Despite normal arteriogenic and angiogenic responses, hind limb perfusion recovery and necrotic and fibroadipose tissue clearance are impaired in matrix metalloproteinase 9-deficient mice

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Objective: **The relative contributions of arteriogenesis, angiogenesis, and ischemic muscle tissue composition toward reperfusion after arterial occlusion are largely unknown. Differential loss of bone marrow-derived cell (BMC) matrix metalloproteinase 9 (MMP9), which has been implicated in all of these processes, was used to assess the relative contributions of these processes during limb reperfusion.**

Methods: **We compared collateral growth (arteriogenesis), capillary growth (angiogenesis), and ischemic muscle tissue composition after femoral artery ligation in FVB/NJ mice that had been reconstituted with bone marrow from wild-type or MMP9$^{-/-}$ mice.**

Results: **Laser Doppler perfusion imaging confirmed decreased reperfusion capacity in mice with BMC-specific loss of MMP9; however, collateral arteriogenesis was not affected. Furthermore, when accounting for the fact that muscle tissue composition changes markedly with ischemia (ie, necrotic, fibroadipose, and regenerating tissue regions are present), angiogenesis was also unaffected. Instead, BMC-specific loss of MMP9 caused an increase in the proportion of necrotic and fibroadipose tissue, which showed the strongest correlation with poor perfusion recovery. Similarly, the reciprocal loss of MMP9 from non-BMCs showed similar deficits in perfusion and tissue composition without affecting arteriogenesis.**

Conclusions: **By concurrently analyzing arteriogenesis, angiogenesis, and ischemic tissue composition, we determined that the loss of BMC-derived or non-BMC-derived MMP9 impairs necrotic and fibroadipose tissue clearance after femoral artery ligation, despite normal arteriogenic and angiogenic vascular growth. These findings imply that therapeutic revascularization strategies for treating peripheral arterial disease may benefit from additionally targeting necrotic tissue clearance or skeletal muscle regeneration, or both. (J Vasc Surg 2015;61:1583-94.)**

Clinical Relevance: **Asymptomatic peripheral arterial disease can be attributed to endogenous compensation for widespread occlusions, including the luminal growth of collateral arteries (ie, arteriogenesis), capillary network expansion by angiogenesis, and the repair of ischemically injured tissue. Here, we examined the relative roles of these three processes in establishing reperfusion by using models involving matrix metalloproteinase 9. Mice with matrix metalloproteinase 9-deficient bone marrow-derived cells exhibited impaired reperfusion; however, neither angiogenesis nor arteriogenesis was affected. Instead, impaired reperfusion was strongly correlated with high necrotic and fibroadipose tissue composition, stressing the importance of stimulating concomitant muscle repair in therapeutic revascularization strategies.**

Peripheral arterial disease (PAD) is caused mainly by atherosclerotic lesions and is widely prevalent in the aged population (20% asymptomatic and 6% symptomatic prevalence in those aged >65 years).[1] Arterial occlusions can ultimately lead to the symptomatic consequences of intermittent claudication and critical limb ischemia, both significant causes of morbidity and mortality. Asymptomatic disease can be attributed to the endogenous capacity of some patients to compensate for widespread occlusive disease. Thus, one potential treatment for established PAD in symptomatic patients is to therapeutically stimulate neovascularization.

Endogenous compensation for arterial occlusion comes from a confluence of three processes:

First, the pre-existing small arterial pathways that originate upstream of and bypass the occlusion(s) undergo arteriogenesis (ie, outward remodeling), thereby decreasing upstream resistance and increasing flow downstream.[2]

Second, in the tissue downstream of the occlusion(s), hypoxia induces the expansion of the capillary network by angiogenesis to improve blood flow distribution. Adaptive arteriogenesis and angiogenesis can both ameliorate the symptoms of PAD, and using a myoglobin-overexpressing transgenic mouse model, we have recently shown that impaired angiogenesis can lead to a deficit in reperfusion, even in the presence of normal collateral

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arteriogenesis.[3] Diseases in patients that impair both processes are known to contribute to symptomatic PAD.[4,5]

Third, although often overlooked,[6] the tissue injured by the ischemia must be cleared and undergo regeneration to return to functional capacity. In both clinical PAD and analogous animal models (ie, hind limb ischemia), the ischemic tissue is located in the lower limbs. Previously overlooked differences in necrotic tissue clearance and myocyte regeneration in response to ischemia[7] may be as important as the more widely studied elements of collateral structure and vascular remodeling differences.[8,9]

Ultimately, all three elements must work together to allow for full reperfusion and recovery; however, they are rarely examined in tandem.

In this study, we examined the relative roles of arteriogenesis, angiogenesis, and ischemic tissue composition in establishing functional reperfusion by focusing on models involving matrix metalloproteinase 9 (MMP9), an enzyme hypothesized to regulate all three processes. Bone marrow-derived cell (BMC)-specific loss of MMP9 impairs reperfusion,[10-13] with BMC-derived MMP9 regulating neovascularization in response to ischemic injury through mobilization of endothelial progenitor cells[12,13] and angiogenesis.[10] BMC-derived MMP9 may also be involved in arteriogenesis, although the results are conflicting.[14,15]

MMP9 is primarily BMC-derived, with neutrophils being the most prevalent MMP9 cell source.[11,16,17] However, given that neutrophils have a limited role in the angiogenic[18] and arteriogenic processes[19,20] in response to arterial occlusion, the role of MMP9 may lie in regulating the clearance of injured tissue and muscle regeneration. MMP9-depedent extracellular matrix degradation and remodeling allow for proper satellite cell activation and muscle regeneration during injury.[21] One complication in assessing these elements individually is that altered angiogenesis and impaired skeletal muscle regeneration are often correlated. Detailed analyses of both are required to dissect underlying impairment(s).[18]

Given this evidence, we hypothesized that the primary role of MMP9 in ischemic muscle injury due to arterial occlusion is neither through arteriogenesis nor angiogenesis but instead is through tissue clearance and regeneration. Consistent with this hypothesis and contrary to previous findings,[10,13,22] we found no angiogenic or arteriogenic impairments in MMP9-deficient mice. Rather, impaired reperfusion was best correlated with enhanced necrotic and fibroadipose tissue composition, providing evidence that nonvascular remodeling function may serve as an additional target for PAD revascularization therapies.

METHODS

All animal protocols were approved by the University of Virginia Institutional Animal Care and Use Committee and conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Animals and BMC transplantation. Wild-type (WT) control (FVB/NJ) and MMP9$^{-/-}$ (FVB.Cg-*Mmp9*^{tm1Tvu}/J) male mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and housed in the animal facilities at the University of Virginia. BMC transplantations were performed as previously described.[23,24] Donor mice were anesthetized by an intraperitoneal injection of 120 mg/kg ketamine, 12 mg/kg xylazine, and 0.08 mg/kg atropine and euthanized by cervical dislocation. Host mice were anesthetized by inhaled isoflurane before intravenous bone marrow injection. Further details are provided in the Supplementary Methods, online only. Henceforth, bone-marrow chimeric mice are identified using a "BMC donor strain→host strain" nomenclature.

Femoral artery ligation model, perfusion measurements, and tissue processing. Mice for these procedures were anesthetized by an intraperitoneal injection of 120 mg/kg ketamine, 12 mg/kg xylazine, and 0.08 mg/kg atropine. Femoral arterial ligation (FAL) was performed to induce collateral remodeling in the gracilis adductor muscle and produce a moderate level of ischemia in the downstream tissue in a method similar to that previously described.[23,25,26] To control for the effect of the surgery, all mice received a sham operation on the contralateral, in which the femoral artery was exposed but not ligated. Animals received injections of buprenorphine for analgesia immediately post-FAL and 8 to 12 hours later.

Laser Doppler perfusion imaging was performed to monitor blood flow recovery in response to FAL, as previously described, at days 2, 4, 7, 10, and 14 post-FAL before terminal harvesting for tissue cross-sectional and collateral artery analysis.[23,25] Time points are noted as relative to FAL, which is noted as day 0. All animals received unilateral FAL in the left limb and a sham (control) operation in the right limb to account for the effects of surgical intervention.

For tissue and collateral artery morphology analysis, mice were anesthetized at day 3, 7, 10, or 14 post-FAL, and the gracilis muscle vasculature was vasodilated by superfusing the tissue with adenosine by using a simple drip system. Mice were euthanized by anesthetic overdose and perfusion-fixed by cardiac cannulation. Gracilis and calf muscles, including the gastrocnemius and plantaris muscles, were harvested. Further details are provided in the Supplementary Methods, online only.

Collateral artery network analyses. Perfusion-fixed gracilis muscles were immunofluorescently stained for α-smooth muscle actin, whole-mounted, and imaged with a fluorescence microscope. Diameter was measured along the two primary collateral artery pathways that span the gracilis muscle. Further details are provided in the Supplementary Methods, online only.

Cross-sectional analyses. Paraffin-embedded gracilis muscles were cross-sectioned and stained with hematoxylin and eosin (H&E) for measurements of cross-sectional collateral structure or with picrosirius red for collagen content. Additional gracilis muscle sections were immunostained for cluster of differentiation (CD) 45 (panleukocyte marker), MMP9, CD31 (endothelial cells), or