Skeletal muscle reperfusion injury is mediated by neutrophils and the complement membrane attack complex

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Kyriakides, Constantinos, William Austen, J r., Yong Wang, J oanne Favuzza, Lester Kozbik, Francis D. Moore, J r., and Herbert B. Hechtman. Skeletal muscle reperfusion injury is mediated by neutrophils and the complement membrane attack complex. Am. J. Physiol. 277 (Cell Physiol. 46): C1263–C1268, 1999.—The relative inflammatory roles of neutrophils, selectins, and terminal complement components are investigated in this study of skeletal muscle reperfusion injury. Mice underwent 2 h of hindlimb ischemia followed by 3 h of reperfusion. The role of neutrophils was defined by immunodepletion, which reduced injury by 38%, as did anti-selectin therapy with recombinant soluble P-selectin glycoprotein ligand-immunoglobulin (lg) fusion protein. Injury in C5-deficient and soluble complement receptor type 1-treated wild-type mice was 48% less than that of untreated wild-type animals. Injury was restored in C5-deficient mice reconstituted with wild-type serum, indicating the effector role of C5–9. Neutrophic C5-deficient animals showed additive reduction in injuries (71%), which was lower than C5-deficient neutrophil-replete mice, indicating neutrophil activity without C5a. Hindlimb histological injury was worse in ischemic wild-type and C5-deficient animals reconstituted with wild-type serum. In conclusion, the membrane attack complex and neutrophils act additively to mediate skeletal muscle reperfusion injury. Neutrophil activity is independent of C5a but is dependent on selectin-mediated adhesion.

ischemia; inflammation; complement activation; selectins; murine

LOWER TORSO ISCHEMIA OCCURS during aortic or peripheral vascular surgery, following trauma or as a result of an embolic or thrombotic event. Therapeutic attempts to restore blood flow, whether by surgery or thrombolysis, result in a reperfusion injury locally to the muscle and in a remote injury to other organs such as the lungs. The muscle injury is characterized by endothelial damage and permeability edema, which, if unchecked, may lead to a compartment syndrome and tissue necrosis (10).

Both polymorphonuclear leukocytes (PMNs) and the complement system are thought to be important mediators of reperfusion injury (7, 19, 29). After ischemia, local leukosequestration of activated neutrophils occurs with generation of reactive oxygen species and elastase (29). Accordingly, injury can be moderated in part by experimental neutrophil immunodepletion (26). Neutrophil-endothelial cell interaction occurs via adhesion molecules in an orderly and sequential fashion. The selectins are thought to be responsible for the initial slowing and rolling of PMNs in the microvessels. This is followed by the formation of firm adhesion molecules (ICAM)-1 and -2. Subsequently, the PMNs transmigrate out of the microcirculation (22). Inhibition of this sequence of events and diminution of injury have been particularly successful using anti-integrin therapies (11, 16, 23).

Blockade of complement activation with a soluble recombinant form of complement receptor type 1 (sCR1) has been shown to reduce the infarct size following myocardial ischemia (28). These observations were similar to those in a rat model of lower torso and gut ischemia, where reperfusion injury was significantly reduced with sCR1 (6, 10). Recently, identification of the classical complement pathway as the primary mediator in hindlimb and gut reperfusion injury has been established by the observation that IgM-deficient (RAG1−/−) or C4 genetic knockout mice are protected from these ischemic events (27, 31).

In this study of hindlimb ischemia-reperfusion, it is hypothesized that complement-mediated injury is C5-9 dependent. Utilizing C5-deficient mice, we examine the relative inflammatory contributions of the C5b-9 and C5a terminal complement components in mediating injury. Furthermore, the anti-neutrophil effects of selectin blockade are investigated.

MATERIALS AND METHODS

Mice Two congenic strains, C5-deficient (B10.D2/oSnJ) and wild-type (B10.D2/nSnJ) mice, purchased from Jackson Laboratories (Bar Harbor, ME), were used in all experiments. All studies were conducted using males because of reports of intermediate C5 levels in females of the deficient strain (14).

Hindlimb model of ischemia-reperfusion injury. Mice aged 8–12 wk and weighing 25–30 g were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg) and underwent 2 h of hindlimb ischemia followed by 3 h of reperfusion. After a 2-min period of hindlimb elevation to minimize retained blood, bilateral rubber bands (Latex O-Rings) were applied above the greater trochanter, using the McGivney Hemorrhoidal Ligator (Miltex Instrument). Sham mice did not undergo ischemia. Five minutes before rubber band release, animals received 1 µCi of 125I-labeled albumin (ICN, Irving, CA) in 0.3 ml of 0.9% saline via tail vein injection. Hydration was maintained by intravenous infusion of 0.1 ml of 0.9% saline during each hour of reperfusion. Mice were maintained in a supine position and kept anesthetized by intermittent intraperitoneal pentobarbital sodium injections. They were covered throughout the experiment to maintain body temperature. Experimental mortality was <10% in mice undergoing ischemia-reperfusion and was zero in animals undergoing ischemia alone (10).
undergoing sham injury. After euthanasia by an intraperitoneal pentobarbital sodium overdose (90 mg/kg), blood was aspirated from the right ventricle through a midline sternotomy, and its gamma radioactivity was counted (Packard, Downer’s Grove, IL). Muscle was harvested from both hindlimbs, its radioactivity was measured, and then the muscle was dried to a constant weight in a gravity convection oven (Precision Scientific Group, Chicago, IL) at 90°C for 72 h. Extravasation of 125I-albumin was used to assess the hindlimb vascular permeability index (PI), which was determined by the ratio of radioactivity per gram of dry muscle to radioactivity per gram of blood. In addition, hindlimb muscle harvested from these experiments was used for immunohistochemical analysis of IgM and complement deposition.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Human Services, Publication no. 85–23 (National Institutes of Health), revised 1985).

PMN depletion. Mice were rendered neutropenic by a tail vein injection of rabbit anti-mouse PMN antibody (20 mg/kg; Accurate, Westbury, NY) 16 h before ischemia. Venous blood samples for total leukocyte counts and differential counts were taken before ischemia and at the time of euthanasia.

Anti-selectin therapy with P-selectin glycoprotein ligand-1g. Mice were treated with a recombinant (r) soluble form of P-selectin glycoprotein ligand 1-g (PSGL-1g) fusion protein (Genetics Institute, Cambridge, MA) administered intravenously 5 min before reperfusion. Each animal received 1 mg/kg of rPSGL-1g, a dose previously determined to effectively inhibit neutrophil-endothelial selectin interaction (18). In addition, rPSGL-1g was examined at varying concentrations to test its ability to activate the classical complement pathway using an in vitro murine hemolytic assay.

Complement inhibition with sCR1. Both classical and alternative pathways of complement activation were inhibited by an intravenous bolus of sCR1 (AVANT Immunotheapeutics, Needham, MA) administered 5 min before reperfusion. Each animal received 20 mg/kg of sCR1, a dose previously determined to effectively inhibit complement activation (6, 28).

Wild-type serum reconstitution of C5-deficient mice. Blood was aspirated from the right ventricle of wild-type, complement-sufficient mice following euthanasia. The blood was collected in a glass tube and allowed to clot for 5 min. After centrifugation at 5,000 g for 5 min at 0°C, the serum was decanted and kept on ice. C5-deficient mice were infused with 0.5 ml of wild-type serum 16 h before ischemia.

Immunohistological analysis. Immunoperoxidase labeling of IgM and C3 was performed on paraformaldehyde-fixed cryostat sections of hindlimb muscle using goat anti-mouse IgM (Sigma Chemical) or goat anti-mouse C3 (5 mg/ml; Organon Teknia, Durham, NC) and a standard avidin-biotin protocol (8). Immunostaining of hindlimb muscle samples from sham animals was used as controls. Injury was assessed semiquantitatively by evaluating the presence of edema, disruption of normal muscle architecture, and individual myocyte detachment by a pathologist (L. Kozbiak) in a blinded fashion. A score from 0 to 3 was assigned, corresponding to normal, mild (<25% of sample area showing injury), moderate (25–50% of sample area showing injury), and severe (>50% of sample area showing injury), respectively.

Statistical analysis. Results are presented as means ± SE. Groups were subjected to one-way ANOVA, and, when significance was found, Student’s t-test with the Bonferroni correction for multiple comparisons was applied. Percentage reduction in PI was calculated after subtraction of the background value determined in animals that had not undergone ischemia (sham).

RESULTS

Reperfusion of the ischemic skeletal muscle resulted in vascular injury, manifested by the extravasation of radiolabeled albumin. PI in wild-type mice (n = 29) after 3 h of reperfusion was 2.19 ± 0.10, significantly higher than wild-type sham PI of 0.14 ± 0.01 (n = 12, P < 0.05). PI in sham C5-deficient mice (n = 12) of 0.12 ± 0.01 was similar to wild-type sham animals.

Effects of PMN depletion and anti-selectin therapy. Treatment with the anti-neutrophil antibody achieved an 87% neutropenia (Fig. 1; mean absolute PMN count of 129 ± 41 vs. 962 ± 17 cells/µl in neutrophil-replete mice, P < 0.05). After reperfusion, wild-type neutropenic mice (n = 18) had a PI of 1.41 ± 0.07, representing a 38% reduction in permeability (P < 0.05). Similarly, treatment with rPSGL-1g (n = 8) reduced permeability by 38% (P < 0.05; PI = 1.42 ± 0.11), indicating the importance of selectin-mediated neutrophil adhesion. Also, rPSGL-1g, when tested in vitro, failed to induce
red blood cell lysis, showing its inability to activate the classical complement pathway (data not shown).

Role of the terminal complement components in mediating permeability. Complement antagonism with sCR1 (n = 11) reduced permeability by 48%, PI = 1.20 ± 0.06 (P < 0.05; Fig. 2). PI in C5-deficient mice (n = 25) was 1.19 ± 0.12, also representing a 48% reduction in permeability (P < 0.05). This similarity to sCR1-treated animals indicates that permeability is mediated by the terminal complement components. Finally, permeability was 97% restored in C5-deficient mice reconstituted with wild-type serum (n = 11), PI = 2.11 ± 0.16.

Synergy of complement and neutrophils is independent of C5a. To test whether neutrophils are active in the absence of C5a, a group of C5-deficient animals (n = 14) were neutrophil depleted (mean absolute PMN count of 109 ± 54 cells/µl) 16 h before ischemia (Fig. 2). The PI in this group of animals was 0.72 ± 0.06, a 44% reduction compared with ischemic C5-deficient PMN-replete mice, PI = 1.19 ± 0.05 (n = 25, P < 0.05) indicating neutrophil activity without C5a. These C5-deficient neutropenic animals had a 71% reduction in permeability compared with 38% in PMN-depleted injured wild-type and 48% in injured C5-deficient mice (P < 0.05), indicating an additive role for neutrophils and complement in mediating reperfusion injury.

Immunohistological analysis. Hindlimb muscle samples snap frozen in optimal cutting temperature compound 3 h after reperfusion were stained for IgM and C3 (Fig. 3). Ischemic wild-type and C5-deficient animals reconstituted with wild-type serum demonstrated colocalization of IgM and C3 on the endothelium. There was associated edema and muscle cell detachment and deformation, indicative of severe injury (Table 1). Tissue staining for C3 and IgM and architectural injury were negligible in ischemic C5-deficient and wild-type animals treated with sCR1. There was no IgM or C3 staining observed in C5-deficient or wild-type sham groups.

**DISCUSSION**

A central role for neutrophils in mediation of the local events in reperfusion injury after tissue ischemia has been documented in a number of organs including skeletal muscle (9, 29). In this study, neutropenic mice had a 38% reduction in local injury, similar to previous observations of a 36% reduction of injury measured in rats made neutropenic (26). Neutrophil adhesion to the microvascular endothelium is thought to be a prerequisite in the sequence of events leading to the release of cytotoxic proteases and oxygen-derived free radicals, as well as PMN diapedesis and sequestration within posts ischemic tissues (29). The adhesion sequence of events starts with leukocyte rolling and tethering on the endothelial cell, which is facilitated by the selectin family of adhesion molecules (22). P-selectin stored in Weibel-Palade bodies is rapidly translocated on the endothelial cell surface following stimulation by various chemoactivators (5). E-selectin is upregulated on the endothelium in response to the cytokines interleukin-1 and tumor necrosis factor-α (TNF-α) (1). L-selectin is constitutively expressed on the leukocytes and is rapidly shed following their activation (24). Each selectin binds to a glycoprotein counterreceptor expressing sialylated Lewis X (SLX)-related oligosaccharides such as PSGL-1 (17). After this initial selectin interaction, leukocyte β2-integrins upregulated by agents such as C5a, TNF-α, and platelet-activating factor form firm adhesions with endothelial cell ICAM-1 and -2 (21). Local chemoattractants such as the complement fragment C5a and the arachidonic acid metabolites, leukotriene B4 and thromboxane A2, produced in high concentrations at the site of endothelial injury, are potent stimuli for PMN generation of H2O2 and elastase and can facilitate PMN diapedesis (30). The experimental use of anti-selectin immunotherapy has been shown to moderate skeletal muscle reperfusion injury (20). Weiss reported in a rat model of hindlimb ischemia-reperfusion that anti-selectin therapy in the form of a P-selectin antibody reduced permeability by 26%, less than the 36% noted with infusion of the oligosaccharide SLX at a dose of 10 mg/kg, a more general selectin antagonist (26). Our data confirm that broad selectin antagonism offered by a soluble form of the selectin
counterreceptor PSGL-1 reduces local muscle permeability to the same extent as PMN depletion (38%), suggestive of an overlapping function of the selectin adhesion molecules. Furthermore, in our model rPSGL-Ig abrogated PMN-mediated injury at one-tenth of the dose of SLX, suggesting a more potent selectin antagonism. Reports in the literature also describe that anti-β₂-integrin therapy is effective in moderating skeletal muscle reperfusion injury (2, 16).

The dependence of skeletal muscle reperfusion injury on complement activation is also confirmed in these studies with a 48% reduction in permeability in mice treated with sCR1. This is in accordance with published reports where experimental complement inhibition with sCR1 has ameliorated local reperfusion injury of the hindlimb, gut, and heart in mice as well as other animal species (6, 10, 27, 28, 31). Activation of complement can induce local injury by several mechanisms. First, released anaphylatoxins C3a and C5a enhance infiltration and activation of neutrophils, leading to vascular leakage (13). Second, covalently deposited iC3b on endothelial cell membranes acts as a chemoactivator signaling for a neutrophil oxidative burst via CD11b (12). Third, integration of the membrane attack complex C5b-9 into the endothelial cell membrane can act as a pore, leading to unchecked ion flux, which in turn could lead to second messenger signaling, enzyme

Table 1. Semi-quantitative evaluation of skeletal muscle histological injury

<table>
<thead>
<tr>
<th>Groups</th>
<th>Injury Score</th>
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<tbody>
<tr>
<td>Wild-type sham</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Wild-type injured + sCR1</td>
<td>1.25 ± 0.25*</td>
</tr>
<tr>
<td>Wild-type injured</td>
<td>2.57 ± 0.14</td>
</tr>
<tr>
<td>C5D sham</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>C5D injured</td>
<td>1.11 ± 0.26*</td>
</tr>
<tr>
<td>C5D injured + wild-type serum</td>
<td>2.58 ± 0.27</td>
</tr>
</tbody>
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Values are means ± SE. *P < 0.05 compared with wild-type injured. sCR1, soluble complement receptor type 1; C5D, C5 deficient.
activation and potential osmotic lysis (4, 19). This possibility is supported by evidence showing deposition of the C5b-9 membrane attack complex in infarcted regions of human myocardium as well as reperfused areas of gut and skeletal muscle following experimental rat gut and hindlimb ischemia, respectively (3, 7, 25).

In this study, C5-deficient animals demonstrated a 48% reduction in permeability following reperfusion. In addition, permeability was 97% restored in C5-deficient mice reconstituted with wild-type serum. Together with the data showing deposition of the C5b-9 complex, these observations indicate the central role of the C5-9 terminal complement components in mediating injury. Moreover, permeability was further reduced in C5-deficient mice that were neutrophil depleted, indicating that C5a is not needed for PMN activation in this setting.

Additional support for the role of the terminal complement components in mediating injury is provided by the histological injury score of ischemic hindlimb skeletal muscle. Histological injury was worst in ischemic wild-type and C5-deficient mice reconstituted with wild-type serum, as was the PI. Conversely, ischemic C5-deficient mice with significantly lower PI also demonstrated less histological injury. The colocalization of IgM and C3 has been previously shown, and, together with protection from injury noted in C4 knockout mice, indicates a role for the classical pathway of complement (27). The ischemic event is hypothesized to cause binding of IgM natural antibody to exposed novel antigenic determinants in reperfused endothelial cells as a result of alterations in the plasma membrane following ischemia (27). These membrane alterations could come about by a variety of mechanisms such as a reduction in phospholipid biosynthesis, or by activation of Ca2+-dependent phospholipases and proteases, or by endothelial cell expression of new epitopes either preformed or newly synthesized (15). Formation of this pentameric IgM natural antibody-antigen complex facilitates activation of C1 and the classical complement pathway. Complement activation allows for the formation of the C5b-9 complex and perturbation of the endothelial membrane, leading to cellular injury and presumably exposure of more antigenic determinants. This may explain why there is much more immunostaining of IgM and C3 in wild-type animals and C5-deficient mice reconstituted with wild-type serum, possibly reflecting a greater overall injury compared with injured C5-deficient mice (31).

Finally, the additive role of complement and neutrophils in mediating skeletal muscle reperfusion injury was demonstrated by a 71% reduction in permeability in neutropenic C5-deficient animals, which was greater protection than neutropenia or C5 deficiency alone. The therapeutic implication is that treatment should target both the terminal complement components as well as the neutrophils.

In conclusion, skeletal muscle reperfusion injury is mediated by the membrane attack complex and neutrophils in an additive fashion. The inflammatory role of the neutrophil is independent of C5a.

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