

Reperfusion Injury to Skeletal Muscle Affects Primarily Type II Muscle Fibers¹

Rodney K. Chan, William G. Austen Jr., Shahrul Ibrahim, Grace Y. Ding, Nicola Verna, Herbert B. Hechtman, and Francis D. Moore Jr.²

Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Submitted for publication February 22, 2004

Introduction. The injury caused by reperfusion of ischemic skeletal muscle is mediated by the membrane attack complex of complement (C) [1]. This C activation results from local classical pathway activation after deposition of IgM in injured muscle, an event analogous to C deposition in the mucosa of the gut during reperfusion [2]. Our past analysis has indicated that the injury is not uniform even within a single microscopic section. This study was performed to elucidate the exact site of IgM and C deposition on muscle injured by ischemia and reperfusion.

Materials and methods. C57Bl/6 mice were subjected to 2 h of tourniquet-induced hindlimb ischemia followed by reperfusion for 0–6 h. Three muscle groups (vastus, gastrocnemius, and soleus) of varying fast-myosin content were compared for muscle fiber damage and C deposition. Adjacent paraffin-embedded cross-sections were immunostained to correlate C3 deposition with muscle fiber type as defined by monoclonal antibodies.

Results. Muscle injury after ischemia and reperfusion is not uniform and not all fibers in the same microscopic field are affected. Damaged fibers are also those to which IgM and C bind. Immunostaining for slow-twitch (Type 1) or fast-twitch (Type 2) fibers reveals that injury and C3 deposition is confined to Type 2 fibers with lower myosin content. A correlation of Type 2 fiber content and degree of muscle injury showed that the predominantly fast-twitch vastus muscle had the greatest number of damaged fibers per $\times 10$ field (28.2 ± 12.4) when compared to the mixed

fiber-type gastrocnemius muscle (20.5 ± 5.3) and the mixed, but slow-twitch enriched soleus muscle (17.3 ± 11.8).

Conclusion. Complement activation and skeletal muscle reperfusion injury occurs predominantly on Type 2 fibers with low myosin content. This suggests that attempts to control the post-reperfusion inflammation will likely produce substantial muscle recovery. Furthermore, the basis of IgM deposition and complement activation may be revealed in the comparison of the two muscle fiber types. © 2004 Elsevier Inc. All rights reserved.

Key Words: reperfusion; fast-twitch; skeletal muscle ischemia; complement; antibody.

INTRODUCTION

Skeletal muscle ischemia and reperfusion is an important clinical problem that may result in significant morbidity and mortality. Its local effects can lead to compartment syndrome, rhabdomyolysis with renal failure, and limb loss. There is also a remote injury component that can result in the systemic inflammatory syndrome with respiratory and renal compromise, a feature shared also with mesenteric and other major organ reperfusion injuries.

The inflammatory basis of reperfusion injury has been established with immunological studies. Mice deficient in complement C3 are spared both local and remote injury after lower extremity ischemia [1]. To a similar extent, the administration of a C1 or C3 inhibitor [3, 4] or the use of the classical pathway-specific C4 knockout mouse will result in spared local and remote injury. This classical pathway activation is known to be triggered by IgM immune complexes. Antibody-deficient *rag*^{-/-} animals and natural antibody deficient *cr2*^{-/-} animals have attenuated injury. Recon-

¹ This article contains data that were accepted for presentation at the 37th Annual Meeting of the Association for Academic Surgery.

² To whom correspondence and reprint requests should be addressed at Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. E-mail: fmoore@partners.org.

stitution of these mice with normal IgM or natural antibody producing peritoneal B-1 cells (but not normal IgG) will reproduce the injury phenotype in intestinal ischemia-reperfusion [5].

It has been noted that the histological appearance of reperfusion injury to skeletal muscle [6] as well as other organs including the heart [7], kidney [8], brain [9], and liver [10] is in a patchy distribution with sparing of tissue amid cellular necrosis. Explanation as to why neighboring tissue exposed to the same ischemic insult and subsequent reperfusion might exhibit different degrees of injury is lacking.

It is our experience that this patchy distribution is also a key feature in our model of skeletal muscle ischemia reperfusion fibers. Fast-twitch (Type 2) myosin fibers are glycolysis-dependent muscle cells of locomotion; slow-twitch (Type 1) myosin fibers are capable of sustained contraction and are the muscle cells of posture. In this study, we hypothesize that this injury pattern is due to varying sensitivity of muscle bundles depending on myosin content. Studies of fast and slow myosin-rich fibers following ischemia and reperfusion exist in the literature but the conclusions are conflicting. Using immunohistochemical techniques, we are able to correlate C3 deposition as a marker of injury to Type 2 muscle fibers with low myosin content.

MATERIALS AND METHODS

Animals

Male C57/Bl6 mice purchased from Taconic Farms (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 (120 mg/kg) and xylazine (20 mg/kg) and underwent 2 h of hindlimb ischemia followed by a variable period of reperfusion. After a 2-min period of hindlimb elevation to minimize retained blood, 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 hindlimbs of the animals were harvested at the end of the reperfusion period following euthanasia by an overdose of ketamine/xylazine injection. The hindlimbs harvested were used for vital staining, histological and immunohistochemical analysis for IgM, C3 deposition, and fast and slow myosin ($n = 8$). The vastus, gastrocnemius, and soleus muscle groups were dissected separately and analyzed for degree of injury ($n = 4$).

Immunohistochemistry

Hindlimbs were fixed in 4% paraformaldehyde in PBS for 18 h. The gastrocnemius muscle was embedded in paraffin and sections were cut.

These were baked at 60°C for 1 h prior to deparaffinization and hydration. Sodium citrate (10 mM) pH 6.0 heated to 100°C was used to for antigen retrieval. For IgM staining, the sections were blocked in 1.5% horse serum for 1 h, incubated in 1:25 dilution of biotinylated goat anti-mouse IgM (Jackson Laboratory, Bar Harbor, ME) for 1 h and developed with Vectorstain ABC (Vector Labs, Burlingame, CA). No primary antibody or biotinylated anti-mouse IgG was used to assess for non-specific staining or spontaneous peroxidase activity, respectively. For C3 staining, 5% rabbit serum was used for blocking and 1 μ g/mL goat anti-mouse C3 (gift from Lester Kobzik, M.D.) was used overnight at 4°C. Rabbit anti-goat IgG (1:50) was added and after 1 h the reaction was developed with goat peroxidase–anti-peroxidase complex (Sternberger Monoclonals, Lutherville, MD). For fiber typing, monoclonal mouse anti-fast myosin 1:800 (Clone MY-32, Sigma) and mouse monoclonal anti-slow myosin 1:10,000 (Clone NOQ7.5.4D, Sigma) were used as primary antibodies in conjunction with a mouse-on-mouse kit (Vector Labs).

To assess the viability of the reperfused muscle, nitroblue tetrazolium (NBT, Sigma), a histochemical stain for mitochondrial enzyme activity, was used as previously described [11]. Briefly, 2-mm transverse sections of snap-frozen tissue were placed in a solution of NBT dye (0.033%) and NADH (0.133%, Sigma) at room temperature for 10 min. These were then fixed in 4% paraformaldehyde in PBS and visualized at $\times 4$ magnification.

For the correlation of C3 staining with fiber type classification, murine hindlimbs ($n = 8$) were harvested after 2 h of ischemia and 1 h of reperfusion. Muscle types were classified based on 50 C3-stained fibers/slide.

Pathology

JB-4 glycol methacrylate embedded sections were also stained with Masson Trichrome for histological assessment of muscle fiber integrity ($n = 4$ /muscle group). Fifty individual muscle fibers were counted in each histological section, and the extent of injury was expressed as the number of damaged fibers/50 fibers counted.

Statistics

Results are presented as means \pm standard error in the text and figures. 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.

RESULTS

Animals Subjected to Ischemia and Reperfusion Demonstrate a Patchy Pattern of Injury

After 2 h of ischemia and 3 h of reperfusion, there is a diffuse but non-uniform pattern of muscle fiber disruption, tissue edema, and rhabdomyolysis (Fig. 1). A similar pattern of injury is revealed when fresh tissue is stained for mitochondrial oxidative activity, in this case, using loss of functioning mitochondria as another indicator of injury (Fig. 2). Similarly, immunohistochemical staining for IgM and C3 deposition also revealed a patchy checkerboard distribution of injury (Fig. 3). By all three techniques of visualizing injured muscle, the same number of fibers are abnormal and in the same pattern. By staining serial sections, it was noted that fibers staining positive for IgM deposition also stained positive for C3 deposition (Fig. 3). Thus, the inflammatory component of the reperfusion injury has the same overall uneven distribution as

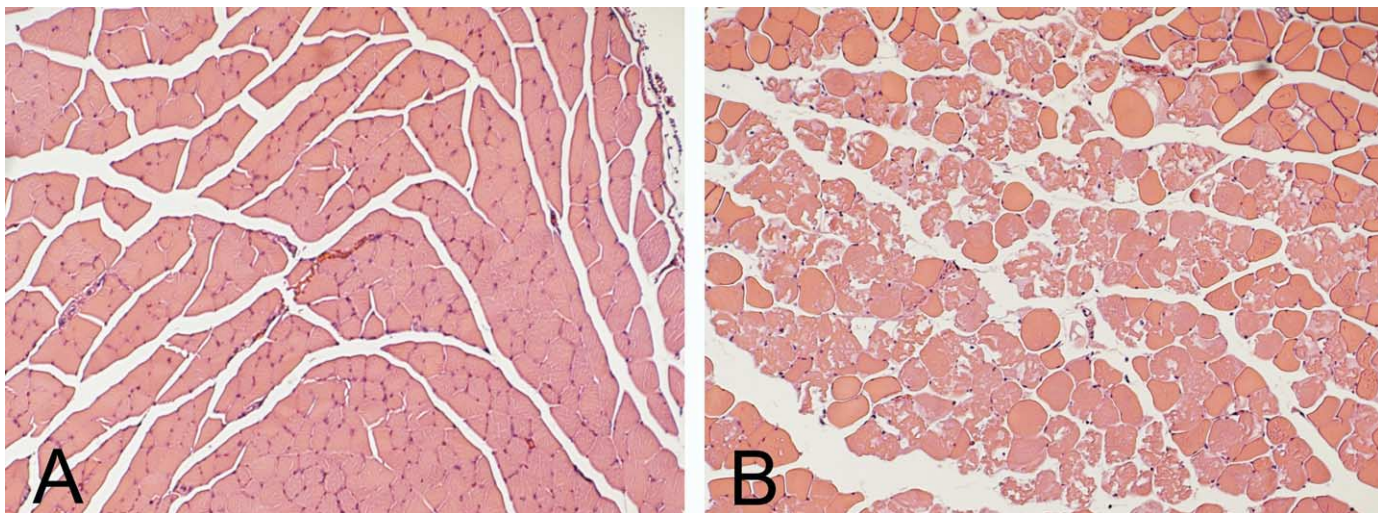


FIG. 1. $\times 10$ microscopic section of murine hindlimb skeletal muscle subjected to 2 h of ischemia and 3 h of reperfusion, stained with H&E (B). This demonstrates widespread rhabdomyolysis in a patchy and non-uniform distribution in comparison to uninjured control (A).

that seen with conventional histology or by functional staining for loss of intracellular enzyme.

IgM and Complement Deposition are Confined to Type 2 Fast Twitch Muscle Fibers with Low Myosin Content

To determine whether reperfusion injury, as defined by the deposition of inflammatory mediators, was confined to a particular fiber type, tissue sections were stained for fast-twitch and slow-twitch myosin content with monoclonal antibodies. Adjacent serial sections were stained for C3. C3 deposition occurred almost exclusively on Type 2 muscle fibers with low myosin content (Fig. 4). Among 50 fibers counted with C3 deposition in mouse hindlimbs after 1 h of reperfusion, 42.4 ± 4.80 were fast twitch with low myosin content; 2.22 ± 2.20 were fast twitch with

high myosin content and 5.34 ± 3.84 were slow twitch. There is a far significantly greater deposition of C3 on fast twitch fibers with low myosin content, $P < 0.01$ (Fig. 5).

Overall Muscle Reperfusion Injury Correlates with Muscle Content of Type 2 Fibers

In analyzing the vastus, gastrocnemius, and soleus muscle groups, the predominantly fast-twitch vastus muscle had the greatest number of damaged fibers per $\times 10$ field (28.2 ± 12.4) when compared to the mixed fiber-type gastrocnemius muscle (20.5 ± 5.3) and the mixed, but slow-twitch enriched soleus muscle (17.3 ± 11.8) (Fig. 6). While there is a trend toward greater injury

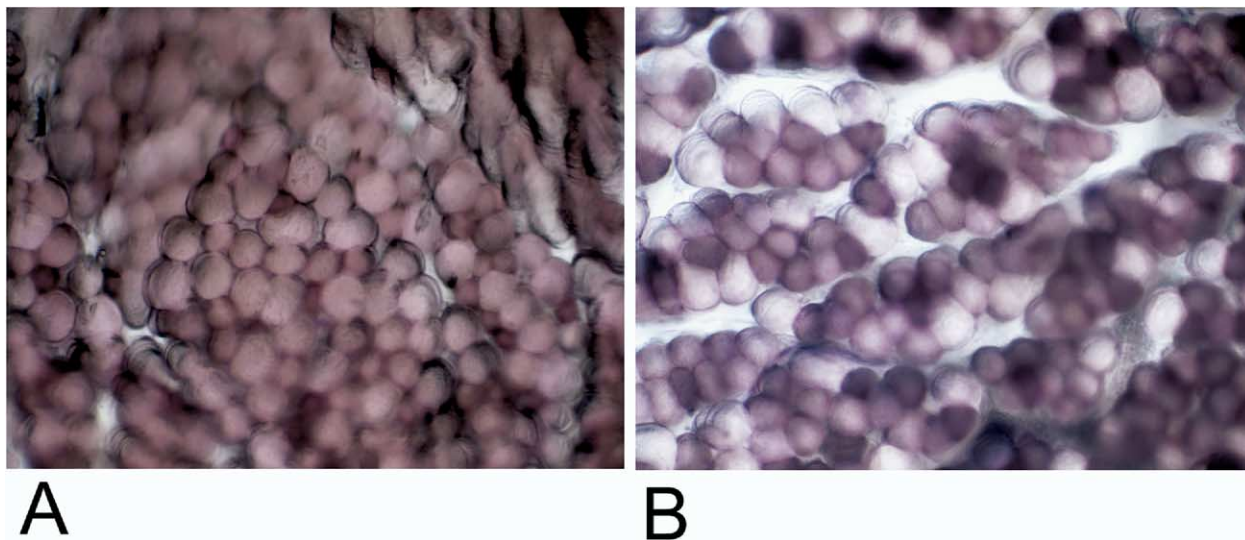


FIG. 2. $\times 10$ microscopic section of normal (A) and reperfused (B) murine hindlimb stained with the vital dye, nitroblue tetrazolium. Injured tissue (B) shows selective loss of viability, varying fiber by fiber.

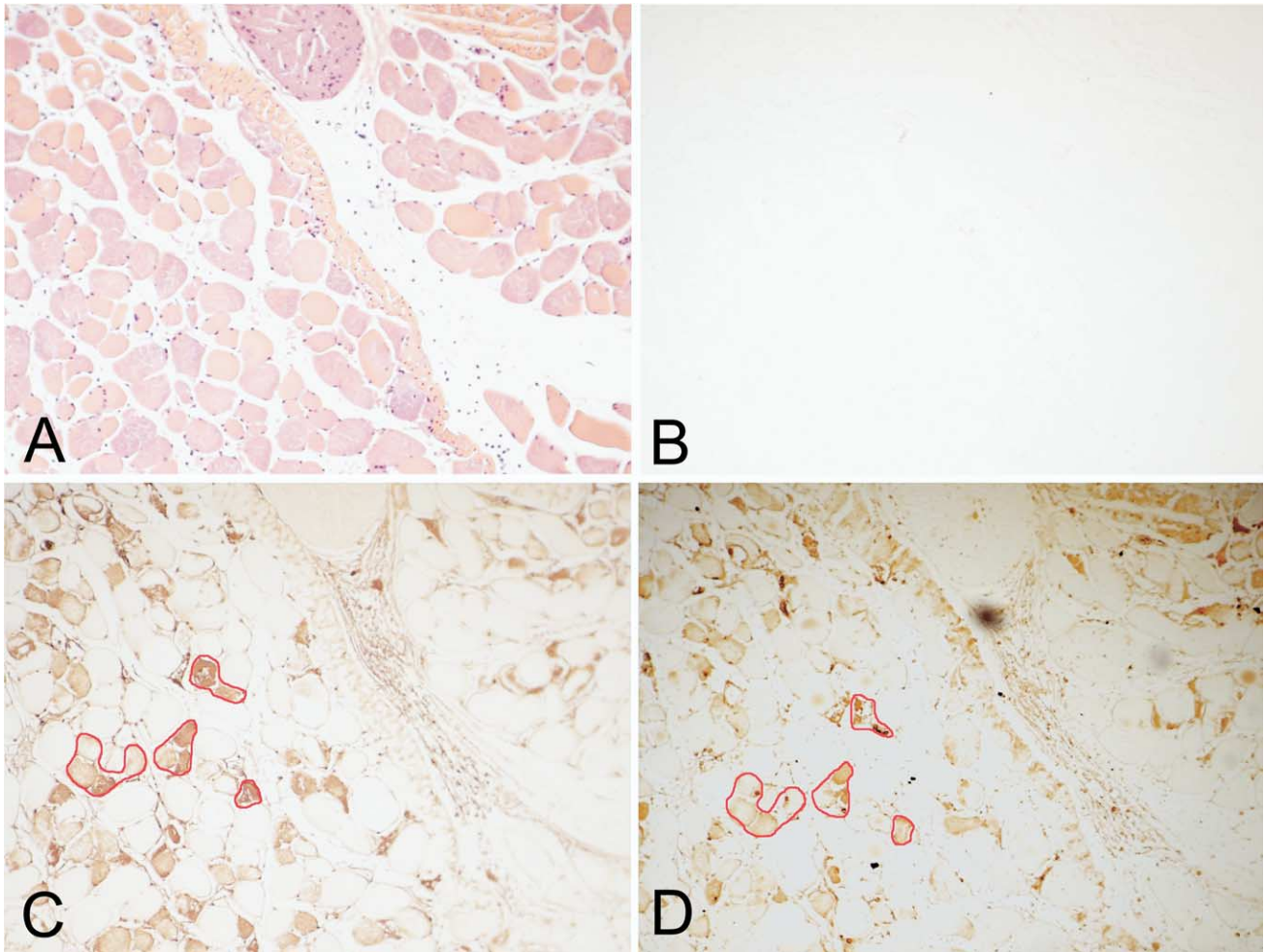


FIG. 3. Serial sections of reperfused murine hindlimb muscle at $\times 10$ magnification stained with H&E (A), immunoperoxidase with non-specific goat IgG (B), immunoperoxidase with horse anti-mouse IgM (C), and immunoperoxidase with goat anti-mouse C3 (D). Red highlights denote examples of contiguous muscle fibers seen on two different slides. IgM deposition and complement C3 are localized to the same muscle fibers.

in the fast twitch rich vastus muscle, this did not reach statistical significance ($P = 0.22$).

DISCUSSION

Complement activation resulting from deposition of IgM natural antibody on injured tissue initiates skeletal muscle reperfusion injury [12]. This is postulated to be on the basis of expression of ischemia-generated neo-epitopes for which there are corresponding preformed natural antibodies. As ischemia is induced throughout the entire limb by tourniquet occlusion at the proximal thigh, one would expect that the injury would be both diffuse and uniform. Yet, microscopic analysis of injured hindlimb sections show that injured muscle bundles lie literally adjacent to uninjured muscle bundles (Fig. 1). This is the case whether we identify injury by fiber disruption, alteration on H&E, or loss of mitochondrial activity or deposition of the in-

flammatory proteins, IgM, and C3. This heterogeneity does not appear to have any relationship to proximity to vessels. Thus, muscle bundles that have experienced identical periods of ischemia show different degrees of reperfusion injury. As a result, it is logical to investigate whether there is a known cellular basis on which the muscle bundles might be distinguished otherwise.

Skeletal muscle bundles are known to be composed of one of two types of myosin. Muscles which are predominantly slow twitch (Type 1) are those used for sustained contraction and posture, such as the cremaster. Muscles which are predominantly fast twitch (Type 2) are those used for locomotion and have higher glycolytic capacity. These two types of myosin types are definable by monoclonal antibodies. Type 2 bundles can be further divided into high- and low-myosin content. Most, if not all, muscles have all fiber types to varying degrees depending on muscle function. The muscles of a murine hindlimb demonstrate this phe-

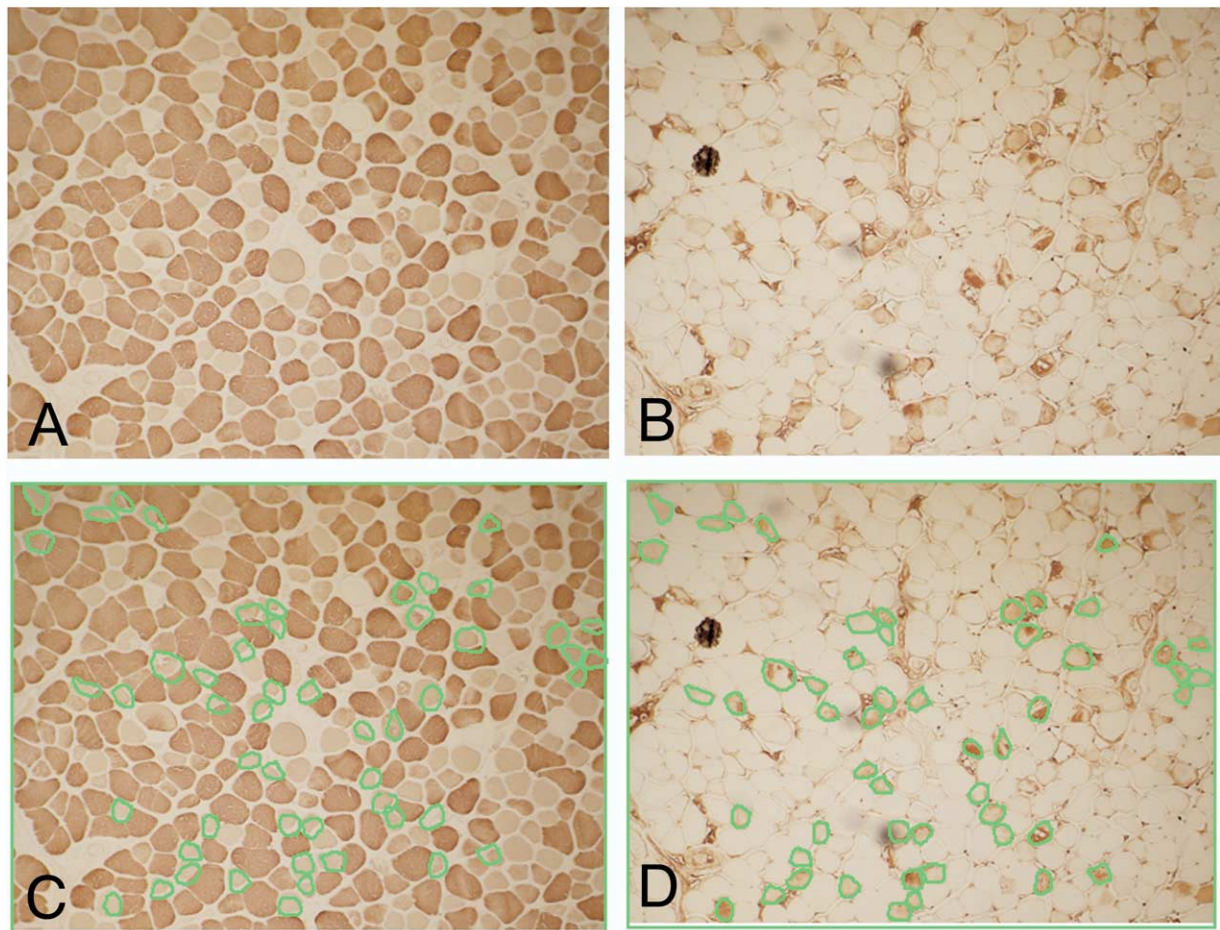


FIG. 4. Serial sections of murine skeletal muscle subjected to 2 h of ischemia and 1 h of reperfusion stained with anti-fast heavy chain myosin (A) and anti-mouse C3 (B). Green highlights denote examples of corresponding muscle fibers on serial sections. C3 deposition is confined to Type 2, low-myosin content fibers.

nomenon. Vastus is predominantly fast twitch; gastrocnemius is a mixture; and soleus is predominantly slow twitch. Thus, in a single hindlimb subjected to reperfusion injury, we had the opportunity to study the distribution of injury relative to the distribution of fast and slow twitch fibers.

Within the gastrocnemius muscle, we observed a correlation between injury and muscle fiber type. Differing techniques of fixation and tissue preparation for each analysis prevent us from displaying each type of analysis on successive sections. Thus, we cannot definitively produce a series of images which establish that a given disrupted fiber (loss of mitochondrial oxidative activity) also had IgM or C3 deposition. However, the similarities in the pattern of observed abnormality and in the number of fibers injured by each criterion strongly suggest that this is indeed the case. Furthermore, the dependency of reperfusion injury on the presence of natural IgM and complement indicate that reperfusion is localized to areas where those proteins are deposited. In fact, a patchy and persistent binding of IgM to injured muscle can be seen within minutes of

the induction of ischemia [13], indicating that it is the localization of the IgM binding that initiates the reperfusion injury. In other words, the same fibers exhibit all three types of criteria for injury: histological damage, inflammatory protein deposition, and loss of mitochondrial oxidative enzymes. We then used the technique of analysis which could provide serial sections, immunoperoxidase staining for binding of specific antibodies, and compared serial sections stained for either C3 or muscle fiber type. Inflammatory injury appeared almost completely confined to fast-twitch, low-myosin content fibers (Fig. 4). The finding that this Type 2 fiber is the site of injury is supported by the correlation that we observed between overall muscle content of fast-twitch fibers and degree of muscle injury from reperfusion, as assessed on conventional histological sections (Fig. 6). Thus, in this model, reperfusion injury to the hindlimb is almost entirely confined to one muscle fiber type: the fast-twitch, low-myosin content phenotype.

Previous studies regarding fiber type and reperfusion injury indicate that fast fibers are more prone to

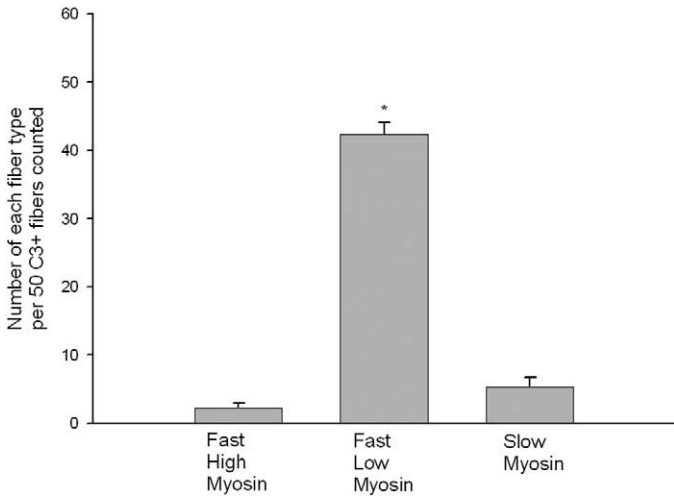


FIG. 5. Number of C3-positive muscle fibers as analyzed by each fiber type according to antibody staining. Results based on 50 fibers counted per murine hindlimb ($n = 8$). C3 deposition occurs primarily on Type 2, low-myosin content fibers. * $P < 0.01$.

injury, although results have been mixed [14]. Comparison of the fast fiber-rich extensor digitorum longus muscle with its slow fiber-rich soleus muscle counterpart demonstrates that the former has delayed recovery and more severely damaged mitochondria, sarcoplasmic reticulum, and myofibrils [15]. There is also a selective decrement in fast-twitch muscle mass after ischemia and reperfusion injury [16]. Muscles containing a greater number of fast twitch fibers had significantly greater necrosis than did those richer in slow-twitch fibers [17]. Surprisingly, several functional studies in the literature suggest that fast-twitch muscles are more resistant to ischemic insults [18]. The end point of these investigations, however, is the function of the remaining fibers, not the degree of injury. After reperfusion, fast-twitch muscle exhibits a greater degree of injury but is still functional, likely due to the low metabolic demand of the surviving fibers. Overall, these studies support our observations.

One explanation for this finding is that fast-twitch muscle has a higher metabolic rate than slow twitch and is, therefore, intrinsically more susceptible to loss of substrate through ischemia. Thus, although the two fiber types both could be injured as a result of ischemia/reperfusion, only the fast twitch is injured clinically due to its greater sensitivity to the particular insult. This explanation does not offer any insight as to why there might be a difference in susceptibility between fast-twitch, high-myosin content fibers, which are not injured, and fast-twitch, low-myosin content fibers, which are. A variant hypothesis is that the hypoxic insult is sub-maximal and that if the ischemia time were increased, then injury to the slow-twitch muscle fibers would be observed. In that regard, it needs to be pointed out that this is a maximal injury for this model, which already causes 5–10% of animals to die during

the reperfusion period. Increased ischemia times produce mortality rates which make these experiments unfeasible.

A second possible explanation for our findings is that injured muscle fibers have enough membrane disruption to non-specifically adsorb our study reagents. Thus the apparent specificity is an artifact of the injury itself. We think that this is not the case for several reasons. First, there is no observable increase in staining with secondary antibody or developing reagents alone, an expected feature were this type of artifact to exist. Second, there is no observable deposition of IgG in injured tissue until very late in the time course of the injury [13]. Third, the binding of IgM by ischemic tissue is observed to be a very early event, as mentioned above, and C3 binding does not begin to appear until perfusion is re-established, a time course which strongly argues that the observations have a biological basis [13]. Finally, uninjured and injured muscle exhibit identical numbers of fast-twitch and slow-twitch fibers. Thus the binding of the monoclonal antibodies used to define these phenotypes is not altered by injury.

The explanation which we favor is that the fast-twitch, low-myosin muscle fiber type is uniquely pre-programmed to respond to injury in a way that is independent of its metabolic rate. Our past observations and reports that reperfusion injury in this model is almost entirely inflammatory and depends on the presence of natural IgM strongly imply that ischemia provokes the expression of neo-antigens for which there are preformed circulating natural IgM. Once the neo-antigens or epitopes are exposed, the preformed IgM binds and potentially initiates complement activation and the ensuing cytotoxic inflammatory cascade. Given that sequence, it is reasonable to suppose that not all tissues and not all cells would

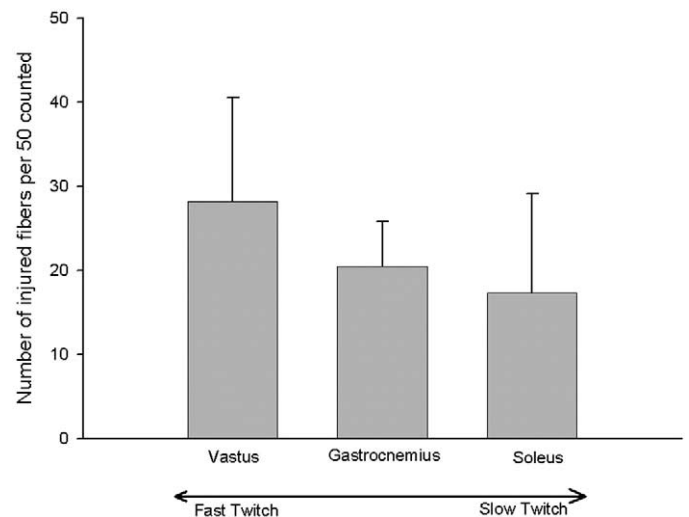


FIG. 6. Number of injured fibers in three different muscle groups with varying fast twitch fiber content. The degree of injury to a muscle corresponds to its content of fast twitch fibers.

contain these neo-antigens. Thus, our hypothesis is that the fast-twitch, low-myosin content fibers possess these injury neo-antigens, and the slow-twitch fibers or the fast-twitch, high-myosin content fibers do not. Whether it is the myosin itself that becomes exposed or whether there are injury antigens that co-segregate with the fast twitch, low-myosin content phenotype remains to be tested.

The finding that one particular fiber type results in preferential deposition of IgM and complement has significant implications for future therapies against reperfusion injury. Control of inflammation will likely lead to meaningful muscle recovery due to the patchy nature of injury and the large number of unaffected fibers. Since the differences in gene expression pattern among these fibers result in the injury phenotype, analysis of specific genes that are differentially regulated might lead to the identity of the IgM binding epitope exposed during ischemia and eventually toward therapeutics aimed against reperfusion injury.

ACKNOWLEDGMENT

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

REFERENCES

1. Weiser, M. R., Williams, J. P., Moore, F. D. Jr. Kobzik, L., Ma, M., Hechtman, H. M., *et al.* Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J. Exp. Med.* **183**(5): 2343, 1996.
2. Williams, J. P., Pechet, T. T., Weiser, M. R., Reid, R., Kobzik, L., Moore, F. D. Jr., *et al.* Intestinal reperfusion injury is mediated by IgM and complement. *J. Appl. Physiol.* **86**(3): 938, 1999.
3. Lindsay, T. F., Hill, J., Ortiz, F., Rudolph, A., Valeri, C. R., Hechtman, H. B., *et al.* Blockade of complement activation prevents local and pulmonary albumin leak after lower torso ischemia-reperfusion. *Ann. Surg.* **216**(6): 677, 1992.
4. Nelson, D. P., Wechsler, S. B., Miura, T., Stagg, A., Newburger, J. W., Mayer, J. E. Jr., *et al.* Myocardial immediate early gene activation after cardiopulmonary bypass with cardiac ischemia-reperfusion. *Ann. Thorac. Surg.* **73**(1): 156, 2002.
5. Reid, R. R., Woodcock, S., Shimabukuro-Vornhagen, A., Austen, W. G. Jr. Kobzik, L., Zhang, M., *et al.* Functional activity of natural antibody is altered in Cr2-deficient mice. *J. Immunol.* **169**(10): 5433, 2002.
6. Pedowitz, R. A., Friden, J., and Thornell, L. E. Skeletal muscle injury induced by a pneumatic tourniquet: an enzyme- and immunohistochemical study in rabbits. *J. Surg. Res.* **52**(3): 243, 1992.
7. Chen, C., Liu, J., Hua, D., Ma, L., Lai, T., Fallon, J. T., *et al.* Impact of delayed reperfusion of myocardial hibernation on myocardial ultrastructure and function and their recoveries after reperfusion in a pig model of myocardial hibernation. *Cardiovasc. Pathol.* **9**(2): 67, 2000.
8. Yamamoto, T., Tada, T., Brodsky, S. V., Tanaka, H., Noiri, E., Kajiya, F., *et al.* Intravital videomicroscopy of peritubular capillaries in renal ischemia. *Am. J. Physiol. Renal Physiol.* **282**(6): F1150, 2002.
9. Johanson, C. E., Palm, D. E., Primiano, M. J., McMillan, P. N., Chan, P., Knuckey, N. W., *et al.* Choroid plexus recovery after transient forebrain ischemia: role of growth factors and other repair mechanisms. *Cell Mol. Neurobiol.* **20**(2): 197, 2000.
10. Kunz, R., Schoenberg, M. H., Buchler, M., Jost, K., and Beger, H. G. Oxygen radicals in liver ischemia and reperfusion—experimental data. *Klin. Wochenschr.* **69**(21-23): 1095, 1991.
11. Lazarus, B., Messina, A., Barker, J. E., Hurley, J. V., Romeo, R., Morrison, W. A., *et al.* The role of mast cells in ischaemia-reperfusion injury in murine skeletal muscle. *J. Pathol.* **191**(4): 443, 2000.
12. Chan, R. K., Ibrahim, S. I., Verna, N., Carroll, M., Moore, F. D. Jr., and Hechtman, H. B. Ischaemia-reperfusion is an event triggered by immune complexes and complement. *Br. J. Surg.* **90**(12): 1470, 2003.
13. Chan, R. K., Ding, G., Verna, N., Ibrahim, S. I., Oakes, S., Austen, Jr., W. G., *et al.* IgM binding to injured tissue precedes complement activation during skeletal muscle ischemia-reperfusion. In press. 2004.
14. Sternbergh, W. C., and 3rd, Adelman, B. Skeletal muscle fiber type does not predict sensitivity to postischemic damage. *J. Surg. Res.* **53**(5): 535, 1992.
15. Woitaske, M. D., and McCarter, R. J. Effects of fiber type on ischemia-reperfusion injury in mouse skeletal muscle. *Plast. Reconstr. Surg.* **102**(6): 2052, 1998.
16. Carvalho, A. J., Hollett, P., and McKee, N. H. Recovery of synergistic skeletal muscle function following ischemia. *J. Surg. Res.* **59**(5): 527, 1995.
17. Petrusek, P. F., Homer-Vanniasinkam, S., and Walker, P. M. Determinants of ischemic injury to skeletal muscle. *J. Vasc. Surg.* **19**(4): 623, 1994.
18. Gurke, L., Marx, A., Sutter, P. M., Stierli, P., Harder, F., and Heberer, M. Function of fast- and slow-twitch rat skeletal muscle following ischemia and reperfusion at different intramuscular temperatures. *Eur. Surg. Res.* **32**(3): 135, 2000.