Improved Survival of Murine Island Skin Flaps by Prevention of Reperfusion Injury

Soner H. Tatlidede, M.D.
Adrian D. Murphy, M.B.,
M.R.C.S.I.
Michael C. McCormack, B.S.
John T. Nguyen, M.D.
Kyle R. Eberlin, M.D.
Mark A. Randolph, M.A.S.
Francis D. Moore, Jr., M.D.
William G. Austen, Jr., M.D.

Background: Studies have demonstrated that blocking a single specificity of self-reactive immunoglobulin M with a 12–amino acid peptide mimic of the antigen of immunoglobulin M can attenuate murine intestinal and skeletal muscle injury following ischemia and reperfusion. The aim of this study was to ascertain whether peptide (P8) had protective effects in an axial island skin flap model, where tissue loss is attributed to ischemia-reperfusion injury.

Methods: Dorsal lateral thoracic artery island skin flaps (3.5 × 1.5 cm) were elevated in 82 male C57BL/6 mice and rendered ischemic for 10 hours by placing a 7-mm microclamp on the vascular pedicle followed by 7 days of reperfusion. Group I (n = 7), the sham group, had no clamp placed. Group II (n = 21) had clamp placement but no other treatment. Thirty minutes before clamp placement, group III (n = 18) received 0.25 cc of saline intravenously, group IV (n = 18) received 25 μg/0.25 cc P8 peptide, and group V (n = 7) received 25 μg/0.25 cc random 12-mer peptide. Animals in group VI (n = 11) underwent two cycles of 20 minutes of ischemic preconditioning before 10 hours of ischemia. After 1 week of reperfusion, percent necrosis was measured and results were compared using analysis of variance and an unpaired t test.

Results: In animals treated with P8 peptide, flap necrosis was 14.61 ± 2.77 percent. This represents a statistically significant, 56 percent reduction in flap necrosis compared with controls (p < 0.001).

Conclusion: These data demonstrate that prevention of ischemia-reperfusion injury with P8 peptide produces a significant reduction in necrosis of treated flaps. (Plast. Reconstr. Surg. 123: 1431, 2009.)

Restoration of blood flow to ischemic tissue is vital for tissue survival. This reperfusion, however, can trigger a cascade of acute inflammatory events. This leads to increased cellular death resulting in tissue dysfunction and necrosis. Ischemia-reperfusion injury is modulated by complex inflammatory and immunologic signaling pathways that have to date not been fully elucidated. Clinically, ischemia-reperfusion injury is responsible for significant morbidity in the fields of trauma, vascular, transplant, and plastic surgery.

Although ischemia-reperfusion injury is multifactorial, one of the earliest events in the process is related to the binding of circulating natural antibodies and the activation of complement. First described in myocardial reperfusion by Hill and Ward, complement activation was shown also to involve the release of C3α and C5α anaphylatoxins that induce degranulation of mast cells with release of histamine and other chemical mediators. The final common pathway is edema, attraction of

Disclosure: Francis D. Moore, Jr., M.D., is a co-founder of DecImmune Therapeutics, which holds the license to develop this P8 technology. He currently acts as a consultant for DecImmune, Inc. None of the other authors has a financial interest in any of the products, devices, or drugs mentioned in this article.
activated leukocytes, and the formation of membrane-attack complexes that damage membranes, leading to tissue necrosis.3

Attempts to reduce ischemia-reperfusion injury by blocking the complement cascade at various levels have shown some success. Pretreatment of rodents with recombinant soluble complement receptor type 1, a potent global inhibitor of complement, showed profound protection in different organ models.4–10 Both C4-deficient knockout mice and C3-deficient knockout mice were protected from local injury in the hindlimb and intestinal ischemia reperfusion models.11,12 In a myocardial ischemia-reperfusion study, complement inhibition with C1 esterase inhibitor therapy suppressed endothelial adhesion molecule expression and reduced infarct size.13,14 In rat intestinal models15,16 and myocardial models,17 blockade of C5a/C5a with antibodies or inhibitors resulted in protection from ischemia-reperfusion injury.

Zhang et al. isolated ischemia-reperfusion–specific natural immunoglobulin M producing cells using B-1 lymphocyte hybridomas constructed from peritoneal cells. A single immunoglobulin M–producing clone, CM22, was identified that restored ischemia-reperfusion injury in immunodeficient mice (RAG-1−/−) in an intestinal model of ischemia-reperfusion injury with evidence of immunoglobulin M, C3, and C4 complement deposition in damaged tissue.18 In addition, Austen et al. demonstrated restoration of reperfusion injury in antibody repertoire-altered Cr2−/− mice in a hindlimb ischemia-reperfusion model when reconstituted with this specific immunoglobulin M.19

The identification of CM22 as a natural self-reactive antibody led to the search for injury neoantigens. Zhang et al. hypothesized that circulating immunoglobulin M would bind self-antigens exposed during ischemia and that these complexes could be isolated and the antigens identified by proteomic techniques. When the immune complexes in injured tissues of RAG-1−/− mice reconstituted with CM22 antibody were investigated, nonmuscle myosin heavy chain type II A and C were determined to be the involved antigen.20 It has been shown in intestinal tissue and hindlimb that a synthetic peptide (P8) representing a conserved region of nonmuscle myosin heavy chain type II binds immunoglobulin M (CM22) in vitro and blocks reperfusion injury in wild-type mice when given intravenously as a competitive inhibitor.20,21

In the past, there have been many attempts to mitigate ischemia-reperfusion injury through various experimental means. One of the more effective methods of protection has been the process of ischemic preconditioning, whereby tissue is exposed to short cycles of ischemia and reperfusion before critical ischemia. This phenomenon was first described in 1986, when Murry et al. discovered that brief cycles of ischemia and reperfusion within the canine heart exerted a myocardial protective effect from a longer ischemic insult.22 This has been subsequently investigated in large animal models and within various tissue types, including cardiac,23,24 hepatic,25 skeletal muscle,26 musculocutaneous flap,27,28 and skin flap models,29 although the main investigational focus has historically been on cardiac tissue. In this study, we used ischemic preconditioning of the skin flap as a positive control.

In plastic surgery, composite tissues composed of muscle and skin and subcuticular tissues are often used. In addition, as composite tissue transplantation becomes more commonplace, protection of the tissue from ischemia-reperfusion injury will assume greater importance. The aim of our study was to investigate the potential protective effects of the synthetic peptide P8 in an axial island skin flap model and to establish the possibility that tissue loss in this model is attributable to reperfusion injury.

**MATERIALS AND METHODS**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital and followed all the policies outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eighty-two C57BL/6 mice, weighing 22 to 25 g, were used in this experiment. Mice were separated into the following six groups:

**Group I:** sham group (without ischemia) \( (n = 7) \).

**Group II:** control group (10 hours of ischemia) \( (n = 21) \).

**Group III:** saline group (saline injection plus 10 hours of ischemia) \( (n = 18) \).

**Group IV:** CM-22–specific binding 12-mer peptide group (P8 injection plus 10 hours of ischemia) \( (n = 18) \).

**Group V:** control peptide group (control peptide injection plus 10 hours of ischemia) \( (n = 7) \).

**Group VI:** preconditioning group (two cycles of 20 minutes of ischemia followed by 20 minutes of reperfusion before 10 hours of ischemia) \( (n = 11) \).

Mice were anesthetized by intraperitoneal administration of sodium pentobarbital, 60 to 90 mg/kg. Additional doses were given as necessary during surgery. During the surgical procedure,
animals were placed on a heating pad to maintain body temperature at 37°C. The dorsum of each animal was shaved with an electric animal shaver and residual hair was removed thoroughly with depilatory cream. The surgical area was cleansed and treated with povidone-iodine for disinfection.

Dorsal lateral thoracic artery pedicled island skin flaps measuring 3.5 × 1.5 cm were raised on the right dorsolateral area of the mice. The medial border of the flap was on the midline of the dorsum, the lateral border was on the right axillary line, and the cranial and caudal borders were on the infrascapular line and the iliac crest, respectively. The flap contains skin, subcutaneous tissue, panniculus carnosus, and superficial fascia. After flap elevation, a medical-grade silicon sheet (Bioplexus, Ventura, Calif.) was placed on the muscle bed as a barrier to vascular invasion and sutured in place with 7-0 polypropylene (Fig. 1, above, and below, left). In group I, flaps were repositioned and sutured with a continuous 6-0 polypropylene suture and no other procedure was performed. Group II had a flap elevated as before but were subjected to 10 hours of clamp ischemia. Thirty minutes before flap elevation and clamp ischemia, the following treatments were administered by means of tail vein injection: group III, 0.25 cc saline; group IV, 25 μg/0.25 cc peptide P8 (NGNNVNGNRNNN; Biopeptide Co., San Diego, Calif.); group V, 25 μg/0.25 cc random 12-mer peptide (LDKNKDPLNETV). Ischemia was induced by placing a 7-mm microclamp (Fine Science Tools, Inc., Foster City, Calif.) on the vascular pedicle. Group VI underwent two cycles of 20 minutes of ischemia produced by pedicle clamping followed by 20 minutes of reperfusion before the clamp was placed for the 10-hour ischemia time. Interrupted sutures were used at the cranial bor-

---

**Fig. 1.** (Above, left) Magnified image of 3 × 1.5-cm flap mapped on the right dorsolateral area of a C57BL/6 mouse. (Above, right) Magnified image of a murine lateral thoracic artery pedicled island flap raised with pedicle clamp in place. (Below, left) Magnified image of the flap after 7 days of reperfusion in an animal treated with control peptide; the necrotic area is outlined. (Below, right) Magnified image of the flap after 7 days of reperfusion in an animal treated with P8 peptide; the necrotic area is outlined.
ders of the flaps to provide easy clamp removal. Mice were permitted to recover from anesthesia during the ischemia time and were kept in cages, with free access to food and water. At the end of 10 hours of ischemia, clamps were taken out by removing the interrupted sutures on the cranial border of the flap and then resutured.

Mice were returned to their cages, allowed water and chow ad libitum, and kept on a 12-hour light/dark cycle with room temperature between 20° and 22°C. On the seventh postoperative day, the mice were given an overdose of pentobarbital (200 mg/kg intraperitoneally). Flap viability was assessed by drawing the viable and nonviable areas of each flap on transparent paper. The border between viable and nonviable flap areas was determined by visual inspection based on dark color, eschar formation, and lack of capillary refill. The areas were calculated using the ImageJ program (National Institutes of Health, Bethesda, Md.) and the necrotic area was expressed as a percentage of total flap area. All evaluations were carried out by one evaluator (S.H.T.), who was blinded as to the treatment groups.

All groups were compared using analysis of variance and unpaired t test with significance set at p < 0.05. No corrections were made for multiple testing.

**Immunohistochemistry**

In an additional experiment, 16 C57BL/6 mice were subjected to injury with immunohistochemical analysis at an early time point. They were divided into the four ischemia groups, and treatments and surgical procedures were applied as described above. After 10 hours of ischemia and 3 hours of reperfusion, flaps were harvested. The flaps were embedded in paraffin and cut in cross-section followed by deparaffinization. Samples were stained with either anti-immunoglobulin M or anti-C3 antibodies. To assess immunoglobulin M deposition, sections were blocked for 20 minutes and then incubated in a 1:100 dilution of biotinylated goat anti-mouse immunoglobulin M (Jackson Laboratory, Bar Harbor, Maine) for 30 minutes at 25°C. Sections were developed with Vectorstain ABC (Vector Labs, Burlingame, Calif.). To assess C3 deposition, we used a 1:100 dilution of goat anti-mouse C3 (MP Biomedicals, Solon, Ohio) for 30 minutes at 25°C. Biotinylated rabbit anti-goat immunoglobulin G (Sternberger Monoclonals, Lutherville, Md.) was used at a 1:100 dilution and sections were incubated for 30 minutes. After washing, sections were reacted with avidin/streptavidin conjugated to horseradish peroxidase for 30 minutes and then developed with diaminobenzidine.

**RESULTS**

The mean percentage necrosis and the standard error of the means for each group is shown in Figure 2. The percentage of flap necrosis was 1.11 ± 1.11 percent (SEM) in group I, animals with flaps that were not rendered ischemic. Group II, animals with flaps subjected to 10 hours of ischemia, developed 33.14 ± 2.51 percent necrosis by the end of the 7 days of observation. Group IV, animals with ischemic flaps that were treated with P8 peptide, had 14.61 ± 2.77 percent necrosis. This represents a 56 percent reduction in necrosis compared with group II, a 51 percent reduction in necrosis compared with group III (animals treated with saline 29.57 ± 2.99 percent), and a 72 percent reduction compared with group V (animals treated with random peptide 42.05 ± 4.51 percent). Animals in group VI, which underwent two cycles of ischemic preconditioning, had 18.82 ± 1.86 percent necrosis.

Analysis of variance showed statistically significant differences among all groups (p ≤ 0.001). With pairwise comparisons, there was no significant difference between the P8 group and the sham group,
which did not undergo ischemia. There were significant differences between the animals treated with P8 and the other ischemia groups (ischemia alone, ischemia plus saline, and ischemia plus random peptide). There were no significant differences with $2 \times 2$ comparisons between the ischemia alone, ischemia plus saline, and the random peptide groups. There was no significant difference between the protection conferred by ischemic preconditioning (group VI) and that conferred by administration of the P8 protein (group IV). Post hoc power analysis was performed with alpha of 0.05, giving a power of 1.

**Immunohistochemistry**

In the 10 hours of ischemia and 3 hours of reperfusion experiment, there was immunoglobulin M and C3 deposition on the endothelium of medium-sized vessels of the pedicled flaps. Untreated, salinetreated, and random peptide–treated groups had qualitatively similar amounts of deposition. P8-treated animals exhibited a significant qualitative decrease in immunoglobulin M and C3 deposition (Figs. 3 and 4).

**DISCUSSION**

We developed a model of a composite pedicle flap on the dorsum of the common inbred C57BL/6 mouse (Fig. 1). When the pedicle is clamped with a microclip for 10 hours and then observed for 1 week, 30 percent of the flap is lost to necrosis (Fig. 2). This situation simulates loss of free flap tissue perfusion because of microvascular thrombosis, followed by restoration of flow from revision of the anastomosis. Treatment of animals with intravenous saline or with a random 12–amino acid pep-

![Fig. 3. Photomicrographs of immunoglobulin M deposition on medium vessels of murine skin flaps after 10 hours of ischemia and 3 hours of reperfusion, as assessed by anti–immunoglobulin M immunoperoxidase reaction (original magnification, 200×). (Above, left) Group II, no pretreatment; (above, right) group III, pretreatment with intravenous saline; (below, left) group IV, pretreatment with intravenous P8 peptide; (below, right) group V, pretreatment with intravenous random peptide. P8-treated animals showed little or no immunoglobulin M deposition compared with group II, III, or V.](image-url)
tide did not improve tissue loss (Fig. 2). However, treatment of animals with the 12–amino acid P8 peptide significantly reduced tissue loss (Fig. 3). As this peptide was designed to compete with natural injury antigens for the binding site on pathogenic antibodies that cause diverse reperfusion injuries, these data suggest that the loss of tissue in this situation is attributable to reperfusion injury and is potentially reversible with appropriate treatment. The hallmark of reperfusion injury is the significant amplification of injury by deposition of immunoglobulin M, with resultant complement co-deposition and damage. Examination of tissue sections reveal deposition of immunoglobulin M (Fig. 3) and complement C3 (Fig. 4) in the same flap vessels, a process that is reversed by P8 peptide. These data suggest that P8 is acting through its anticipated mechanism and, therefore, that the prevention of tissue loss is a result of preventing reperfusion injury in the flap.

Tissue injury during reperfusion after prolonged periods of ischemia is the result of multiple factors. One of the earliest events involved in the process of ischemia-reperfusion injury is the binding of circulating natural antibody (immunoglobulin M) and the subsequent activation of complement. Previous findings in hindlimb, intestinal, myocardial, and burn models have found that injury is mediated by a local effect of specific natural immunoglobulin M, with subsequent complement activation, and that this effect can be mitigated by intravenous pretreatment with a 12-mer peptide (P8).20,30,31 The results of our study demonstrate that blocking the immunoglobulin M and complement cascade in pedicled skin flaps in mice improves tissue survival.

Fig. 4. Photomicrographs of C3 deposition on medium vessels of murine skin flaps after 10 hours of ischemia and 3 hours of reperfusion, as assessed by anti-C3 immuno-peroxidase reaction (original magnification, 200×). (Above, left) Group II, no pretreatment; (above, right) group III, pretreatment with intravenous saline; (below, left) group IV, pretreatment with intravenous P8 peptide; (below, right) group V, pretreatment with intravenous random peptide. P8-treated animals showed little or no C3 deposition compared with group II, III, or V.
Attempts to inhibit complement activation during ischemia-reperfusion injury have targeted the classic pathway (C1 esterase inhibitor and C1 antagonists), the alternative pathway (C3 and factor B), the lectin pathway (mannose-binding lectin antibodies), and all three pathways (sCRI and anti-C5/C5a). It has been suggested that classic pathway activation is an antibody-independent process and that intracellular moieties exposed by ischemia may bind C1q directly. However, natural immunoglobulin M has been shown to be an essential factor in complement activation; mice deficient in immunoglobulin M were protected from intestinal ischemia-reperfusion injury. The same strain of animals reconstituted with pooled wild-type immunoglobulin M had restoration of injury.

This discovery has led to the hypothesis that ischemia-reperfusion can be activated by recognition and binding of preexisting natural immunoglobulin M to neoantigen expressed by hypoxic cells. Natural immunoglobulin M is the first line of defense against foreign pathogens and is a component of the innate immune system. B-1 cells are the major source of this preexisting or natural immunoglobulin M. These cells are found mainly within the peritoneal and pleural cavities of humans, mice, and other animals. Recent studies using Cr2−/− mice that are deficient in complement receptors 1 and 2, which are important for B-1 cell development, have shown these mice to have markedly decreased ischemia-reperfusion injury. These mice have normal serum concentrations of immunoglobulin M. However, they have markedly reduced levels of natural immunoglobulin M specificities, which explains the decreased injury seen in intestinal ischemia-reperfusion models. This protection is eliminated by intravenous reconstitution of immunoglobulin M from normal mice or by engraftment of knockouts with peritoneal cells from isogenic normal mice.

A similar mechanism of ischemia-reperfusion injury has been demonstrated in the rat, supporting the hypothesis that the role of natural immunoglobulin M in ischemia-reperfusion injury is highly conserved and is similar across mammalian species. There is limited but increasing evidence to suggest that a similar innate immunologic response is responsible for ischemia-reperfusion injury in humans. Recently, Zhang et al. have shown that human natural immunoglobulin M can induce ischemia-reperfusion injury in a murine intestinal model. It has also been shown that immunoglobulin M along with complement and C-reactive protein is deposited in injured human myocardium following acute myocardial infarction.

In 2004, Zhang et al. identified a specific natural antibody [immunoglobulin M (CM22)] that initiates intestinal and skeletal muscle reperfusion injury in antibody-deficient mice. This discovery made it possible to examine the initiation of acute ischemia-reperfusion injury and to isolate and characterize the self-antigen involved. It is likely that the pathogenic property of this immunoglobulin M is inherent in its binding site; therefore, it is likely that there is a specific antigen(s) on the cells expressing the injury phenotype to which the immunoglobulin M binds. This led to the identification of a highly conserved region within nonmuscle myosin heavy chain type II A and C as the target for natural immunoglobulin M and initiator of reperfusion injury in murine skeletal and intestinal models. A review by Bresnick suggests that ischemia-reperfusion injury is initiated at the endothelium surface similar to that found in the intestinal model. Nonmuscle myosin heavy chain type II is the major self-ligand in ischemic skeletal muscle and that the nonmuscle myosin heavy chain type II epitope is mobilized to the cell surface by hypoxia-related events. Although these experiments suggest that nonmuscle myosin heavy chain type II is the major antigen exposed during reversible reperfusion injury, other epitopes are likely exposed on the surface of injured endothelium. However, in the absence of specific natural immunoglobulin M, nonmuscle myosin heavy chain type II is insufficient to initiate inflammation.

Zhang et al. isolated synthetic peptides to bind pathogenic immunoglobulin M (CM22) and block reperfusion injury in wild-type mice. They accomplished this using an M13 phage display library consisting of random 12-mer amino acid sequences that were screened with the specific immunoglobulin M. Ten phage clones were isolated. One of these clones, P8, bound with the highest affinity. All three isoforms of nonmuscle myosin heavy chain type II included a motif that shows similarity with the P8 sequence. These results suggest that immunoglobulin M (CM22) binding to phage P8 is specific for the peptide region and that the synthetic peptide could be used as a mimotope for the actual antigen.

Interestingly, Zhang et al. found no exact matches when the amino acid sequence of the 12-mer peptide from the P8 clone was compared with that of all known mammalian protein sequences. However, P8 is homologous to a number of protozoan antigens. The specificity of the in-
teration of P8 with immunoglobulin M in the prevention of reperfusion injury was reinforced when random peptides of similar size did not exhibit this protection.41

Although P8 was shown to be effective at lower concentrations in intestine,20 Chan et al. used 20 μm of peptide to block skeletal muscle ischemia reperfusion.31 Because blood supply to the skin is not as abundant as to skeletal muscle and the flap that we raised would contain a significantly lower proportion of circulating blood than hindlimb, we chose to use 25 μg of these 12-mer peptides in our study.

It has been shown that ischemia alone is not sufficient to produce visible histologic muscle injury. However, structural evidence of muscle fiber damage can be seen after only a short period of reperfusion. Immunoglobulin M deposition has been demonstrated as early as 5 to 10 minutes after ischemia and continued throughout reperfusion. Complement C3 deposition is not seen during the ischemic period but increases dramatically once reperfusion begins.18

Because immunoglobulin M deposition starts during ischemia and before reperfusion, the timing of peptide administration is imperative. For attenuation of reperfusion injury to skeletal muscle, Chan et al. used P8 intravenously 30 minutes before ischemia.41 Similarly, in an intestinal model, Zhang et al. pretreated wild-type mice with P8, 5 minutes before reperfusion, to test whether this peptide represented a mimotope for a major self-antigen. This pretreatment before reperfusion blocked any apparent injury.20 We chose pretreatment 30 minutes before ischemia, similar to Chan et al. As this peptide is being administered in a preventative capacity before any injury occurs, it mirrors the elective surgical experience rather than that of traumatic ischemia-reperfusion injury.

In our study, skin flaps were analyzed by immunohistochemistry with antibodies to immunoglobulin M and C3. P8-treated animals exhibited a significant qualitative decrease in immunoglobulin M and C3 deposition compared with all other groups. These results are in accordance with the findings of Chan et al., who reported that P8-treated skeletal muscle did not show any immunoglobulin M or C3 deposition after reperfusion,41 and with those of Zhang et al., who determined that P8-treated animals did not show immunoglobulin M, C3, or C4 deposition in the microvilli in an intestinal ischemia-reperfusion model.20 As demonstrated in other investigations,11,41 our study showed that immunoglobulin M and C3 deposition is seen mainly on the endothelium. This was shown in all groups except the flaps treated with P8.

Although it is reported that, during ischemia, exposure of other forms of myosin heavy chain (such as smooth muscle) is possible,20 we could not detect any immunoglobulin M or C3 deposition on the panniculus carnosus in this study. Despite the absence of the panniculus carnosus layer in human skin, the lack of immunoglobulin M or C3 deposition seen in these experiments led us to believe that this model closely correlates with ischemia-reperfusion injury as seen in human skin flaps.

Chan et al. reported a 41 percent reduction in injury with P8 treatment compared with random peptide in a skeletal muscle model of ischemia-reperfusion injury.41 In our study, there was a 65 percent reduction in necrosis compared with the control peptide group and a 56 percent reduction in necrosis compared with the saline control group. In comparison with previously published hindlimb and intestinal models, our skin flap model showed a higher degree of protection with the P8 peptide.20,41 This apparent increased efficacy of P8 in preventing ischemia-reperfusion injury in skin compared with hindlimb and intestine may reflect an increased susceptibility to immunoglobulin M–mediated ischemia-reperfusion injury in skin flaps.

These results lead us to conclude that blockage of a specific immunoglobulin M can protect against ischemia-reperfusion injury in an axial skin flap. This type of protection could be useful in improving results in free flap transfer and composite tissue transplantation.