Increasing Survival of Ischemic Tissue by Targeting CD47

Jeff S. Isenberg, Martin J. Romeo, Mones Abu-Asab, Maria Tsokos, Anna Oldenborg, Loretta Pappan, David A. Wink, William A. Frazier, David D. Roberts

Abstract—Thrombospondin-1 (TSP1) limits the angiogenic and vasodilator activities of NO. This activity of TSP1 can be beneficial in some disease states, but endogenous TSP1 limits recovery of tissue perfusion following fixed ischemic injury in dorsal skin flaps in mice. Using mice lacking the TSP1 receptors CD36 or CD47, we now show that CD47 is the necessary receptor for limiting NO-mediated vascular smooth muscle relaxation and tissue survival following ischemic injury in skin flaps and hindlimbs. We further show that blocking CD47 or TSP1 using monoclonal antibodies and decreasing CD47 expression using an antisense morpholino oligonucleotide are effective therapeutic approaches to dramatically increase survival of soft tissue subjected to fixed ischemia. These treatments facilitate rapid vascular remodeling to restore tissue perfusion and increase skin and muscle viability. Thus, limiting CD47-dependent antagonism of NO-mediated vasodilation and vascular remodeling is a promising therapeutic modality to preserve tissues subject to ischemic stress. (Circ Res. 2007;100:712-720.)

Key Words: nitric oxide  ■  thrombospondin-1  ■  ischemic tissue survival  ■  CD47  ■  therapeutics

Tissue viability requires continuous perfusion, which in turn depends on vascular tone, sufficient intravascular volume, and adequate blood oxygenation. The contractile status of arterial smooth muscle is the major determinant of vascular tone, with venous tone playing a lesser role. Underperfusion of soft tissues is the leading cause of tissue necrosis and secondary delayed wound healing in surgical patients. The complications incurred can be substantial and life threatening. Complications of inadequate tissue perfusion are multiplied in the elderly and patients with hypertension and diabetes because of the general vasculopathies associated with these disease processes.

Current therapies to improve vascular perfusion combine surgical vessel manipulation/bypass with vasodilators that relax vascular smooth muscle cells (VSMCs). The bioactive gas NO is a potent vasodilator that activates soluble guanylate cyclase. The increased cGMP activates cGMP-dependent protein kinases and thereby decreases VSMC sensitivity to intracellular Ca2+, leading to relaxation of contractile proteins.

We recently reported that NO/cGMP signaling in VSMCs and endothelial cells is potently inhibited by the secreted protein thrombospondin-1 (TSP1). We further showed that endogenous TSP1 limits the ability of NO to increase skeletal muscle perfusion and blood oxygen levels in vivo. Following surgically induced acute ischemia in random dorsal skin flaps, endogenous TSP1 also limits tissue survival and recovery of tissue oxygenation. Ischemic tissue survival could be improved by increasing NO levels using isosorbide dinitrate, but the degree of tissue necrosis in treated wild-type mice remained higher than in TSP1-null mice, which achieved essentially complete flap survival following this treatment.

To further improve survival of ischemic stress in wild-type mice, we explored therapeutic approaches that target the TSP1 receptors mediating its antagonism of NO signaling. Although ligation of the antiangiogenic TSP1 receptor CD36 by antibodies or TSP1-derived peptides is sufficient to inhibit NO/cGMP signaling in endothelial and VSMCs, we recently found that CD36 is not necessary for this activity of TSP1. Instead, engaging the TSP1 and SHP-1 receptor (signal regulatory protein-α/SHPS1) is necessary and sufficient for TSP1 to inhibit NO-driven responses in both endothelial and VSMCs.

We now show that CD47 is also the critical TSP1 receptor that regulates vascular responses to NO in skin and hindlimb ischemia models. We demonstrate that CD47-null but not CD36-null mice are protected from necrosis caused by acute ischemia. We further show that suppressing CD47 expression by local application of an antisense CD47 morpholino oligonucleotide or locally applying CD47 or TSP1 blocking antibodies dramatically reduces tissue loss resulting from fixed ischemia.

Materials and Methods

Animals
C57BL/6 wild-type, TSP1-null, CD36-null, and CD47-null mice were allowed ad libitum access to water and standard chow and...
maintained in a pathogen-free environment in accordance with guidelines established by Animal Care and Use Committees of the National Cancer Institute and Washington University.

**Cells and Reagents**

Human aortic VSMCs were obtained from Clonetics (Walkersville, Md) and cultured in standard growth medium according to the recommendations of the manufacturer. Wild-type, TSP1-null, and CD47-null aortic VSMCs were prepared as described. Rat anti-murine CD47 monoclonal antibody, Ab 301, was prepared as described. cGMP was measured using an immunoassay obtained from Amersham BioSciences (Piscatway, NJ). TSP1 was prepared from human platelets obtained from the NIH blood bank as previously described. Diethylyamine NONOate (DEA/NO) and diethyltriamine NONOate (DETA/NO) were provided by Dr Larry Keefer (National Cancer Institute, Frederick, Md). Type I collagen (Vitrogen) was from Inamed (Fremont, Calif). The TSP1 monoclonal antibody clone A6.1 was purchased from Neomarkers/Laboratory/gen) was from Inamed (Fremont, Calif).

**Vascular Index Determination**

Random myocutaneous flap wound beds and/or ischemic hindlimb vastus medialis muscle was assessed at indicated time points (72 hours or 7 days postoperation) for visible alterations in vascularity under ×5 magnification. An arbitrary, although strictly applied, definition of countable vessels was used to highlight both individual vessels and vascular ramifications. In any given vascular plexus visible by ×5 magnification, a vessel was defined as that segment traversing two branches. Visible vessels without ramifications and branches were counted once. Treatment status and genetic background of tissue images was not known by the reviewer.

**Mitochondrial Viability Assay**

Mitochondrial viability of hindlimb muscle biopsies was assessed by the reduction of a tetrazolium salt to water insoluble formazan through mitochondrial oxidation as described. Tissue biopsy samples were weighed and incubated in 3 mL of PBS supplemented 1:10 with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium stock (MTT) (Promega, Madison, Wis) for 3 hours in the dark at 37°C, washed with distilled water, and blotted dry. The formazan salt was extracted in 3 mL of 2-propanol for 6 hours in the dark at 37°C. Absorbance for 200-μL aliquots was determined at 425 nm on a microplate reader. Muscle samples were dried at 90°C and weighed again. Results were expressed as absorbance normalized to dry tissue weight.

**Laser Doppler Imaging of Hindlimb Perfusion**

Laser Doppler blood-flow imaging was used to assess the extent of hindlimb perfusion following arterial ligation using a Doppler imager (Moor Instruments Inc, Wilmington, Del), which measures the flux of blood (blood×area ×time ). Body temperature was maintained at 37°C. Mice were scanned immediately before and after surgery and 7 days after surgery. Equal areas of the control and ischemic limbs from the same anatomical region of the limbs were compared. To control for ambient light and temperature, calculated perfusion was expressed as the flux ratio between the ischemic and nonischemic limbs.

**Histology**

Sections of excised skin wounds were cut parallel to the long axis of each flap to include the entire length of the tissue sample, fixed in 10% buffered formaldehyde, paraffin embedded, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. Grading of inflammatory cell infiltrate and necrosis was also performed for each section. Immunohistology of morpholino and control tissue samples was performed as previously described with a primary monoclonal antibody to CD47 (clone B6H12, Lab Vision Corp., Fremont, Calif).

**Statistics**

Statistical significance was calculated with the Student’s t test or 1-way or 2-way ANOVA as appropriate, with a probability value <0.05 taken as significant using a standard software package (Origin 7, Origin Labs, Northampton, Mass). All in vitro experiments were repeated a minimum of 3 times.

**Results**

**CD47-Null Mice Demonstrate Increased Soft Tissue Survival of Fixed Ischemia**

Dorsally located random myocutaneous McFarlane flaps in wild-type mice demonstrated significant necrosis at 7 days
In contrast, random flaps in CD47-null mice demonstrated minimal to no tissue necrosis in response to the same ischemic challenge. The response of CD47-null animals to ischemia resembled that of TSP1-null mice.20 In contrast, CD36-null flaps demonstrated areas of necrosis similar to, and in some instances greater than, wild-type flaps.

CD47 Is Necessary for Exogenous TSP1 to Limit NO-Stimulated VSMC Relaxation

FCS-driven VSMC contraction of type I collagen gels was robust in both wild-type and CD47-null VSMCs, and exogenous NO comparably delayed contraction in both (Figure 1C). As previously reported,20 relaxation of precontracted VSMCs by NO was completely inhibited by addition of exogenous TSP1 in wild-type cells, but this response to TSP1 was absent in CD47-null VSMCs (Figure 1C). Similar results were found for VSMCs precontracted using sphingosine 1-phosphate (Figure 1D). Therefore, CD47 is the TSP1 receptor that mediates its inhibition of VSMC relaxation by NO.

Antibody Engagement of CD47 or Sequestration of TSP1 Increases Ischemic Tissue Survival Independent of CD36

Treatment with the function blocking anti-murine CD47 antibody 301 (10 μL of 4 mg/mL stock in 90 μL of PBS) but not with a control IgG2α resulted in essentially complete tissue survival in wild-type myocutaneous flaps (Figure 2A and 2B). A similar increase in survival was observed using CD36-null mice, further indicating that this response is independent of CD36 (Figure 2B). We also tested an anti-human TSP1 antibody known to recognize an epitope that is conserved in murine TSP1 (clone A6.1,34) as a strategy to sequester endogenous TSP1. Tissue survival was enhanced in wild-type flaps by treatment with this TSP1 antibody (Figure 2C; P<0.05).

Antisense Knockdown of CD47 Prevents TSP1 Inhibition of NO-Stimulated VSMC Relaxation

Because blocking the CD47 receptor preserved ischemic tissue, we examined the effectiveness of temporarily reducing CD47 expression to enhance tissue survival. To first validate this approach in vitro, human aortic VSMCs were pretreated with a CD47 morpholino oligonucleotide or a control morpholino. Western blotting showed a dose-dependent decrease in CD47 protein following treatment with the CD47 morpholino but not in cells treated with the control morpholino (Figure 3A). Based on these results, VSMCs were pretreated with 10 μmol/L CD47 morpholino and seeded into 3D collagen gels, and the gel contraction measured (Figure 3B). As in untreated cells, FCS-induced gel contraction of CD47 morpholino-treated VSMCs was relaxed by exogenous NO, but TSP1 was not able to block this relaxation (Figure 3B). Control morpholino-treated VSMCs remained sensitive to TSP1 inhibition of NO-stimulated relaxation (results not shown).

CD47 Knockdown Prevents TSP1 Inhibition of NO-Driven cGMP Accumulation

We further confirmed suppression of CD47 signaling by assessing cGMP levels in the morpholino-treated cells. Human aortic VSMCs were pretreated with the CD47 or control morpholinos, and cGMP accumulation in response to NO±TSP1 was determined. Knockdown of CD47 using the CD47 morpholino rendered the human aortic VSMCs essentially blind to the inhibitory effects of TSP1 on NO-driven cGMP accumulation (Figure 3C). Conversely, control morpholino-treated cells retained sensitivity to inhibition of...
an NO-stimulated cGMP flux by TSP1. Control experiments with untreated VSMCs demonstrated complete inhibition of an NO-driven cGMP flux by exogenous TSP1 (data not shown) and decreased basal and stimulated levels in the presence of CD47 as previously reported.23

CD47 Knockdown Increases Tissue Survival of Ischemic Myocutaneous Flaps

The CD47 morpholino was designed to complement a sequence in the CD47 mRNA that is conserved between the murine and human transcripts. Wild-type C57BL6 mice undergoing mobilization of dorsal McFarlane flaps received either the CD47 morpholino (10 μmol/L in 250 μL of PBS with 125-μL volumes injected in the flap and wound bed, respectively) or the control morpholino. To validate CD47 knockdown in vivo, immunohistochemical staining of soft tissue flaps treated with the CD47 morpholino was performed and demonstrated decreased protein staining compared with untreated controls (Figure 3D). Remarkably, wild-type flaps treated with the CD47 morpholino showed essentially 100% survival (Figure 3D). In contrast, treatment of flaps with the control morpholino resulted in necrosis comparable to that with untreated wild-type flaps. Treatment with vehicle alone or vehicle plus delivery agent (Endoporter, GeneTools) did not alter tissue survival (data not shown). Quantification of wound bed vascularity showed substantial increases in visible blood vessels following CD47 morpholino treatment relative to treatment with the control morpholino (Figure 3E) or untreated wound beds (data not shown).

CD47-Null Flaps Demonstrate Increased Numbers of Patent Blood Vessels Compared With Wild-Type Flaps

Histologic inspection of sections from CD36-null and CD47-null flaps harvested 72 hours postoperation found evidence of early ischemic necrosis in CD36-null flaps with epithelial loss, hair follicle drop out, and thinning and coagulation of the dermis (Figure 3F and 3G). CD47-null flaps demonstrated normal cutaneous architecture, with only a modest inflammatory cell infiltration at the inferior aspects of the flap units.

Endogenous TSP1 Limits Tissue Survival Under Fixed Hindlimb Ischemia

Although the random dorsal flap is a useful model of ischemic injury, the limited thickness of murine myocutaneous tissue may facilitate diffusion of oxygen from the wound bed into the ischemic flap. Therefore, large composite tissue units might not obtain similar tissue protection in the absence...
of TSP1 or CD47. To better model a complex 3D ischemic injury, we examined fixed ischemic insult secondary to proximal ligation of the external iliac and common femoral arteries in hindlimbs. Tissue survival was dramatically increased in the absence of TSP1 (Figure 4A). The clinical findings in ischemic hindlimbs correlated with the increased muscle perfusion of TSP1-null limbs induced by an NO challenge.

The clinical assessments of hindlimb survival were confirmed by quantifying mitochondrial viability in limb muscle using MTT reduction and normalizing to mitochondrial function in the untreated contralateral muscle (Figure 4B). Mitochondrial function decreased to $23.5\pm5\%$ of control in wild-type ischemic hindlimbs but remained at $58.5\pm6\%$ of control in TSP1-null hindlimbs ($P<0.05$). Increased muscle viability was consistent with increased vascular remodeling in the treated TSP1-null hindlimbs (Figure 4C). These findings correlated with histologic findings of muscle cell necrosis and inflammatory cell invasion of wild-type hindlimbs as compared with TSP1-null limbs (Figure 4D).

Consistent with the flap ischemia model, hindlimb ischemia was well tolerated in CD47-null mice as compared with wild-type or CD36 nulls (Figure 5A). Both CD47-null and TSP1-null animals showed minimal to no clinical evidence of tissue necrosis. In contrast, wild-type and CD36-null hindlimbs demonstrated necrosis of skin, muscle, and acral parts, including the toes, in response to vessel ligation. Similar to the TSP1-null mice, muscle mitochondrial viability was significantly increased under a fixed ischemic insult in CD47-null limbs (Figure 5B) compared with wild-type or CD36-null animals. Both CD47 and TSP1-null animals exhibited significantly increased vascular remodeling following vascular ligation as compared with wild-type and CD36-null animals (Figure 5C).

Most surprisingly, treatment of ischemic wild-type hindlimbs with a CD47 morpholino recapitulated the TSP1/...
CD47-null phenotype with essentially no tissue necrosis and increased muscle mitochondrial viability (Figure 5D). A control morpholino did not protect ischemic wild-type hindlimbs from tissue necrosis.

Laser Doppler analysis of ischemic hindlimb perfusion was performed in wild-type and transgenic mice. All animals demonstrated equal and profound decreases in hindlimb perfusion in the immediate postoperative interval. However, CD47-null animals demonstrated statistically greater levels of perfusion compared with wild-type animals by the end of the first postoperative week (Figure 6). Also, in keeping with clinical findings, TSP1-null animals demonstrated increased perfusion compared with CD36 null (data not shown).

**Figure 5.** Tissue survival in ischemic hindlimbs is limited by CD47 but not by CD36. A, CD47-null and CD36-null mice underwent ligation of the proximal arterial inflow of the left hindlimb as described. At postoperative day 7, tissue necrosis scores were determined, animals were euthanized, and tissue was harvested for analysis of mitochondrial viability and histology. B, Mitochondrial viability of muscle from the tibialis anterior was determined via conversion of MTT reagent to the formazan salt and quantified. C, Using ×5 magnification, visible vessels on the surface of the vastus medialis were quantified using ×5 magnification. Results represent the mean ± SD of 16 animals (4 sex- and age-matched pairs of CD47-null and CD36-null animals). *P<0.05. D, Wild-type animals underwent vascular ligation as described in Materials and Methods and received either a CD47 or control morpholino (125 μL of 10 μmol/L morpholino in PBS to thigh musculature). *P<0.05 vs perfused (B and D).

**Discussion**

We recently reported that NO-stimulated VSMC contraction in vitro and acute vasodilation in vivo are effectively blocked by TSP1. VSMCs express 2 TSP1 receptors that can mediate its inhibition of NO signaling: CD36 and CD47. CD36 is necessary for inhibition of angiogenesis by TSP1 in the cornea, but inhibition of NO signaling by TSP1 in vascular cells requires CD47, not CD36. The cell surface glycoprotein CD47 is expressed by all vascular cells and has been ascribed roles in regulating integrin–matrix protein interactions, self-recognition, and immunity. Using CD47-null cells and morpholino knockdown of CD47, we now show that CD47 is required for TSP1 to block NO-stimulated relaxation of VSMCs. We further show that this pathway limits the ability of soft tissue to survive ischemic injury. Under conditions of soft tissue ischemia, CD47-null mice resemble TSP1-null mice in lacking significant tissue necrosis. Conversely, CD36-null flaps and ischemic hindlimbs,
which express TSP1 and CD47, demonstrate tissue necrosis comparable with that of wild-type mice. Thus, CD47 is the dominant receptor through which TSP1 limits survival of ischemic soft tissues, whereas CD36 does not play a significant role in this pathology.

The increased tissue survival in TSP1-null and CD47-null flaps correlated with increased blood vessel density in tissue sections as compared with wild-type and CD36-null sections. Angiogenesis may contribute to revascularization under acute ischemic challenge. However, angiogenesis may not be rapid enough to account for the increased random flap and hindlimb survival of tissue ischemia in CD47 nulls or induced by CD47 blockade or suppression. Differences between wild-type and CD47 mice could be detected based on clinical markers of viability and perfusion in ischemic flaps and hindlimbs immediately following surgery. Electron paramagnetic resonance tissue oxygen measurement in ischemic flaps of CD47-null mice (J.S. Isenberg, unpublished results, 2006) showed similar recovery rapid recovery as previous reported for TSP1-null mice. Combined with the ability of endogenous TSP1 to acutely limit an increase in muscle oxygenation stimulated by NO, the acute recovery of blood flow we observe by preventing TSP1/CD47 signaling may result from blood vessel remodeling in addition to angiogenesis. The increased number of visible vessels in treated flap wound beds and on the surface of the vastus medialis following proximal femoral ligation also suggests rapid adaptation by existing vascular networks. Such changes were found within 24 hours postoperatively. Angiogenic neovascularization and arteriogenesis, as opposed to vascular dilation, require time intervals from days to weeks. Such dramatic and immediate effects in tissue vascularity must arise through a process of dilation and remodeling of existing vascular networks, permitting increased flow across a preexisting vascular plexus rather than formation of new vessels. The dramatic increase in perfusion in response to fixed ischemia in the corresponding null mice reflects the central role TSP1 and CD47 play in controlling these vascular responses.

Ligation of the external iliac and common femoral arteries supplying the hindlimb creates fixed ischemia in a complex composite structure that includes cutaneous, muscle, and osseous tissues and has greater metabolic needs than cutaneous flaps. TSP1 and CD47-null mice were protected from significant tissue loss and necrosis of acral parts relative to wild-type and CD36-null animals, confirming that eliminating TSP1/CD47 signaling is sufficient to allow survival of fixed ischemia in a complex composite tissue as well as in skin flaps. Differences in tissue survival correlated with mitochondrial viability in biopsies taken from the tibialis anterior muscle. This muscle unit, by virtue of its distal location in the lower limb vascular runoff, provided a reliable barometer of limb perfusion. Analysis of limb perfusion with laser Doppler imaging further confirmed the clinical and muscle mitochondrial findings. Despite exhibiting comparable initial losses of limb perfusion following vascular ligation, recovery of perfusion was always greater in the TSP1 and CD47-null limbs compared with wild-type and CD36-null mice.

These data from transgenic mice suggested that CD47 could be a useful therapeutic target for improving survival of ischemic tissues. We confirmed this by demonstrating that treating wild-type mice with a CD47 blocking antibody or knockdown of CD47 using a morpholino dramatically increases tissue survival from ischemia. These results show that temporary suppression of TSP1/CD47 signaling is sufficient to prevent loss of ischemic tissue and suggest that similar approaches may be effective for treating patients subject to acute ischemic stress attributable to injury or surgical reconstruction. The CD47 antibody that we used is specific for murine CD47, but a number of anti-human CD47 antibodies exist that could be evaluated for preventing necrosis of ischemic tissues.
ischemic tissues. The CD47 morpholino hybridizes to a sequence that is conserved between human and murine CD47, and we have verified that it suppresses CD47 expression in human VSMCs and murine tissue. Pending additional pre-clinical testing, this morpholino may be an effective therapeutic in humans.

The strategies we present to block TSP1/CD47 signaling may have broad clinical relevance. The responses in cutaneous flaps show that CD47 morpholino and antibody treatments can effectively prevent tissue loss caused by ischemia in skin. Despite the potential difficulties in delivering agents to composite tissues, treatment with the CD47 morpholino in the hindlimb ischemia model resulted in a similar degree of tissue protection. Treatment resulted in decreased limb necrosis manifest clinically as protection from necrosis of toes, skin and muscle ulceration, and limb shrinkage. Muscle mitochondrial viability was significantly improved in treated limbs relative to the control limbs.

These results establish an important pathophysiological role for TSP1/CD47 signaling to limit NO-driven responses in VSMCs and tissue perfusion. In the absence of CD47, tissue subjected to a fixed ischemic insult can approach 100% survival, suggesting that CD47 is the dominant TSP1 receptor for mediating its effects on survival of ischemic stress. The absence of CD47 relieves vascular beds from the inhibitory actions of TSP1 on NO signaling. Therapeutic intervention targeting TSP1 or CD47 can improve tissue survival with potential to circumvent the significant morbidity and mortality caused by ischemic tissue necrosis. Therapeutic modalities based on modulation of TSP1 and CD47 could have profound clinical utility for individuals through increasing tissue perfusion and eliminating ischemia as a limiting factor in wound healing and tissue reconstruction.

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Disclosures
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