A Monoclonal Antibody to the Adherence-promoting Leukocyte Glycoprotein, CD18, Reduces Organ Injury and Improves Survival from Hemorrhagic Shock and Resuscitation in Rabbits

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Abstract

Leukocytes have been shown to play an important role in the development of isolated organ injury after experimental ischemia and reperfusion. To examine the role of leukocytes in generalized ischemia-reperfusion injury we used the MAb 60.3 (directed to the human leukocyte adherence glycoprotein, CD18) to block leukocyte adherence functions in a rabbit model of hemorrhagic shock and resuscitation. In control animals subjected to 1 h of shock (mean blood pressure 45 torr and mean cardiac output 30% of baseline) followed by resuscitation, only 29% survived 5 d. All had gross and histologic evidence of injury to lungs, liver, and gastrointestinal mucosa. In contrast, 100% of the MAb 60.3-treated animals survived 5 d ($P < 0.01$) and organ injury was absent or markedly attenuated. The control animals also had a persistent acidosis, lost more weight, and had evidence of continued gastrointestinal bleeding in contrast to MAb 60.3-treated animals. We conclude that increased leukocyte adhesiveness plays an important role in the development of multiple organ injury and death after generalized ischemia-reperfusion and that this injury may be significantly reduced by blocking leukocyte adherence functions with the MAb 60.3.

Introduction

The organ injury that results from ischemia and reperfusion determines the outcome of many important clinical disorders including myocardial infarction, stroke, mesenteric and peripheral vascular disease, organ transplantation, and circulatory shock. A number of recent investigations into the mechanisms of ischemia-reperfusion injury have focused on oxygen-derived free radicals and their production of microvascular

injury (reviewed in reference 1). The role of leukocytes in the pathogenesis of ischemia-reperfusion injury has recently been suggested by studies demonstrating a significant injury reduction in neutrophil-depleted animals (2, 3). Since circulatory shock followed by resuscitation is in essence whole-body ischemia-reperfusion, it is not surprising that injury to multiple organs is frequently a consequence. Clinical studies of the multiple organ failure syndrome in trauma patients have shown a close correlation with prior hypovolemic shock (4). An important role of the neutrophil has been postulated in this setting as well (5, 6), yet this has not been critically examined.

Neutrophils may exert damaging effects through several mechanisms. After activation, neutrophils generate and release toxic oxygen metabolites, numerous proteases, and phospholipase products, all of which may result in vasomotor changes, endothelial injury, and loss of vascular integrity (reviewed in reference 7). Increased neutrophil adhesiveness is a critical, early step in the sequence of events leading to neutrophil-mediated injury. Increased adhesiveness results in neutrophil adherence to endothelium, migration into tissues, and neutrophil aggregation. In the setting of ischemia-reperfusion, these events may combine to produce microvascular occlusion, tissue injury, and death.

Recent studies have provided insight into the cellular and molecular mechanisms that mediate neutrophil adhesiveness. Specifically, the human neutrophil membrane glycoprotein heterodimer, CD11b/CD18 (Mac-1) has been shown to play an important role in mediating adhesiveness (reviewed in reference 8). MAbs to these glycoproteins have been shown to inhibit adherence-dependent leukocyte functions. We have previously shown in vitro that an MAb, 60.3, directed to a function-related epitope on CD18 (9), inhibits neutrophil spreading on plastic, chemotaxis, aggregation, adherence to endothelial monolayers, and endothelial injury (7-9). We recently demonstrated that MAb 60.3 blocks chemotaxin-induced neutrophil adherence to microvascular endothelium, the subsequent accumulation of neutrophils, and associated plasma leakage in vivo (10).

To test the hypothesis that leukocytes play an important role in the development of generalized ischemia-reperfusion injury, we examined the effect of blocking leukocyte adhesive functions with MAb 60.3 on the development of multiple organ injury after hemorrhagic shock and resuscitation in rabbits.

Methods

14 female New Zealand white rabbits (1-1.5 kg) were anesthetized with ketamine (30 mg/kg i.v.). Using sterile technique, central venous and

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thermistor-tipped aortic catheters (model 94-011, American Edwards Laboratories, Santa Ana, CA) were placed through an open femoral approach with local 1% lidocaine supplement. Arterial BP, central venous pressure, and core temperature were monitored continuously. Periodic determinations were made of: arterial blood gases, hematocrit (Hct),¹ white blood cell count (WBC), and relative thermodilution cardiac output (CO) using a model 9310 cardiac output/lung water computer (American Edwards Laboratories).

After recovery from anesthesia, animals were pretreated in a randomized, blinded fashion with either 2 mg/kg of MAb 60.3 or an equal volume of sterile saline as a control. This dose of MAb 60.3 had been previously determined by immunofluorescence to completely saturate CD18 binding sites (11). 30 min later, blood was withdrawn from the venous catheter into heparinized (10 U/ml) polypropylene syringes to maintain a mean BP of 45 torr and a mean CO of 30% of baseline for 1 h. Animals were then resuscitated with the entire volume of shed blood plus lactated Ringer's, titrated to restore normal CO. This resuscitation was continued for 3 h at which time the catheters were removed, again using sterile technique, under light ketamine (5–10 mg/kg) anesthesia with local 1% lidocaine supplement.

Animals were then returned to their cages where they were maintained on a standard diet of food and water ad libitum. Animals were followed with daily weight, Hct, and arterial blood gases to 5 d, at which time survivors were killed with an overdose of pentobarbital. Necropsy was performed on all animals within 6 h of death. Organs were examined grossly for evidence of injury and sections were fixed in paraffin and stained with hematoxylin and eosin for examination by light microscopy.

The experimental protocol was reviewed and approved by the University of Washington Animal Care Committee.

Statistics. Survival data were analyzed by constructing Kaplan-Meier survival curves for treatment and control groups (12). These curves were then compared by logrank test. Other data were expressed as mean $\pm$ SD and significance determined by unpaired *t* test, comparing treatment and control groups. Significance was attributed to $P < 0.05$.

MAb. MAb 60.3 is a murine IgG_{2a} antibody that recognizes a functional epitope on CD18 (9). MAb 60.3 was prepared as previously described (13) and used as a 3-mg/ml solution in sterile saline. This solution had no detectable endotoxin contamination by limulus assay.

Results

The ischemic insult. The ischemic insult, as measured by BP, CO, and degree of acidosis during the period of shock, was similar between the two groups (Figs. 1 and 2). To maintain the same level of BP and CO, the MAb 60.3-treated animals required more blood to be removed and had a lower Hct at the end of the ischemic period, compared with control animals (Fig. 1).

Response to resuscitation. After reinfusion of the shed blood there was a rapid return of BP and CO to near baseline levels in both groups (Fig. 1). The control animals initially had an elevated BP and depressed CO relative to the MAb 60.3-treated animals, reflecting an increased total peripheral resistance. At the end of the 3-h resuscitation period, however, both groups had similar BP and CO. The control animals tended to require more crystalloid resuscitation fluid, compared with the MAb 60.3-treated group ($63 \pm 25\%$ of calculated total blood

Figure 1. BP (mean), relative thermodilution cardiac output (expressed as percent of baseline), volume of blood removed (expressed as percent of total blood volume), and Hct are represented on the ordinate. Time, including the 1-h period of shock and 3-h period of resuscitation, is represented on the abscissa. Total blood volume was calculated as 7% of body weight. All data points represent means of the seven control animals (open circles) or seven MAb 60.3-pretreated animals (closed circles) with error bars representing ± 1 SD. Values significantly different at $P < 0.05$ by *t* test are designated by an asterisk.

1. Abbreviations used in this paper: CO, cardiac output; Hct, hematocrit; WBC, white blood cell.

Figure 2. Serum bicarbonate concentration on the ordinate is plotted against time on the abscissa with "0" h representing the beginning of the 1-h period of shock. Bicarbonate concentration was calculated from arterial pH and PCO₂ using the Henderson-Hasselbalch equation. Data points represent means of the control animals (open circles) or MAb 60.3-pretreated animals (closed circles) with error bars representing ± 1 SD. Values significantly different at $P < 0.05$ by *t* test are designated by an asterisk.

volume vs. $33 \pm 34\%$ of total blood volume in the MAb 60.3-treated group) but this difference was not statistically significant ($P = 0.1$). Associated with this tendency toward increased volume requirement in the control animals was an elevated Hct compared with the MAb 60.3-treated animals at the end of the resuscitation period (Fig. 1). The WBC counts in both groups fell to about one-half baseline by the end of the ischemic phase, yet returned to baseline level by the end of the resuscitation period. There were no significant differences in WBC counts between the two groups at any measurement point (data not shown).

Late sequelae and survival. Only two of the seven control animals (29%) survived 5 d after resuscitation with two deaths occurring within the first 24 h and one each on days 2, 3, and 4. In contrast, all seven MAb 60.3-treated animals survived 5 d ($P < 0.01$). The control animals remained acidotic for 3 d. By

Table I. Effect of MAb 60.3 on Survival and Degree of Injury after Hemorrhagic Shock and Resuscitation

	Control	60.3	P
Survival	2/7	7/7	<0.01
Δ Weight (%)	-17 ± 10	$+1 \pm 13$	<0.05
Δ Hct	-12 ± 6	-5 ± 5	<0.05
Organ injury			
Lung	Moderate	Moderate	
Liver	Severe	Mild	
Gastric mucosa	Severe	Mild	

MAb 60.3 improves survival and reduces weight loss, blood loss, and organ injury after hemorrhagic shock and resuscitation. Animals were pretreated with either saline (control) or MAb 60.3 (2 mg/kg) and then subjected to 1 h of hemorrhagic shock followed by resuscitation. Survival was assessed to 5 d. Weight loss and Δ Hct represent changes from preinjury to time of death. Organ damage was assessed subjectively by gross and histologic examination at necropsy.

comparison, most of the MAb 60.3-treated animals rapidly corrected their acidosis within the first 24 h (Fig. 2). The control animals also had evidence of progressive cachexia in contrast to the MAb 60.3-treated animals who actually gained weight on average (Table I).

At necropsy, all of the control animals had evidence of significant injury to lungs, liver, and gastrointestinal mucosa, characterized by intravascular and extravascular accumulation of neutrophils, endothelial injury, and loss of vascular integrity with edema, hemorrhage, and necrosis. The gastric mucosa was particularly susceptible to injury in the control animals and in all cases the stomach was grossly hemorrhagic. In the MAb 60.3-treated animals the liver and gut injuries were either absent or markedly attenuated. Histologic sections of stomach, liver, and lung, typical of both groups when killed at 24 h, are shown in Fig. 3. Consistent with the ongoing blood loss in the control animals, their Hct fell significantly more than the MAb 60.3-treated animals (Table I).

In contrast to the reduction in gastrointestinal injury seen in the MAb 60.3-treated animals, there was no significant difference in the pulmonary injury. Both groups typically had evidence of patchy atelectasis and loss of vascular integrity with intraparenchymal hemorrhage and alveolar hyalin. However, neither group had a substantial impairment in oxygenation or ventilation as measured by arterial PO₂ or PCO₂, nor were there significant differences between the two groups (data not shown).

Discussion

An association between leukocytes and ischemic organ injury has been known for many years. Until recently, however, leukocytes were thought to have a role only in the healing process that followed ischemic injury (14). Recent studies, using neutrophil-depleted animals, have provided evidence of a more integral leukocyte role in the actual pathogenesis of ischemia-reperfusion injury (2, 3, 15).

There are several mechanisms by which neutrophils might cause tissue injury in the setting of ischemia-reperfusion. Because they are larger and less deformable than erythrocytes, neutrophils may plug the small capillaries as perfusion pressure drops (16). When neutrophil adhesiveness increases, either in response to direct neutrophil stimulation, or by endothelial-mediated mechanisms (8), this situation is worsened. Neutrophils may then actively adhere to endothelium and to each other (aggregation), finally occluding the larger post-capillary venules and ultimately resulting in the "no-reflow phenomenon" (15, 16). Once adherent to endothelium, neutrophils may then release proteases, toxic oxygen metabolites, and vasoactive substances. Together these may produce endothelial injury with subsequent loss of vascular integrity, edema, hemorrhage, and tissue injury (7). Studies in intestine (17) and myocardium (3, 18) have shown a dramatic increase in tissue leukocytes with microvascular plugging by leukocytes shortly after ischemia and reperfusion. These studies have also shown a marked reduction in neutrophil accumulation and tissue injury by inhibitors of AA metabolism (18) or by inhibitors of oxygen free radicals (17). This suggests that the deleterious effects of AA metabolites and oxygen free radicals in ischemia-reperfusion injury may be due, at least in part, to their ability to generate or activate neutrophil chemoattractants.

This is supported by studies demonstrating that the superoxide radical is capable of activating a latent neutrophil chemotactic factor in plasma (19).

Morphologic studies have demonstrated that neutrophil adherence to endothelium is a critical early event in the process leading to neutrophil-mediated inflammation and tissue injury (20). The MAb 60.3 has proven to be a highly effective agent at blocking this critical step. We have previously shown that MAb 60.3 in vitro produces virtually complete inhibition of stimulated neutrophil adherence to cultured endothelium (21). Similarly in vivo it produces a nearly complete inhibition of chemotaxis-induced emigration and tissue accumulation of neutrophils (10).

In this study of generalized ischemia-reperfusion, we found that selectively blocking leukocyte adherence functions with the MAb 60.3 reduced organ injury and significantly improved survival. This indicates that leukocytes, through increased leukocyte adhesiveness, play an important role in the development of the organ injury associated with generalized ischemia and reperfusion.

In interpreting these results it is important to recognize the highly selective nature of MAbs. In this case, the MAb 60.3 binds to a function-related idotype of CD18 (9), which has been found only on leukocytes. Because of this, any differences between treatment and control groups must be attributed to leukocytes. Since neutrophils comprise the vast majority of circulating leukocytes and since they are primarily involved in the acute phase of injury, it is likely that neutrophils are primarily responsible for the leukocyte-mediated injury. Our results, however, do not rule out the possibility that other leukocytes or other leukocyte adherence functions may also be involved, as CD18 is present on all leukocytes.

Saline was used as a control in these studies rather than an Ig. This is because available isotype-matched control antibodies do not bind to rabbit neutrophils and because all batches of nonspecific murine IgG tested were contaminated with endotoxin, producing fever and leukopenia in the animals. It is unlikely that the protective effects of MAb 60.3 are due to nonspecific Ig effects as we have previously shown that Fab₂ fragments of MAb 60.3 are as effective as the intact antibody in preventing leukocyte adherence to endothelium in vivo (10). It is also unlikely that MAb 60.3 provided any protection by increasing plasma oncotic pressure since the amount of IgG administered would be expected to increase serum protein concentration by only 0.05%.

The most prominent injury we observed was to the gastrointestinal system. This is not surprising since the intestinal mucosa and hepatic parenchyma are known to be very susceptible to ischemia. The gastrointestinal injury was also the injury that showed the greatest reduction by MAb 60.3. In addition to the qualitative reduction in injury (as assessed histolog-

ically) there was also a significant protection by MAb 60.3 with respect to acidosis, gastrointestinal bleeding, and the animals' ability to maintain their weight. We have observed a similar protective effect of MAb 60.3 in an isolated feline small bowel model of ischemia-reperfusion (22).

It is interesting to note that a pulmonary injury was observed at necropsy in both groups, though neither group had a significant impairment of oxygenation or ventilation. One interpretation of the ineffectiveness of MAb 60.3 at reducing this injury is that the pulmonary injury in this model is leukocyte-independent. Recently, some doubt has been cast on the role of neutrophils in the pulmonary injury associated with the adult respiratory distress syndrome (reviewed in reference 23). It is possible that the injury we observed is of a similar nature. Another possibility is that leukocyte adherence to pulmonary endothelium is not mediated by CD18 and therefore is not blocked by MAb 60.3.

Though this study strongly suggests that leukocytes play a central role in the development of organ injury after ischemia and reperfusion, the question of when leukocytes exert their damaging effects remains unanswered. There is a body of evidence that suggests that after limited ischemia, substantial injury may occur as a result of reperfusion (reviewed in reference 24). Our results suggest that at least part of the leukocyte-mediated injury occurs during the ischemic phase. This is based on the observation that, at the end of the ischemia phase, the MAb 60.3-treated animals had a significantly lower Hct and that they had required more blood to be removed to maintain the same BP and CO as the control animals. This suggests that by the end of the ischemic phase, the control animals had lost their ability to compensate and were already entering the period of intravascular fluid loss, whereas the MAb 60.3-treated animals were still compensating by drawing extravascular fluid into the intravascular space. After resuscitation, this difference became more pronounced as the control animals showed a higher rate of intravascular fluid loss, evidenced by relative hemoconcentration and a tendency to require more crystalloid.

We have demonstrated in this study of generalized ischemia-reperfusion that blocking leukocyte adherence functions with the anti-CD18 MAb 60.3 results in a marked reduction of multiple organ injury and an improvement in survival rates. It is, therefore, reasonable to postulate that a similar protective effect may prevail with ischemia-reperfusion injury to other isolated organs such as brain, heart, kidney, muscle, and skin. Additional studies will be required to examine issues such as safety, side effects, duration of action, etc. If further studies confirm this protective effect in other models, our findings may then be relevant to the study and perhaps therapy of the many important clinical disorders that result from ischemia and reperfusion.

Figure 3. A histologic comparison of organ injury in rabbits treated with saline (control) or MAb 60.3 then subjected to 1 h of hemorrhagic shock followed by resuscitation. For this comparison, animals were killed at 24 h, specimens fixed immediately, stained with hematoxylin and eosin, and photographed at 200 $\times$. (A) Section through liver of control rabbit showing hepatocyte necrosis and neutrophil infiltration (arrows). (B) Section through liver of identically injured rabbit, treated with MAb 60.3, showing no organ injury. (C) Severe

stomach injury produced in a control rabbit. The luminal epithelium (L) is completely destroyed and the tissue infiltrated with leukocytes (arrows). (D) Normal epithelial stomach lining (Ep) of MAb 60.3-treated rabbit. (E) Section through lung of control rabbit showing leukocyte infiltration (arrows) and diffuse hemorrhage. (F) Lung of MAb 60.3-treated rabbit. Cellular infiltration exists as in control rabbit, with only slightly less hemorrhage.

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