IgM Binding to Injured Tissue Precedes Complement Activation during Skeletal Muscle Ischemia–Reperfusion


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Submitted for publication December 22, 2003

Background. Skeletal muscle reperfusion injury is mediated by IgM natural antibodies and by complement activation, as shown by the attenuation of reperfusion injury seen in mice with no natural IgM [1] and in mice deficient in complement C3 and C4 [2]. We postulate that tissue, when ischemic, expresses neoantigens to which preformed natural IgM antibodies bind, in turn producing harmful complement activation and reperfusion injury.

Materials and methods. C57Bl/6 mice were subjected to 2 h of tourniquet-induced hind limb ischemia followed by variable periods of reperfusion. Two hours of ischemia and 3 h of reperfusion produced severe muscle necrosis and edema. Deposition of IgM and C3 in tissue was assessed using immunohistochemistry on both frozen and Formalin-fixed tissue samples.

Results. IgM binding to the endothelium and muscle bundles of the hind limb began during the ischemic period and continued throughout reperfusion up to 6 h. C3 deposition was not present during ischemia and, in contrast, began to appear at 1 h of reperfusion and increased progressively thereafter.

Conclusions. These data demonstrate that IgM binding to ischemic tissues precedes the damaging complement activation by a significant period of time. This has important therapeutic implications when considering anti-inflammatory therapy for reperfusion injury.

Key Words: ischemia/reperfusion; skeletal muscle; timecourse; antibody; complement.

INTRODUCTION

Skeletal muscle ischemia reperfusion is an important clinical problem that may result in significant morbidity and mortality. Its local effects result in muscle fiber disruption and edema leading to compartment syndrome and limb loss. Remotely, reperfusion injury results in a systemic inflammatory syndrome with resulting respiratory compromise and myoglobin-induced renal failure [3].

The classical complement pathway and IgM natural antibodies are critical in the pathogenesis of skeletal muscle reperfusion injury. The central role that complement plays was demonstrated initially by the attenuation of injury achieved with the complement inhibitor sCR1 [4] and that seen in C3 knockout mice [2]. To a similar extent, the purely classical pathway-deficient C4-/− mice are spared local and remote pulmonary injury [2], establishing that the pathway to complement activation is antibody-initiated.

Antibody-deficient rag-/- animals and natural antibody-deficient cr2-/- animals also prove to have significant reductions in hind limb reperfusion injury [1, 5]. Rag-/- mice lack all classes of antibody. Cr2-/- animals lack the CD21/CD35 complement receptor 2, which results in both a decreased repertoire of CD5− B-1 cells and an altered repertoire of circulating IgM. While the total IgM concentration remains the same, these mice lack IgM natural antibodies produced by peritoneal B-1 cells. Reconstitution of these mice with normal IgM or natural antibody producing peritoneal B-1 cells will recreate the injury phenotype after ischemia–reperfusion. This is also the case with rag-/- mice. Reconstitution with IgG, however, fails to recreate the injury in either antibody-deficient strain [1].

We postulate that ischemic tissue expresses one or more neoepitopes that are recognized by circulating natural IgM. The resulting membrane-bound antigen...
antibody complex is a potent activator of complement which triggers the sequence of events culminating in the tissue injury of reperfusion. The purpose of this study is to examine the time course of IgM binding in the injured tissue in comparison to the time course of complement deposition. We now show that IgM deposits during ischemia and precedes the complement activation. Complement activation occurs only after the onset of reperfusion. This observation strengthens our hypothesis.

MATERIALS AND METHODS

Animals

Male C57/Bl6 mice (Taconic Farma, Germantown, NY) were used for all experiments. 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 Institute of Health), revised 1985].

Animal Experiment

Mice aged 6–8 weeks, weighing 25–30 g, were anesthetized with intraperitoneal ketamine (10 mg/kg) and xylazine (20 mg/kg) and underwent 2 h of hind limb ischemia followed by a variable period of reperfusion. Longer periods of ischemia result in excess mortality of mice during the experimental period. After a 2-min period of hind limb elevation, bilateral double rubber bands (Latex O-rings) were applied above the greater trochanter using the McGivney Hemorrhoid Ligator (Miltex, York, PA). Sham mice did not undergo banding. Mice were maintained in a supine position and kept anesthetized throughout the course of the experiment. The hind limbs of the animals were harvested at the end of the reperfusion period following euthanasia by an overdose of ketamine/xylazine by injection. The hind limbs harvested were used for histological and immunohistochemical analysis for IgM and complement C3 deposition.

Permeability Studies

Five minutes before tourniquet release, animals received 1 μCi of 125I-labeled human albumin (ICN, Irving, CA) in 0.3 cc of normal saline via tail vein injection. After the reperfusion period, blood was aspirated from the right ventricle and its gamma radioactivity counted (Packard, Downers Grove, IL) and weighed. Muscle was harvested from both hind limbs, its radioactivity measured, and then it was dried to a constant weight in a gravity convection oven (Precision Scientific Group, Chicago, IL) at 100°C for 2 nights. Extravasation of 125I-labeled albumin was used to assess the hind limb vascular permeability index, which was determined by the ratio of radioactivity per gram of dry muscle to radioactivity per gram of blood.

Pathological Processing and Immunohistochemistry

For frozen sections, the gastrocnemius muscle was dissected and immersed in OCT and then snap frozen a dry ice/2-methylbutane bath. For permanent sections, the hind limbs were fixed in 4% paraformaldehyde in PBS for 18 h and changed to PBS (Sigma, St. Louis, MO) and kept at 4°C. The gastrocnemius muscle was embedded in paraffin and sections were cut. Sections were baked at 60°C for 1 h prior to deparaffinization and hydration; 10 mM sodium
citrate, pH 6.0, heated to 100°C was used for antigen retrieval. For IgM staining, the sections were blocked in 1.5% horse serum (Vector Labs, Burlingame, CA) for 1 h, incubated in a 1:25 dilution of biotinylated goat antimouse IgM (Jackson Laboratory, Bar Harbor ME) for 1 h, and developed with the Vectorstain ABC reagent. Exclusion of primary antibody and biotinylated antimouse IgG only were used to assess nonspecific staining or peroxidase activity. For C3 staining, 5% rabbit serum was used for blocking and goat antimouse C3 (a gift from Lester Kobzik, Harvard School of Public Health, Boston, MA) was used at a concentration of 1 μg/cc overnight at 4°C. Rabbit antigoat IgG (at 1:50) was used for 1 h and the reaction was developed with a goat peroxidase–antiperoxidase complex (Sternberger Monoclonals, Lutherville, MD). To maximize the stain visibility, no counterstaining was used.

**Scoring**

Histological damage was scored on a 5-point system (0–4) by blinded observers where 0 = no injury, 1 = disruption of <10% of fibers, 2 = 10–50% of muscle fibers disrupted, 3 = >50% of muscle fibers disrupted, and 4 = 100% of muscle fibers disrupted and unrecognizable muscle structure. Briefly, a microscopic grid was placed over a ×10 microscopic section, and injury was determined by counting the total number of boxes with injured fibers over the total number of boxes occupied by muscle bundles.

The immunohistochemical deposition of C3 and IgM was scored on a similar 5-point system (0–4), where 0 = no deposition and 4 = deposition on all fibers.

**RESULTS**

**Animals Subjected to Ischemia Alone Showed No Signs of Injury, while Progressive Injury Was Seen Only after the Start of Reperfusion**

As shown previously, ischemia alone is not sufficient to produce histological muscle injury. Microscopic analysis of mouse hind limb cross sections with 2 h of ischemia showed no structural evidence of muscle fiber damage (Fig. 1B) and was similar to that in uninjured controls (Fig. 1A). After 1 h of reperfusion, an increase in muscle diameter was seen corresponding to cellular swelling. Structural disruptions were seen in a few isolated fibers (Fig. 1C). By 3 h, there was a diffuse but non-uniform pattern of muscle fiber disruption, tissue edema, and rhabdomyolysis (Fig. 1D). There was little observable infiltration of neutrophils.

**Progressive IgM Staining Begins during Ischemia and Progressive C3 Deposition Begins during Reperfusion**

IgM deposition was demonstrated by immunohistochemistry as early as 5 min into ischemia and increased progressively in intensity and in distribution until the end of the 2-h ischemic period (Figs. 2A(i) and 2A(ii)). There was continued IgM deposition throughout reperfusion (Figs. 2A(iii–vi) and 4B) which began to diminish late in reperfusion, likely a secondary effect from the diminishing number of intact fibers. In contrast, complement C3 deposition was not demonstrated at all or only in small amounts during the ischemic period (Figs. 2B(i) and 2B(ii)) and then increased dramatically after the start of reperfusion (Figs. 2B(iii–vi) and 4C). These changes were seen in both the paraffin sections and the cryostat sections (Fig. 3).

In the cryostat sections, IgM deposition was localized to the rim of the muscle fibers, while C3 staining was seen throughout the fiber (Fig. 3). This pattern of staining was also seen in the paraffin sections (Fig. 2).
corresponding paraffin section, however, the rimlike staining of IgM was localized to cells surrounding the muscle fibers. These were most likely endothelial cells, although their exact nature still needs further characterization. Intense IgM staining was seen in postcapillary venules during ischemia (Fig. 3A(iii)) and complement deposition was seen in these venules following reperfusion only (Figs. 3B(ii) and 3B(i)).

Previous studies have shown that the endothelial surface is the likely site of initial IgM deposition [2]. Our data support this hypothesis due to early IgM deposition around and not within muscle fibers. Pentameric IgM has an estimated molecular weight of 900 kDa, too large to cross an intact endothelium. Complement deposition and injury at the endothelial surface at the onset of reperfusion would result in impaired endothelial integrity, thus allowing IgM access to the interstitium. This would also produce tissue edema, as water, electrolyte, and other proteins also cross this barrier. Figure 5 shows the time-dependent increase in

FIG. 3. Immunoperoxidase identification of IgM binding (A) and C3 binding (B) on cryostat sections of murine hind limb at 2 h of ischemia (i), 2 h of ischemia and 1 h of reperfusion (ii), 3 h of reperfusion (iii), and 6 h of reperfusion (iv) (original magnification, ×10). (Color version of figure is available online.)
endothelial permeability after reperfusion, as assessed by extravasation of a smaller protein, radiolabeled albumin.

For each stained section, the corresponding negative control lacking primary antibody, as well as the negative control using nonspecific goat IgG, was performed and did not show any staining (data not shown). Immunohistochemistry for murine IgG showed minimal deposition late in ischemia corresponding to areas of IgM/C3 deposition but variable and trivial staining during reperfusion (Fig. 4D). This may be the result of nonspecific general permeability to macromolecules as a result of injury.

DISCUSSION

Ischemia alone is not sufficient to lead to histological evidence of injury. Reperfusion is required, an observation that has led to the term “reperfusion injury.” This is shown again in our murine hind limb model (Fig. 1). Intuitively, however, ischemia–reperfusion should be a metabolic injury and histological changes should be seen during ischemia. One might then argue that the injury in this model is simply submaximal and that histology is insensitive to the changes occurring. However, longer periods of ischemia lead to such significant mortality that few animals survive to be studied. Given that, one must postulate instead that there is a tissue change that occurs during ischemia that then triggers far more damage upon reperfusion. The putative agent of this damage is the innate immune or inflammatory system, as shown in multiple studies where reperfusion injury is attenuated by pretreatment of animals with various anti-inflammatory agents. The dependence of reperfusion injury on complement activation [6–10], specifically classical path-

FIG. 4. (A) Amount of histological muscle damage, (B) IgM deposition, (C) C3 deposition, and (D) IgG deposition over an increasing period of ischemia and reperfusion.
way [2] activation, has been well documented in a number of organs, including intestine, heart, liver [11–13], and skeletal muscle. The dependence on the presence of IgM natural antibodies is a new finding by our investigative group [1] and by the Holers group in Denver [14]. This has led to the hypothesis that ischemia induces expression of neoepitopes in involved tissue, epitopes which are recognized by circulating IgM. With the onset of reperfusion, complement activation ensues, followed by tissue injury. If one is to base a therapy on interfering with the IgM–epitope interaction, it becomes important to know whether IgM binding significantly precedes complement activation or whether they are simultaneous.

Here, we demonstrate that IgM binding indeed precedes complement activation in a time course study of skeletal muscle ischemia reperfusion. In Fig. 2, it is shown that the initial deposition of IgM begins surprisingly early, at 5 min, so early as to suggest that there is no metabolic basis for reperfusion injury in terms of depletion of cellular substrate. There is no accompanying C3 deposition until after perfusion has been reestablished. This temporal pattern is also seen in Fig. 3. This indicates that there is sufficient IgM present in tissue to lead to detectable binding in the absence of blood flow. This suggests that the involved IgM may be relatively abundant. In contrast, sufficient complement components to produce detectable activation are not present without blood flow. The temporal relationships are shown graphically in Fig. 4, supporting the conclusion that there is a definite sequence of events in the production of reperfusion injury. IgM leads to binding of IgM, followed by complement activation and cell injury with reperfusion.

Other models of lower extremity ischemia reperfusion have been reported which use vascular occlusion instead of tourniquet occlusion. An advantage to tourniquet-induced ischemia is that all feeding vessels to the hind limb are occluded with the exception of the intramedullary artery that runs inside the femur; there are no muscle collaterals. In comparison to models that occlude only the femoral artery, there is a greater amount of muscle mass at risk for local and remote reperfusion injury. Furthermore, models with significant collaterals in place could exhibit reperfusion injury throughout the period of occlusion. Thus the tourniquet model provides the best opportunity to study the temporal sequence of events, as it has virtually total arterial occlusion through the entire hind limb. Also, there is a theoretical possibility of the confounding effects of venous hypertension since flow through the femoral vein will also be stopped by the tourniquet. However, in the absence of any gross or histological changes following ischemia alone (Fig. 1B), clinically significant venous congestion is not likely present and would not contribute to a preferential delay in complement activation.

The deposition of IgM and C3 is demonstrated both on endothelium and on muscle bundles, indicating a possible common mechanism of injury, such as expression of a neoepitope. Were ischemia in this circumstance to be a metabolic injury, one might expect preferential localization to the more metabolically active muscle. Further demonstration of the endothelial injury is seen in the rapid accumulation of extravascular fluid after reperfusion injury in the same mice, as demonstrated by the extravasation of radiolabeled albumin in Fig. 5. Thus, reperfusion injury is simultaneous in two different hind limb cell types, muscle and endothelium, and they share a common dependency on IgM and complement, strongly suggesting a common mechanism of injury.

This temporal sequence of IgM binding and complement activation has significant consequences when deciding when and what drug should be administered. There are three general categories of patients with clinical reperfusion injury—patients who have yet to undergo ischemia and reperfusion, patients who have undergone ischemia but not reperfusion, and those who have undergone both ischemia and then reperfusion. The first category includes those who are about to undergo various surgical procedures, such as coronary artery bypass, free tissue transfer, aortic surgery requiring arterial cross clamping, and lower extremity bypasses. These patients might best be treated with the prophylactic use of an anti-IgM strategy, such as a soluble ischemia antigen or a genetically engineered IgM molecule that lacks the ability to activate complement.

The second category includes patients who have been ischemic but have not undergone reperfusion, such as a patient who has suffered an acute myocardial infarction before angioplasty, lower extremity clot before thromboembolectomy, or an occluded lower extremity bypass.
graft before graft revision. In this situation, IgM binding has already occurred and complement inhibitors would be the drug of choice.

The final category of patients has already suffered a degree of reperfusion injury and the use of inhibitors of downstream targets would avoid further injuries. Examples of this category include hypotensive trauma patients who have already been resuscitated and previously ischemic limbs that have resumed perfusion.

IgM natural antibodies and complement activation are critical in the mediation of reperfusion injury. Characterization of their temporal sequence of binding and activation is important for rational drug design and administration. In the future, it will be important to elucidate upstream and downstream effectors to develop multiple therapies to most effectively curb injury.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Grant P50 GM52585 and the Trauma Research Foundation.

REFERENCES


