Reperfusion Injury of Ischemic Skeletal Muscle Is Mediated by Natural Antibody and Complement

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Summary

Reperfusion of ischemic tissue induces an acute inflammatory response that can result in necrosis and irreversible cell injury to both local vascular endothelium and parenchyma. To examine the pathogenesis of ischemia/reperfusion injury, we have used mice deficient in complement components C3, C4, or serum immunoglobulin in a hindlimb model of ischemia. We found that mice homozygous deficient in C3 or C4 were equally protected against reperfusion injury based on a significant reduction in leakage of radiolabeled albumin out of the vasculature. This demonstrates that classical pathway complement is an important factor in the initiation of inflammation following reperfusion. Furthermore, mice deficient in serum immunoglobulin were equally protected and this protection could be reversed by reconstitution with serum from normal mice. Thus, this report describes a novel mechanism for reperfusion injury that involves antibody deposition and activation of complement leading to inflammation permeability.

While the pathogenesis of reperfusion injury is not completely understood, the complement system is thought to be involved since there is clear evidence of deposition of complement components C5-9 after reperfusion of hypoxic tissue (1, 2) and injury can be inhibited in part by pretreatment with soluble complement receptor type 1 (sCR1) (3–6). Weisman et al. found that pretreatment with sCR1 substantially reduced influx of neutrophils, deposition of C5-9 and myocardial infarct size in a rat model of myocardial ischemia (3). Similar studies have used sCR1 to block or reduce inflammation after reperfusion of ischemic rat hindlimb or intestine and of mouse skeletal muscle (cremaster) (4–6). Complement receptor type 1 (CR1; CD35), which is normally expressed by human erythrocytes, B lymphocytes, granulocytes, and macrophages, binds activated C3 (C3b) and C4 (C4b) leading both to dissociation of the complement system's C3 and C5 activating enzymes and to proteolytic degradation of C3b and C4b by serum factor I. When administered in a soluble form, recombinant sCR1 functions as a highly effective inhibitor of serum complement activation (7).

To clarify the role of complement and gain insight into the pathogenesis of ischemia/reperfusion injury, we have examined novel strains of mice deficient in either complement components C3 or C4 in a mouse model of hindlimb ischemia reperfusion. Both strains of mice were equally protected from vascular injury demonstrating that injury is mediated by the classical pathway. Moreover, mice totally deficient in antibody, i.e., recombination-activation gene 2

deficient (RAG-2−/−) (8), were equally protected and protection could be abrogated by reconstitution with fresh mouse serum. These results support the hypothesis that ischemia/reperfusion injury is initiated by natural antibody binding to hypoxic endothelium and activation of the complement system.

Materials and Methods

Reconstitution of Antibody-deficient Mice. RAG2−/− mice were reconstituted by i.v. administration of 0.5 ml of pooled serum from control mice 15 h before ischemia. Strain FVB mice were used as controls since the RAG2−/− allele had been backcrossed onto that background. Circulating Ig levels were confirmed as ~50% normal by ELISA. Briefly, 96-well plates were coated with rabbit anti–mouse Fc (diluted in carbonate buffer, pH 9.5) and following blocking with 5% milk protein, dilutions of serum from control mice or reconstituted RAG2−/− mice were added for 2 h at room temperature. After wash, a detection antibody (alkaline–phosphatase conjugated goat anti–mouse IgG or IgM) was added for 1 h followed by color substrate (Sigma 105; Sigma Chem. Co., St. Louis, MO). Serum samples and detection antibodies were diluted with 5% milk protein in phosphate-buffered saline, pH 7.5. Following addition of substrate, O.D. 540 was measured in a Micro-titer plate reader.

Hindlimb Model of Ischemia/reperfusion Injury. Mice underwent 2 h of hindlimb ischemia and 3 h of reperfusion as described (4). Bilateral tourniquets were placed above the greater trochanter of pentobarbital anesthetized mice (20–35 g). Sham controls did not undergo ischemia. 5 min before tourniquet release animals re-
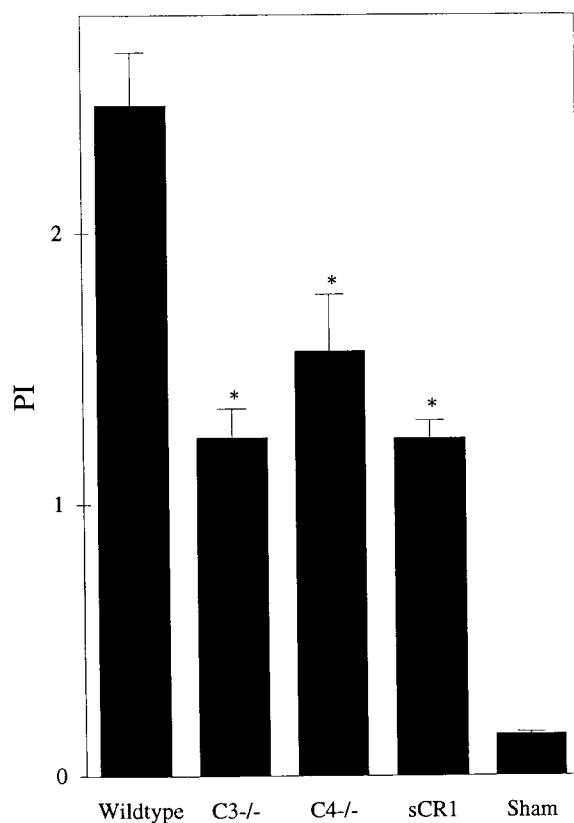
Figure 1. Ischemia/reperfusion injury is mediated by classical pathway of complement. Mice underwent 2 h of hindlimb ischemia and 3 h of reperfusion. Hindlimb P.I. was reduced significantly (P <0.05) in C3$-$/$-$, C4$-$/$-$ and sCR1 treated compared to complement sufficient littermates. The group treated with sCR1 received 20 mg/kg before tourniquet release. The hindlimb permeability index (P.I.) was determined by the ratio (CPM/g dry muscle)/(CPM/g blood). Error bars represent the standard error of the mean and significance at P <0.05 is indicated by an asterisk.
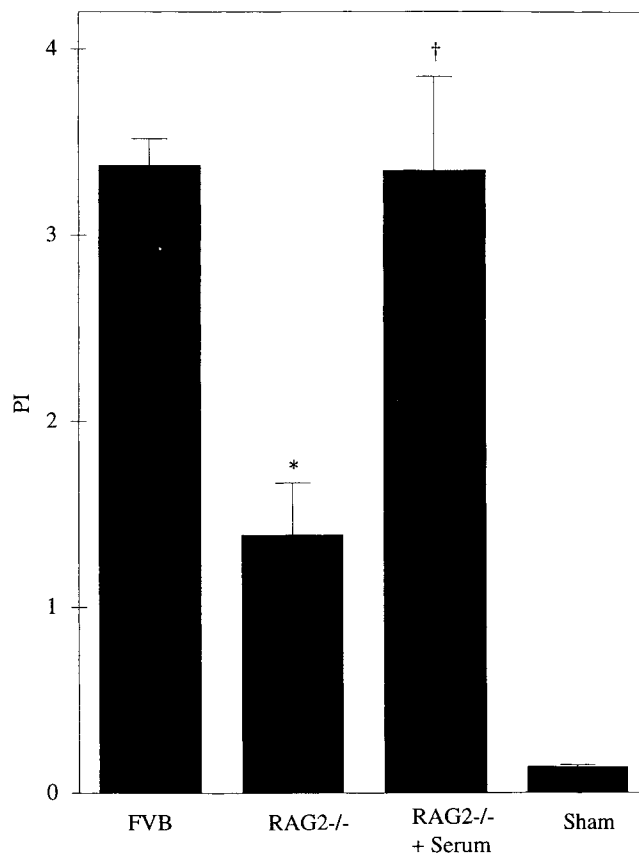
Figure 2. Antibody is important in ischemia/reperfusion injury. Ischemia/reperfusion injury was induced in RAG2$-$/$-$ or control mice as described in Fig. 1. RAG2$-$/$-$ mice (strain FVB background) were reconstituted by i.v. administration of 0.5 ml of pooled serum from control mice (strain FVB) 15 h before ischemia. RAG2$-$/$-$ mice had a significant reduction in P.I. (P <0.05) (indicated by asterisk) which was reversed by addition of normal serum (P <0.05).

ceived 1 μCi ^{125}I-labeled albumin intravenously. The group treated with sCR1 received 20 mg/kg at the same time as radiolabeled albumin. Tourniquets were removed and limb reperfusion was confirmed by return of pink color. Animals received 0.1 ml of 0.9% saline intraperitoneal during each hour or reperfusion. Following euthanasia hindlimb muscle was harvested bilaterally, its radioactivity measured, and dried to a constant weight at 90°C for 3 d. The hindlimb permeability index (P.I.) was determined by the ratio (CPM/g dry muscle)/(CPM/g blood).

Statistics. Groups were subjected to analysis of variance followed by Student's *t*-test with the Bonferroni correction for multiple comparisons.

Immunohistological Analysis. Immunoperoxidase labeling of IgM, IgG and C3 was performed on paraformaldehyde-fixed cryostat serial sections of hindlimb muscle using goat anti–mouse IgM (Sigma), goat anti–mouse IgG (Sigma) or goat anti–mouse C3 (5 μg/ml) (Organon Teknia, Durham, NC) and a standard avidin-biotin protocol (9).

Results and Discussion

Mice deficient in either C3 (C3$-$/$-$) or C4 (C4$-$/$-$) were constructed using the technique of homologous re-

combination in embryonic stem cells (ES) and both strains had undetectable levels of serum C3 (10) or C4 (Fischer, M.B., M. Ma, S. Goerg, X. Zhou, J. Xia, O. Finco, S. Hans, G. Kelsoe, R.G. Howard, T.L. Rothstein et al., manuscript submitted for publication), respectively.

The extent of complement-mediated damage in the hindlimb model of ischemia/reperfusion injury was examined by comparing the complement-deficient mice, i.e., C3$-$/$-$ and C4$-$/$-$, with normal littermates. Hindlimbs of treated animals were made ischemic for 2 h followed by 3 h of reperfusion. Subsequently, both right and left hindlimbs were harvested for analysis. Injury, i.e., vascular leakage, was evaluated by administering radiolabeled albumin immediately before reperfusion and comparing the level of radioactivity in dry muscle to that of blood.

Reperfusion of ischemic skeletal muscle resulted in endothelial cell injury characterized by vascular leakage with permeability edema. The muscle permeability index (P.I.) of normal complement-sufficient mice (n = 19) was 2.47 ± 0.19 (mean ± standard error) compared to sham animals, 0.15 ± 0.01. In contrast, C3$-$/$-$ (n = 13) animals had a significant (P <0.05) reduction in vascular leakage, i.e., P.I.
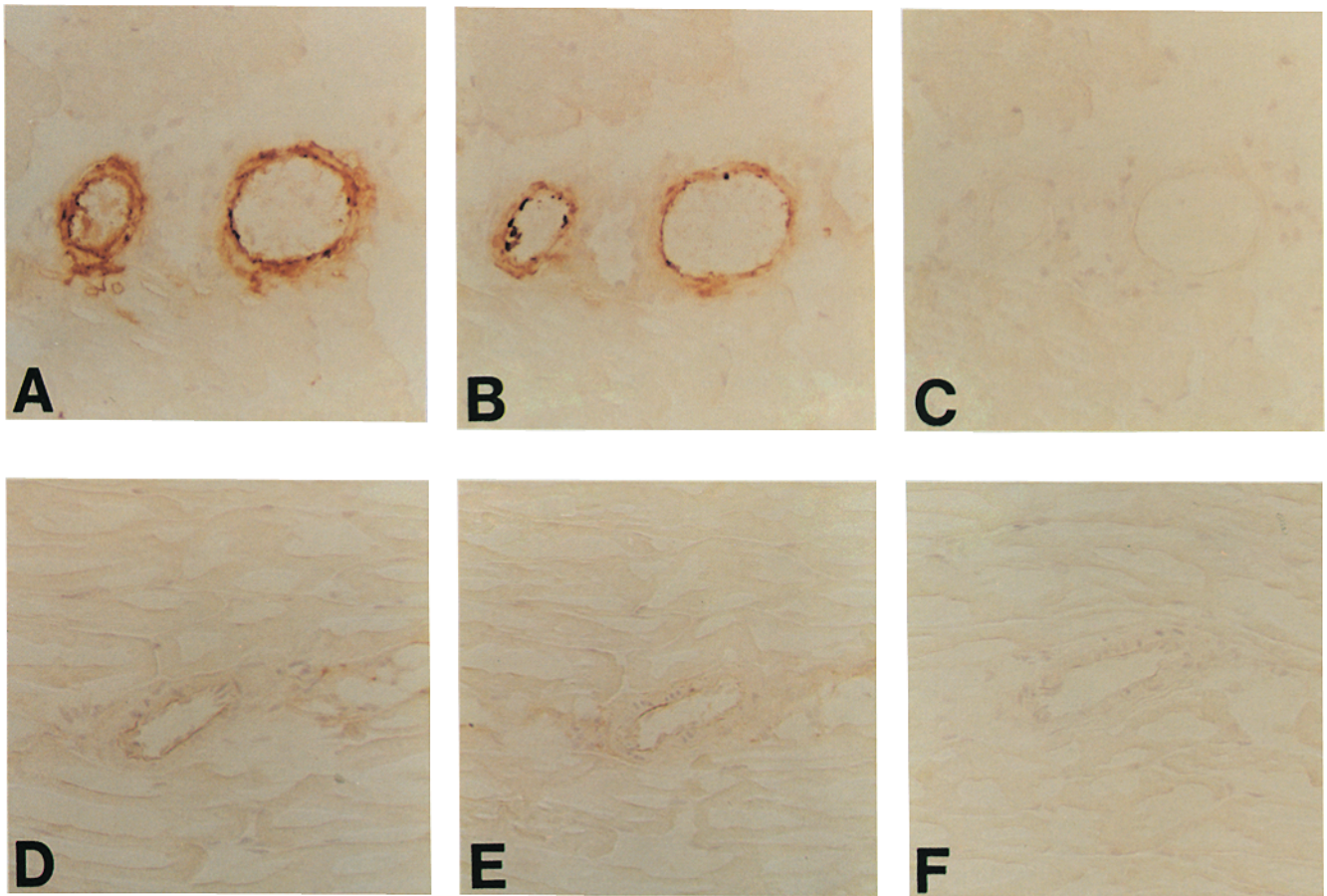
Figure 3. Ischemia/reperfusion injury results in deposition of IgM (*A*) and C3 (*B*) on vascular endothelium. Immunoperoxidase labeling of IgM and C3 was performed on paraformaldehyde-fixed cryostat serial sections of hindlimb muscle using goat anti–mouse IgM and C3 (5 μg/ml) and a standard avidin-biotin protocol (9). No staining was seen with irrelevant goat IgG (5 μg/ml) (*C*) or with muscle samples from sham mice (*D–F*, anti-IgM, anti-C3 and irrelevant goat IgG, respectively). Results were similar in three mice analyzed per group. Original magnification, 200×.

of 1.25 ± 0.11 (Fig. 1). Thus, deficiency in C3, which impairs both pathways of complement, resulted in a 50% reduction in injury. To correlate this result with earlier reports that used sCR1 to block C3 activation, complement-sufficient mice ($n = 12$) were treated with sCR1 immediately before reperfusion. Treatment with sCR1 reduced vascular injury, i.e., 1.25 ± 0.06, to a level similar to that observed in the C3−/− mice (Fig. 1).

The complement system can be activated by either the classical or the alternative pathways (11). While the classical pathway is generally activated by antibody, the alternative pathway can activate spontaneously and is tightly regulated by "regulators of complement activation" (12, 13). Therefore, as a first step towards defining the mechanism of complement-mediated ischemia/reperfusion injury, it was important to determine which pathway was being activated by hypoxic endothelium. To test the hypothesis that classical pathway was involved, C4−/− mice, which have an intact alternative pathway but can not form classical pathway C3 convertase (C2aC4b), were compared in the hindlimb model. Interestingly, the C4−/− mice ($n = 12$) were protected to a similar degree as the C3−/−− and sCR1-

treated mice, i.e., P.I. of 1.56 ± 0.21 (Fig. 1). Thus, the extent of vascular leakage due to complement injury is mediated by activation of the classical and not the alternative pathway.

A principal mechanism for activation of classical pathway of complement is serum antibody. To determine if the complement-mediated injury observed in the hindlimb model was initiated by antibody, mice deficient in recombination-activation gene 2 (RAG2−/−) (8) were analyzed. The antibody-deficient mice were protected to a similar level as the C3−/− and C4−/− mice, i.e., 1.82 ± 0.25 ($n = 8$) vs. 3.45 ± 0.17 ($n = 11$) for RAG2−/− and FVB controls, respectively (Fig. 2).

Since RAG2−/− mice also lack mature B and T lymphocytes, it was important to demonstrate that the reduction in vascular permeability was not a cellular effect. To distinguish serum from cellular effects, RAG2−/− mice were reconstituted (intravenously) with 0.5 ml of pooled normal mouse serum and treated as described above in the hindlimb model. Analysis of the mice, demonstrated that pooled serum restored the level of injury to that of normal FVB controls, i.e., 3.45 ± 0.50 ($n = 5$) vs. 3.45 ± 0.17 ($n =$

11), respectively (Fig. 2). Thus, expression of normal serum immunoglobulin is required for ischemia/reperfusion injury in the hindlimb model.

To further evaluate the nature of the antibody involved in injury to hypoxic endothelium, sections of reperfused or sham control hindlimb tissues were stained with antisera specific for mouse IgG or IgM. As shown in Fig. 3 A, IgM was deposited on vascular endothelium of hindlimb harvested from reperfused mice but absent in sham controls (Fig. 3 D). Although staining for IgG did not appear specific in the reperfused hindlimb, a role for IgG can not be excluded (data not shown). This result demonstrates that IgM binds to local vascular endothelium following reperfusion of ischemic hindlimb and supports the finding above that reconstitution of RAG2−/− mice with normal serum restores their inflammatory response.

Further support for a role of antibody and complement in mediating injury, comes from the results of immunostaining sections of injured hindlimb with antibody to C3. C3 deposition colocalized with IgM on the local vascular endothelium of reperfused complement-sufficient mice (Fig. 3 B) but was absent in sham controls (Fig. 3 E) and treated C3−/− mice as expected (data not shown). In addition, C3 deposition was seen on the skeletal muscle of reperfused hindlimbs of control mice but it is not clear if this represents C3 activated by classical or alternative pathway since deposition of IgM was not observed in this region.

The most probable explanation for the observations that deficiency in either classical pathway complement, i.e., C3−/− or C4−/−, or serum antibody impairs ischemia/reperfusion injury by a similar level (50%), is that inflammation is initiated by deposition of antibody on hypoxic endothelium. While the remaining 50% of vascular permeability is not explained by these results, it seems highly unlikely that the observed complement- and antibody-mediated injuries are independent of each other. Two major mechanisms by which antibody initiates inflammation are activation of classical pathway complement, i.e., binding of C1q, or binding to Fc receptors (FcR) on leukocytes. The later effector mechanism has been described for the IgG and IgE isotypes but not for IgM (14). Thus, binding of IgM in the absence of complement would not be expected to induce inflammation and vascular leakage.

A possible explanation for the binding of IgM antibody in reperfused vessels is that novel antigenic determinants may be exposed as a result of subtle alterations in the plasma membrane following ischemia. Two possible mechanisms for hypoxia-mediated membrane disruption that are not mutually exclusive are: (a) a reduction in phospholipid biosynthesis, or (b) activation of Ca^{++}-dependent phospholipase and proteases. Alternatively, hypoxia induces endothelial cell release of preformed products or synthesis of novel membrane-associated proteins (15). These novel determinants could serve as targets for binding of circulating natural (preexisting) antibody. Although the function of

natural antibodies is not clear, it is generally held that they represent the product of germ-line genes (16) and they are often implicated in autoimmune vasculitis (17). Anti-endothelial cell (anti-phospholipid) antibodies have been found in sera of normal individuals and it has been postulated that alteration in the membrane of endothelial cells whether by exposure of existing negatively charged phospholipids or by induction of new determinants results in specific binding (17–21). Thus, in the proposed model, reperfusion of hypoxic vessels results in binding of natural antibody and activation of the classical pathway of complement.

Previous studies examining activation of complement in various models of ischemia/reperfusion have found evidence in support of both pathways. For example, Lindsay et al. found that classical but not alternative pathway of complement was consumed in their model of hindlimb ischemia in the rat (4). In contrast, studies using a mouse skeletal model (cremaster) of ischemia/reperfusion concluded that alternative pathway was responsible for injury since serum from mice treated with sCR1 retained classical but not alternative pathway activity in an in vitro hemolytic assay (6). Similarly, in a pig myocardial infarct model of reperfusion injury, activated factor B (alternative pathway) was found in serum of ischemic but not sham controls within 15 min after reperfusion (22). While it is possible that the pathogenesis of injury varies in each model, a unifying explanation is that classical pathway complement is required for initial activation, i.e., by formation of C3 convertase, and the alternative pathway is subsequently activated and components such as factor B are consumed. Other differences such as blocking of classical pathway in vivo but not in vitro could be explained by the sensitivity of the different assays.

Activation of complement has varied effects on initiation of local injury. Integration of the terminal components of complement C5–C9 in the membrane of vascular endothelium disrupts cell function and leads to injury as discussed above (1, 2). In addition, release of anaphylatoxins C3a and C5a induce vascular leakage and enhance infiltration and activation of neutrophils (11). Moreover, adhesion and extravasation of neutrophils is enhanced by covalent deposition of iC3b at the site of activation (23). A role for neutrophils in ischemia/reperfusion injury has been demonstrated as depletion or blocking of neutrophil adhesion with antibody to B_2 integrins or L-selectin reduces inflammation (24–27). It is likely that neutrophil-mediated injury is in part independent of complement activation and this would explain the residual vascular leakage in the complement- and antibody-deficient mice.

To our knowledge this is the first demonstration that serum antibody is directly involved in ischemia/reperfusion injury. Thus, the novel concept that is emerging is that serum antibody recognizes neo-determinants on damaged endothelium (owing to hypoxia in our model) and on binding to the cell surface initiates inflammation by activating the classical pathway of complement.

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