The anti-ischemic effect of blood flow preservation in TNFR-1^{-/-} mice persisted for at least 7 days (Figure 1). The calculated ischemic score assessed 1, 3, and 7 days after ischemia was significantly lower in TNFR-1^{-/-} mice compared to WT mice (Figure 2). Although no mortality was seen in either group, 14 days later left leg amputation occurred in 50% of WT mice while no amputation was observed in TNFR-1^{-/-} mice.

Effects of TNFR-1 on ischemia-induced cell death

TUNEL staining was used to detect cell death in the calf muscle of the ischemic limb. One day after surgery, there were 11.1 ± 1.0 TUNEL-positive cells per field in the TNFR-1^{-/-} group, which was significantly lower than 14.1 ± 1.6 TUNEL-positive cells per field in the WT group (*p* < 0.05) (Figure 3). The ratio of TUNEL-positive cells to Hoechst 33342-labeled total cells was 0.44 ± 0.02 and 0.58 ± 0.07 in the TNFR-1^{-/-} and WT groups, respectively (*p* < 0.05) (Figure 3).

An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180–200 bp units, producing a characteristic ladder pattern on an agarose gel. No clear DNA laddering was observed in the sham-operated limbs of both groups (Figure 4A); however, typical apoptotic laddering appeared in the ischemic left limbs. The DNA fragmentation was much less in TNFR-1^{-/-} mice compared with the WT group (Figure 4A).

To further verify the apoptotic cell death in the ischemic limb, expression of Bax and activated cleaved caspase-3 levels was examined using Western blot 1 day after ischemia. The protein levels of both Bax and caspase-3 were attenuated in the absence of TNFR-1 (Figure 4B).

Effect of TNFR-1 on angiogenic response to ischemia

The mitotic marker BrdU was injected after ischemic surgery to track proliferating cells. Although the total capillary endothelial cell density was similar between TNFR-1^{-/-} and WT mice 21 days after ischemia, there were fewer BrdU-positive endothelial cells in the TNFR-1^{-/-} mice when compared to WT mice (4 ± 0 vs 7 ± 1 , *n* = 6, *p* = 0.012). This was also true for the ratios of newly formed endothelial cells to total endothelial cells ($24.4 \pm 0.7\%$ and $36.0 \pm 1.3\%$ in TNFR-1^{-/-} and WT mice, respectively; *n* = 6, *p* = 0.001) (Figure 5).

To further explore the mechanism underlying the impaired angiogenesis caused by the knockout of TNFR-1, Western blot was used to analyze the expression of major angiogenetic factors, including Ang-1, Ang-2, the angiopoietin receptor Tie-2, and VEGF. From 3 days after ischemia, the expression of Ang-1, Ang-2, Tie-2 and VEGF was upregulated in both groups. The expression of Ang-1, Ang-2, and Tie-2 peaked at 7 days after ischemia; the highest expression of VEGF was observed at 1–3 days after ischemia (Figures 6 and 7). Compared with WT controls, TNFR-1^{-/-} animals exhibited a significant down-regulation of Ang-1 and Tie-2 expression at post-ischemia day 7; Tie-2 expression was also down-regulated 3 days after ischemia (Figure 6).

Discussion

APAO is a major form of PAD that often affects the circulation of the lower extremities and its prevalence increases with age.¹⁹ As the disease progresses, eventually 25% of APAO patients will require revascularization and 5% will evolve into critical limb ischemia.^{20,21} APAO often occurs on the basis of atherosclerosis and can lead to limb necrosis and even loss of life.¹ In the present investigation, APAO led to auto amputation in 50% of WT mice while no amputation developed in TNFR-1^{-/-} mice. This observation suggests that the TNF- α /TNFR-1 pathway plays an essential role in ischemia-induced necrotic pathology and amputation, although this does not exclude other mechanisms in the pathogenesis of amputation. The amputation rate following APAO in our study appears higher than in previous reports. Only female rats were used in this investigation, which might account for the difference in amputation rate among studies, although further studies are necessary to confirm this speculation.

We demonstrated that a lack of TNFR-1 preserved the acute blood flow in the leg following ischemia. The mechanism of this enhanced circulation in TNFR-1^{-/-} mice is not known, but the improved perfusion should contribute significantly to the observed decrease in ischemic damage and the lack of amputation in these mice. TNFR-1 deletion resulted in less cell death, including reduced apoptosis. Our finding also identified lower expression of angiogenic factors and angiogenesis in TNFR-1^{-/-} mice in later stages after ischemia, suggesting that the TNF- α /TNFR-1 pathway plays dual roles in peripheral ischemia. During the acute phase of our study, the lack of TNFR-1 signaling had an anti-apoptotic effect and preserved post-ischemia reperfusion, while at later stages, the absence of TNFR-1 signaling resulted in an absence of angiogenesis. We can then infer that TNFR-1 signaling actually has a delayed pro-angiogenic effect, which seems contradictory to a recent report that soluble TNFR-1 (sTNFR-1), acting as an antagonist to TNF- α , significantly increased capillary density 21 days after limb ischemia of rats.²² Several obvious differences between our investigation and the reported investigations can be identified: (1) mice and rats were used in these two studies, respectively; (2) the experiments in rats observed an acute effect after one post-ischemic injection of sTNFR-1, while our study was performed on mice of TNFR-1-knockout mice; (3) the degree of TNFR-1 deletion in TNFR-1^{-/-} mice and sTNFR-1 experiments were different. TNFR-1 expression was suppressed by sTNFR-1 in rats but TNFR-1^{-/-} mice had undetectable TNFR-1. It is possible that different levels of TNFR-1 and the expression background of this gene determined the effect on angiogenesis and thus capillary density.

In a wound healing study, skin excisions were prepared in BALB/c (WT) and TNFR-1-deficient mice.²³ At 1–6 days after injury, TNFR-1-deficient mice exhibited increased angiogenesis, collagen content, and re-epithelialization with the increased gene expression of TGF- β 1, CTGF, VEGF, Flt-1, and Flk-1 at the wound sites, resulting in accelerated wound healing. In contrast, leukocyte infiltration, mRNA expression of adhesion molecules, and cytokines were significantly reduced in these mice. The authors suggest that TNFR-1-

mediated signals have some role in promoting leukocyte infiltration at the wound site and negatively affect wound healing, probably by reducing angiogenesis and collagen accumulation.²² As shown in our investigation, the absence of TNFR-1 resulted in a decrease in angiogenesis after limb ischemia. The difference may be due to the different insults tested. In the skin excision model, cell death and tissue repair activity are less extensive and compensatory mechanisms may be able to stimulate angiogenesis.

Despite the specific role of TNFR-1 in angiogenesis and the significant reperfusion difference in early post-ischemia days, the post-ischemia reperfusion at 14–21 days after limb ischemia gradually recovered in both TNFR-1^{-/-} and WT mice. No more reperfusion difference was observed during this time. We view the early enhanced limb perfusion in TNFR-1^{-/-} mice as a result of reduced damage to vasculature in the ischemic limb; on the other hand, the presence of TNFR-1 in WT mice stimulated the delayed response of angiogenesis that improved limb perfusion at the later stage of the experiments. Whether the minimized angiogenesis in the absence of TNFR-1 would lead to a long-term blood flow/functional deficiency and an increased incidence of critical limb ischemia remain to be determined.

TNF- α binds to the cell membrane as a 26 kDa precursor peptide after it is synthesized, and is then cleaved and released by TNF- α convertase as a 17 kDa soluble monomer and finally forms a biologically active homotrimer.²⁴ TNF- α elevation plays an injurious role in congestive heart failure, acute myocardial infarction, and cerebral ischemia.^{25,26} For example, the mortality of acute myocardial infarction in mice was significantly reduced by knocking out the TNF- α gene.²⁷ The underlying mechanisms included attenuated infiltration of inflammatory cells, decreased expression of inflammatory cytokines and suppressed activity of matrix metalloproteinase 9 and 2.²⁷ Most animal studies in acute myocardial infarction or cardiomyopathy suggest that the deleterious effect of TNF- α is likely mediated by TNFR-1.^{28,29} TNFR-1 is ubiquitously expressed on mammalian cells, while TNFR-2 expression is more restricted and is most commonly found in hematopoietic cells, in cells of the immune system, and in endothelial cells.³⁰ Despite the conserved extracellular domains, the cytoplasmic domains of the two receptors lack homology, suggesting activation of different downstream transduction pathways. In the acutely ischemic muscles of PAD patients, there is a drastic upregulation of members of the TNF- α cascade, including TNF- α , TNF- α convertase, TNFR-1, TNFR-2, and the intracellular signaling mediators FADD,¹² but the significance of this upregulation remains unclear. In our investigation, TNFR-1 knockout reduced apoptosis, which is consistent with the previous recognition that TNF- α and TNFR activation can initiate apoptosis.³¹ Activation of Tie-2 by Ang-1 results in the stimulation of endothelial cell survival, sprouting, migration and tubule formation, stabilization and reduction of the permeability of newly formed vessels.^{32–35} Ang-1 can also synergize with VEGF during sprouting angiogenesis *in vivo*.³⁶ TNF- α stimulation of Ang-1 expression occurred via the NF- κ B signal transduction pathway which is downstream of TNFR-1 activation.³⁷ We observed that TNFR-1 knockout showed a belated effect of reducing Ang-1 and Tie-2 expression, suggesting that TNFR-1 activation is a pro-angiogenesis mechanism after limb ischemia.

There are several approaches that may antagonize TNF- α signaling, including anti-TNF antibodies, sTNFR or sTNFR fusion proteins, blockage of TNF biosynthesis with glucocorticoids, pentoxifylline, amrinone, and cyclosporine A, and inhibition of downstream molecules.²⁵ Infliximab and etanercept are two TNF- α blockers approved by the FDA which are used clinically to treat Crohn's disease.³⁸ Some clinical studies found that short-term blockade of TNF- α was beneficial to patients with congestive heart failure but further large-scale and long-term trials failed to prove this effect.³⁹ Considering the dual effects observed in our investigation, it might be predicted that acute and chronic blockades of TNF- α /

TNFR-1 pathway have different results due to its distinct influence on post-ischemia blood flow, cell survival, and angiogenesis.

TNF- α and TNFR-1 were upregulated in human limbs amputated due to acute ischemia.¹² Thus, the role of TNF- α and its receptors in post-ischemic reperfusion and tissue repair is clinically relevant. While the present investigation explored the issue using TNFR-1 knockout mice, it would also be of interest to delineate the role of TNFR-2 in future investigations.

Patients presenting with ALI continue to have a particularly severe short-term outlook both in terms of loss of the leg and mortality, with 30-day amputation rates of between 10% and 30% and a mortality rate of around 15%.² A patient with an embolic cause for an ischemic leg is at a higher risk of death because of the associated underlying cardiac disease, whereas patients with a thrombotic cause are more likely to lose a limb.² The fact that overall mortality rates after intervention for acute ischemia have not improved dramatically over the past 30 years no doubt reflects the severity of the underlying diseases in these high-risk patients. Our investigation suggests that timely and temporary blockade of TNFR-1 may be explored as a therapeutic strategy for ALI as a pharmacological alternative to surgery or be used in combination with other non-operative managements such as anticoagulation and fibrinolytic drugs.

In conclusion, we demonstrated here that knockout of TNFR-1 had an acute effect on post-ischemia circulation, prevented the activation of death-related proteins downstream to TNF- α , and attenuated cell death in acute limb ischemia, yet had a negative effect on post-ischemia angiogenesis due to depressed Ang-1 and Tie-2 signaling pathway. In any event, the absence of post-ischemia amputation in TNFR-1^{-/-} mice demonstrates a powerful protective action of blocking the TNF- α /TNFR-1 pathway in peripheral limb ischemia. It is possible that targeting TNFR-1 may also have an impact on PAD during chronic situations such as critical limb ischemia, although the exact effect and mechanism remain to be tested.

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Figure 1.

Perfusion changes before and after hindlimb ischemia. Local blood flow was evaluated using a laser Doppler perfusion scanner (The PeriScan PIM 3 System). (A) Representative perfusion images of plantar area. Colors displayed in scale correspond to intervals of perfusion value from 0 (dark) to 10 (red). Red is the highest velocity; green, intermediate; blue, low; and dark, the lowest velocity. (B) The ratio of ischemic to non-ischemic limbs of WT mice (open bars) or TNFR-1^{-/-} mice (filled bars). Data at 14 and 21 days after ischemia were from animals without amputation. $n = 8$ in each group. * $p < 0.05$ compared with WT. The color version of this figure can be viewed at <http://vmj.sagepub.com>

Figure 2.

Ischemic damage in WT and TNFR-1^{-/-} mice. Ischemia-induced leg damage was assessed by the calculated ischemic scores at post-ischemia days 1, 3 and 7. The ischemic damage score was significantly higher in WT mice (open bars) than that in TNFR-1^{-/-} group (filled bars) up to 7 days post-ischemia. * $p < 0.05$ compared with WT mice.

Figure 3. Ischemia-induced cell death. Cell death was identified by TUNEL staining. (**A** and **B**) TUNEL-positive cells in the left calf muscle sections of WT and TNFR-1^{-/-} mice 1 day after ischemia. Blue shows nuclei staining by Hoechst 33342; green depicts TUNEL-positive cells. Bar = 50 μ m. (**C**) The number of TUNEL-positive cells per field. (**D**) The percentage of TUNEL-positive cells versus the total Hoechst 33342-positive cells. Fewer TUNEL-positive cells were counted in the TNFR-1^{-/-} group (filled bars). * $p < 0.05$ compared with WT. The color version of this figure can be viewed at <http://vmj.sagepub.com>

Figure 4.

Apoptosis assessments in ischemic calf muscle. Electrophoresis and Western blot were applied to detect DNA fragmentation, Bax expression and caspase-3 activation. (A) WT L, WT R, TNFR-1^{-/-} L and TNFR-1^{-/-} R represent DNA samples from the left hindlimb of the WT group, right hindlimb of the WT group, left hindlimb of the TNFR-1^{-/-} group and right hindlimb of the TNFR-1^{-/-} group, respectively, 2 days after surgery. (B) Western blot analysis of Bax and cleaved caspase-3 with the samples from adductor muscle 1 day after ischemia. Representative of three assays.

Figure 5.

Post-ischemia endothelial cell proliferation and angiogenesis. Effect of TNFR-1 signaling on post-ischemia angiogenesis was assessed by capillary endothelial cell proliferation. (A and B) Co-staining with the endothelial cell marker CD31 (red) and proliferation marker BrdU (green) appears in orange in the section of ischemic calf muscle from WT and TNFR-1^{-/-} mice 21 days after ischemia. Bar = 50 μ m. (C) Bar graph shows the number of CD31 positive cells (filled bar) and CD31-BrdU co-labeled cells (open bars) per microscopic field. (D) The percentage of BrdU co-labeled CD31-positive cells to total CD31-positive cells. Fewer BrdU labeled endothelial cells were observed in TNFR-1^{-/-} mice than in the WT group. * $p < 0.05$ compared with WT controls. The color version of this figure can be viewed at <http://vmj.sagepub.com>

Figure 6.

Expression of angiogenic factors Ang-1 and Tie-2 in ischemic calf muscle. Western blotting analysis was used to detect the protein level of Ang-1 and Tie-2 in muscle samples from non-ischemic control animals and limb-ischemic animals at 1, 3, 7, 14, and 21 days after ischemia. (A) A representative result of three repeat assessments. (B and C) Summarized results of protein levels of Ang-1 (B) and Tie-2 expression (C) in the ischemic muscle. Open bars represent the WT group and filled bars the TNFR-1^{-/-} group. Values were corrected according to α -tubulin levels as loading controls. * $p < 0.05$ compared with WT mice.

Figure 7. Expression of angiogenic factors VEGF and Ang-2 in ischemic calf muscle. Western blotting analysis detected the protein level of VEGF and Ang-2 in calf muscle samples from non-ischemic control mice and limb-ischemic mice at 1, 3, 7, 14, and 21 days after ischemia. (A) Representative gel of three experiments. (B and C) Protein levels of VEGF (B) and Ang-2 (C) are summarized in the bar graph (open bars = WT group; filled bars = TNFR-1^{-/-} group).