Complications from peripheral vascular disease, including coronary artery disease and myocardial infarction, stroke, and ischemic vascular disease affect some 80% of people over the age of 65. The elderly have significant alterations in vascular anatomy including a loss of vascular networks1 and alterations in vascular response to injury.2 Age is a recognized risk factor for complications after surgery including delayed and incomplete wound healing and tissue loss and accounts for significant morbidity and mortality in this group.3–6 Animal studies have confirmed the impact of aging on wound healing.7–9

Tissue perfusion is regulated through the control of blood vessel diameter, which itself is controlled by the contractile state of vascular smooth muscle cells (VSMCs). Nitric oxide (NO) is a primary and ubiquitous dilator of blood vessels.10 NO is constitutively produced in blood vessels by endothelial nitric oxide synthase (eNOS). NO activates soluble guanylate cyclase (sGC) leading to cGMP production and vasodilation. In aged vascular cells,11 animals, and people, both eNOS expression and NO production12 are decreased.

We recently reported that thrombospondin-1 (TSP1) blocks NO-driven VSMC relaxation in a CD47-dependent manner.13–15 NO-driven alterations in blood flow are substantially greater in the absence of TSP1 or CD47. Given the deleterious effects of aging on the cardiovascular system, we wanted to determine whether blocking of TSP1 inhibition of NO signaling would provide significant tissue protection in senescent animals. Aged WT and apolipoprotein E (apoE)-null mice (with diet-driven vasculopathy) demonstrated increased tissue necrosis in response to a fixed ischemic challenge compared with young animals. In contrast, senescent TSP1-null and CD47-null animals subjected to the same ischemic challenge demonstrated tissue preservation comparable to that in young animals. Suppression of CD47 in senescent animals also resulted in increased tissue survival after ischemic injury.

Materials and Methods

Reagents

Isosorbide dinitrate (ISDN) and L-nitro-N-methyl arginine (L-NAME) were purchased from Sigma. A CD47 morpholino antisense oligonucleotide (CGTCACAGGCAGGACCCACTGCCCA) and a mismatched control morpholino were purchased from GeneTools.

Objective—Decreased blood flow secondary to peripheral vascular disease underlies a significant number of chronic diseases that account for the majority of morbidity and mortality among the elderly. Blood vessel diameter and blood flow are limited by the matricellular protein thrombospondin-1 (TSP1) through its ability to block responses to the endogenous vasodilator nitric oxide (NO). In this study we investigate the role TSP1 plays in regulating blood flow in the presence of advanced age and atherosclerotic vascular disease.

Methods and Results—Mice lacking TSP1 or CD47 show minimal loss of their resistance to ischemic injury with age and increased preservation of tissue perfusion immediately after injury. Treatment of WT and apolipoprotein E–null mice using therapeutic agents that decrease CD47 or enhance NO levels reverses the deleterious effects of age- and diet-induced vasculopathy and results in significantly increased tissue survival in models of ischemia.

Conclusion—With increasing age and diet-induced atherosclerotic vascular disease, TSP1 and its receptor CD47 become more limiting for blood flow and tissue survival after ischemic injury. Drugs that limit TSP1/CD47 regulation of blood flow could improve outcomes from surgical interventions in the elderly and ameliorate vascular complications attendant to aging. (Arterioscler Thromb Vasc Biol. 2007;27:2582-2588.)

Key Words: nitric oxide • thrombospondin-1 • perfusion • ischemia • wound healing

Blocking Thrombospondin-1/CD47 Signaling Alleviates Deleterious Effects of Aging on Tissue Responses to Ischemia

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Original received July 13, 2007; final version accepted September 25, 2007.
Animals
C57BL/6 WT, TSP1-null, and CD47-null mice were maintained in a pathogen-free environment with ad libitum access to standard rat chow and water. ApoE null mice (B6.129P2-Apoe<tm1Unc>/J) were purchased from Jackson Labs (Bar Harbor, Me) and maintained on either a standard rat chow diet or a 40% fat diet (Harlan Teklan, Madison, Wisc). Animals were used at 12 to 18 months of age, except as indicated when young animals (aged 2 to 4 months) were used for control purposes. Care and handling of animals was in accordance with the Animal Care and Use Committees of the National Cancer Institute and of Washington University School of Medicine.

Ischemic Soft Tissue Flap Model
Animals underwent creation of a random myocutaneous (McFarlane) flap as previously described. Some animals received either ISDN (1 mg/mL) or L-NAME (0.5 mg/mL) ad libitum in the drinking water during the postoperative interval.

Estimation of Survival Area in Flaps
The necrotic areas of dorsal myocutaneous McFarlane flaps were determined as previously described.

Laser Doppler Analysis of Tissue Perfusion
Core temperature was monitored via rectal probe and maintained at 37°C by a heated stage. Anesthesia was obtained with 1.5% isoflurane. A MoorL1–2A scanner (Moor Instruments) was used with the following parameters: scan area, 1.6×2.5 cm; scan speed, 4 ms per pixel; scan time, 1 minute 54 sec; override distance, 25 cm. The override distance was 20 cm.

Hindlimb Ischemia
Wild-type, TSP1-null, CD47-null mice underwent ligation of the femoral artery at the level of the inguinal ligament as previously described with a 5-0 nylon ligature placed around the femoral artery and tied, resulting in complete vessel occlusion.

Blood Oxygen Level–Dependent MRI Imaging
WT and TSP1 null mice 14 to 18 months of age underwent in vivo analysis of tissue perfusion and blood flow using blood oxygen level–dependent (BOLD) MRI. MRI images were acquired using a Bruker Biospin 4.7 T scanner and isoflurane anesthesia as previously described. T2 mapping spin echo images were obtained using a multi-slice multi-echo (MSME) sequence with a 10-echo train and an echo time of 15 ms. The scan time for a T2 mapping image set (NEX=1) by the MSME sequence was 10 minutes.

Hind Paw Perfusion Assay
Fourteen-month-old mice underwent treatment of the hind paw with 5% mustard oil (Chem Services Inc) in sesame oil (50 μL to dorsal paw), which is known to induce NO-dependent vasoactive responses.

Morpholino Suppression of CD47
Flap soft tissue units and underling wound beds were injected with control vehicle (normal saline), a CD47 morpholino, or a control morpholino (10 μmol/L) in sterile saline. Flap survival was determined as described.

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. Results were expressed as absorbance normalized to dry tissue weight.

Determination of Tissue cGMP
Skeletal muscle biopsies of equal wet weight were excised, frozen in liquid nitrogen (LN2) and pulverized, and then washed with 6% (wt/vol) trichloroacetic acid (TCA) at 4°C. Homogenates were centrifuged and supernatants washed 4 times with 5 volumes of water saturated diethyl ether. The extracts were lyophilized and resuspended in assay buffer for analysis via immunoassay (Amer- sham, GE Healthcare).

Histology
Tissue units were excised, fixed in 10% buffered formaldehyde, paraffin embedded, and sectioned at a thickness of 5 μm. Sections were then stained with hematoxylin and eosin (H&E). Review of each slide was performed by an independent pathologist blinded to the origin of each tissue slide.

Statistics
Results are presented as the mean±SD of a total of 164 aged animals (12 to 18 months old) of the following genetic type: WT=67, TSP1-null=56, CD47-null=31, apo E-null=10. Where indicated studies were performed in comparable numbers of young WT, TSP1- and CD47-null mice aged 2 to 4 months. Significance was calculated with Student t test and 1-way ANOVA using a soft ware package (Origin) with P<0.05.

Results

Thrombospondin-1 Limits Tissue Necrosis in Aged Animals
Aged (14- to 18-month-old) sex-matched WT and TSP1-null mice underwent McFarlane flap surgery. Wild-type flaps demonstrated near total necrosis with only 20±6% survival obtained at 7 days (Figure 1A and 1B). This is significantly less than the survival seen in young mice of the same background (P<0.05). Flaps in senescent TSP1-null mice demonstrated near total tissue survival with minimal necrosis (6±3%). Survival did not differ significantly from that of young TSP1-null mice (P>0.05). Histological review of WT tissue flaps from senescent mice demonstrated loss of the epidermis and ulceration, coagulative necrosis of subcutaneous collagen, and absence of hair follicles (supplemental Figure Ia and Ic, available online at http://atvb.ahajournals.org). TSP1-null flaps demonstrated normal histology with minimal inflammatory cell infiltration (supplemental Figure Ib and Id).

Nitric Oxide Increases Aged Tissue Survival After Ischemia
WT aged mice that underwent random myocutaneous flaps and received ISDN ad libitum in the drinking water demonstrated a moderate but significant increase in tissue survival 41±5% (P<0.05; Figure 1C). Treatment of WT aged mice with L-NAME further decreased flap survival (8±4% versus 20±6%, P<0.05) compared with untreated (Figure 1A and 1C), whereas L-NAME failed to diminish flap survival in TSP1-null animals. Given the near complete survival of random flaps in untreated aged TSP1-null animals treatment with ISDN did not dramatically alter flap survival (Figure 1C).

Tissue cGMP Is Limited by TSP1 and Age
Skeletal muscle from WT and TSP1-null young (12 weeks old) and aged (18 months old; Figure 1D) or old mice alone (Figure 1E) mice was analyzed for cGMP. Consistent with previous reports, cGMP levels decreased significantly in aged WT animals. In contrast, cGMP was slightly elevated in muscle samples from young TSP1-null mice and did not fall with age. After 24 hours of ischemia, old TSP1-null animals...
demonstrated significantly less decrease in tissue cGMP compared with wild-type (Figure 1E).

**TSP1 Limits Immediate Responses to Ischemia in Senescent Animals**

Mice (14 to 18 months old) underwent random myocutaneous flaps and laser Doppler analysis of tissue perfusion (Figure 2A). TSP1-null and CD47-null flaps demonstrated greater flap perfusion than WT flaps (Figure 2B; supplemental Figure II and movie). Null flaps exhibited less overall loss of tissue perfusion immediately after flap elevation and a progressive increase in tissue perfusion during the postoperative interval. WT flaps demonstrated progressive decreases in flap perfusion and showed no tendency to recovery.

**Thrombospondin-1 Limits Hindlimb Survival of Ischemia in Aged Animals**

Mice (age 14 to 18 months) underwent ligation of the femoral artery. Aged wild-type animals demonstrated minimal remodeling of existing collateral vessels in the ischemic limb and significantly less than young mice (Figure 3A and 3D). In contrast TSP1-null limbs demonstrated extensive vascular remodeling of existing collateral vessels with both young and old animals performing equally well (Figure 3B and 3D). Laser Doppler analysis of hindlimb perfusion 72 hours after ligation demonstrated less restoration of perfusion in wild-type (Figure 3A and 3C) than in TSP1-null limbs (Figure 3B and 3C). Mitochondrial viability of muscle biopsies from hindlimbs was only minimally decreased in limbs undergoing vascular ligation compared with contralateral control limbs in TSP1-null specimens (Figure 3E) Aged WT samples showed a pronounced decrease in MTT signal between ischemic limb muscle compared with normal limb muscle, and between young and old tissue samples (Figure 3E). H&E sections of hindlimbs demonstrated marked sterile necrosis of muscle fibers and increased cell nuclei.

**Loss of TSP1 Immediately Enhances Tissue Perfusion After Hindlimb Ligation in Older Animals**

Aged WT and TSP1-null animals showed a profound decrease in perfusion immediately after vascular ligation of the hindlimb (Figure 4A and 4B). However, the TSP1-null animals showed a progressive increase in hindlimb perfusion compared with WT limbs and had significantly more restoration of flow within the first postoperative hour.
Aged TSP1-Null Animals Demonstrate Enhanced Responses to Exogenous NO in Ischemic Tissue

To further define the role of TSP1/NO signaling in acute responses to tissue ischemia, WT and TSP1-null mice (14 to 18 months of age) underwent ligation of the femoral artery. Three days after the procedure, animals underwent BOLD MRI in the presence of an NO challenge (10 μmol/L DEA/NO). In the absence of TSP1, NO-driven blood flow changes showed a progressive increase to levels slightly above those found in the control limb (Figure 4D). WT animals demonstrated a significant deficit in tissue blood flow responses to exogenous NO challenge after proximal ligation compared with the nonoperated limb (Figure 4C). T2 maps showed dramatic differences in WT animals between control and ischemic hindlimbs (Figure 4c). In contrast, blood flow was the same in ischemic and control hindlimbs in TSP1-null mice (Figure 4d).

CD47 Limits Tissue Survival to Ischemia in Aged Animals

We have reported that the effects of TSP1 on tissue ischemia are mediated by CD47.15 After random myocutaneous flap surgery, tissue survival in senescent CD47-null mice (93±4%) resembled levels obtained in aged TSP1-null animals (Figure 5A). In contrast to the decrease with age in WT animals, flap tissue survival in CD47-null animals did not decrease age (Figure 5A).

TSP1 and CD47 Limit Vasodilatory Responses in Aged Animals

WT and CD47-null mice aged 2 to 4 months underwent mustard oil application to the right hind paw and perfusion measured by Doppler. Young WT and CD47-null animals demonstrated equivalent levels of hind paw perfusion under basal conditions (Figure 5B and 5C). However, on application of mustard oil, CD47-null animals experienced a marked increase of the baseline perfusion as compared with WT. Likewise, young TSP1-null mice demonstrated enhanced perfusion in response to a mustard oil challenge compared with WT (Figure 5D). Senescent CD47- and TSP1-null animals had 20% to 25% greater perfusion compared with WT animals after mustard oil treatment, with persistence of perfusion gains over time (Figure 5E).

Morpholino Suppression of CD47 Increases Aged Tissue Survival After Ischemia

WT mice 14 to 18 months in age underwent random myocutaneous flaps and were treated with control vehicle, a CD47 morpholino (supplemental Figure IVA and IVB) or control morpholino (data not shown) injected directly to the flap and wound bed at the time of surgery. Postoperative tissue survival was increased in animals treated with a CD47 morpholino as compared with control treated animals (91±4% versus 31±6%, respectively; Figure 6A). Control morpholino treated flaps displayed degrees of tissue necrosis comparable to untreated aged WT animals (data not shown). Interestingly, CD47 morpholino-treated WT flaps demonstrated substantial remodeling of existing collateral flap vessels (supplemental Figure IVC). Mitochondrial viability was markedly increased in flaps that received the CD47 morpholino as compared with vehicle, missense morpholino, or untreated flaps (Figure 6B).

Ischemic Tissue Necrosis in Aged Animals With Atherosclerotic Peripheral Vascular Disease Is Minimized by CD47 Suppression

ApoE-null mice 12 to 16 months of age fed a high-fat diet for a minimum of 8 months underwent flap elevation. On postoperative day 7 apoE-null animals demonstrated significantly decreased tissue survival comparable to or even...
slightly worse than changes seen in WT animals of comparable age (supplemental Figure IVD and IVE). Despite diet-induced atherosclerosis, these animals showed increased tissue survival after CD47 morpholino treatment (84±7% versus 5±5%, respectively; Figure 6C). Routine histological staining of an artery from an aged apoE-null animal on a high-fat diet 3 weeks after temporary ligation demonstrated significant neointimal enlargement and plaque formation (supplemental Figure IVF).

Discussion

Altered tissue perfusion secondary to atherosclerotic peripheral vascular disease is a common cause of numerous diseases of the elderly.24,25 Such vasculopathy is endemic in Western societies.26 The consequences of atherosclerotic vascular disease in the elderly are altered blood flow27 and inadequate delivery of nutrients and oxygen to tissues with attendant tissue ischemia, necrosis, and loss. Even in the absence of atherosclerotic changes, age-related decreases in nitric oxide synthase activity28,29 and cellular and tissue cGMP22,23 can impair the ability of senescent vasculature to dilate, thereby limiting the delivery of blood to tissues.

Recently, we demonstrated that NO-driven relaxation of VSMCs is regulated by TSP1.30 In both endothelial and VSMCs,30,31 TSP1 via CD47 blocks the ability of endogenous or exogenous NO to elevate cGMP levels. As a physiological consequence, tissue perfusion in response to NO is significantly greater in the absence of TSP1 or CD47.14 Although the absence of TSP1 or CD47 conferred a survival advantage on ischemic tissues in young animals (10 to 16 weeks of age), it was not clear that ischemia in the elderly or in the presence of age-associated vasculopathy would be ameliorated by blocking the TSP1-CD47 pathway.

In the present study we found that TSP1, in a CD47-dependent manner, limits ischemic tissue survival under conditions of advanced age and atherosclerotic vasculopathy. In the absence of TSP1 or CD47, senescent mice were able to maintain perfusion after an ischemic insult. The acute effects of TSP1/CD47 signaling on perfusion of aged ischemic tissues is consistent with the immediate enhancement of
tissue perfusion in muscle units of TSP1-null animals exposed to exogenous NO. Even more remarkable, senescent TSP1-null animals demonstrate a greater perfusion increase in response to exogenous NO than young WT animals (see Figure 4D). Superior perfusion and tissue survival responses of aged TSP1- and CD47-null animals were observed consistently for random cutaneous flaps, hindlimb vascular ligation, and a noninvasive mustard oil/hind paw assay despite the comparable soft tissue vascular densities in wild-type and null animals. The NO dependence for tissue survival of ischemic injury in WT animals is supported by positive effects of NO supplementation with ISDN and negative effects of NOS inhibition with L-NAME. In the absence of TSP1, tissue survival is less sensitive to NO modulation. This is not surprising because TSP1 limits cGMP accumulation in both vascular cells and tissue (Figure 1D and 1E), and prior studies have documented increased tissue TSP1 levels with age. Thus, we propose that the NO insufficiency of aging may be attributable to increased TSP1 antagonism of NO/cGMP signaling as well as loss of NOS activity.

ApoE-null mice on a high-fat diet develop a hyperlipidemic state analogous to that in humans. Blood vessels of these mice show luminal narrowing and hypertrophy of the medial layers with atherosclerotic plaque. Comparable atherosclerotic changes in people have been associated with decreased local NO production by vascular endothelium. Interestingly, TSP1 expression has been found to increase with age in atherosclerotic blood vessels and in several end organs. Morpholino suppression of CD47 improved ischemic tissue survival in apoE-null animals in the presence of diet-induced atherosclerotic vasculopathy, and also in aged wild-type animals. These results suggest that targeting of TSP1 or CD47 in regional vascular beds could improve tissue perfusion under conditions of both advanced age and peripheral vascular disease.

**Sources of Funding**

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center
for Cancer Research (M.T., M.C.K., D.D.R.); and NIH grants HL54390 and GM57573 (W.A.F.).

Disclosures
None.

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Blocking Thrombospondin-1/CD47 Signaling Alleviates Deleterious Effects of Aging on Tissue Responses to Ischemia

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Arterioscler Thromb Vasc Biol. 2007;27:2582-2588; originally published online October 4, 2007;
doi: 10.1161/ATVBAHA.107.155390

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figures Legend

Figure I. TSP1 impairs ischemic tissue survival in aged animals. Sections of random dorsal McFarlane flaps demonstrate near complete necrosis of wild type flaps with massive inflammatory infiltration and normal architecture with minimal inflammatory component in TSP1-null flaps. H&E sections under low (20X objective) (a, b) and high magnification (40X objective) (c, d).

Figure II. Thrombospondin-1 limits immediate responses to ischemia in senescent animals. WT, TSP1-null, and CD47-null mice 14 – 18 months of age underwent dorsal McFarlane flap surgery and perfusion was determined via laser Doppler every 5 min for the first post-operative hour (A, B). Animals were maintained at 37 ºC on a heated stage. Images and data are representative of 18 mice, 6 of each strain.

Figure III. Thrombospondin-1 limits hind limb survival to ischemia in aged animals. Sections from the tibialis anterior muscle of ischemic WT (A) and TSP1-null (B) hind limbs from aged animals are shown demonstrating hylinized degeneration and drop out of muscle fibers with mononuclear cell infiltration in wild type muscle compared to TSP1-null. H&E sections, 20X objective.

Figure IV. Morpholino suppression of CD47 increases aged tissue survival to ischemia. WT mice 14 – 18 months of age underwent random dorsal flaps. Animals were treated with control vehicle (A), missense control morpholino (data not shown) or a CD47 morpholino (B). Representative image of flap vascular remodeling in WT flaps treated with a CD47 morpholino (C). ApoE-null mice on a high fat diet and of at least 12 months of age underwent random dorsal myocutaneous flaps. Animals received no treatment (D), a vehicle (data not shown) or a CD47 morpholino (E) and flap survival
determined on post-operative day 7. Representative H&E section of artery from an aged apoE-null animal on a high-fat diet three weeks following soft tissue injury, 20x objective (F).
Fig. I

Wild type  TSP 1 -/-

a.  b.

c.  d.
**Fig. II**

A. Flap image

B. Graph showing flux (% control) over time (min) for Wild type, TSP1 -/-, and CD47 -/-.
Fig. IV

Wild type

vehicle + CD47 morpholino Treated flap vessels

A B C

Apo E null Apo E null + CD47 morpholino

D E F