Inhibition of Leukocyte–Endothelial Adherence following Thermal Injury

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Progressive microvascular damage in the tissue adjacent to a cutaneous burn injury results in extension of burn size. The role of neutrophils (PMNs) in the pathogenesis of microvascular injury was investigated by inhibition of PMN adherence to the microvascular endothelium using monoclonal antibodies directed to the leukocyte CD18 adhesion complex or its endothelial ligand, intercellular adhesion molecule-1 (ICAM-1, CD54). A model of thermal injury was developed using New Zealand White rabbits. Under general anesthesia two sets of three full-thickness burns separated by two 5 × 30-mm zones were produced by applying brass probes heated to 100°C to the animals’ backs for 30 sec. Cutaneous blood flow determinations were performed for 72 hr. Blood flow measurements were performed using a laser doppler blood flowmeter (PF3, Perimed, Piscataway, NJ). There were five experimental groups; controls given saline alone, n = 12; animals given monoclonal antibody to the PMN CD18 complex, R 15.7 prior to burn injury (pre-R 15.7, n = 5); animals given R 15.7 30 min after burn injury (post-R 15.7, n = 6); animals given the anti ICAM-1 antibody, R 6.5 prior to burn (pre-R 6.5, n = 6); and animals given the R 6.5 30 min postburn injury (post-R 6.5, n = 6). BF in the marginal “zone of stasis” between burn contact sites was significantly higher in the antibody-treated animals and administration of the antibodies 30 min after injury was as effective as preburn administration in preserving blood flow. At 72 hr post-burn all antibody-treated animals had blood flow in the areas at risk for progression (i.e., the zone of stasis) at or above baseline levels while the control animals had levels equal to 34.7 ± 12% of baseline (P < 0.05 by analysis of variance and Mann–Whitney U test). These results suggest that PMNs play an important role in the pathogenesis of burn wound progression, and that this progression can be attenuated by modulating PMN–EC adherence.

Burn injury initially produces an area of irreversible tissue destruction surrounded by a marginal zone of injury with reduced blood flow. In the postburn period ongoing inflammation and microvascular injury in the zone of stasis result in extension of the area of tissue loss.

Leukocytes, particularly polymorphonuclear neutrophils (PMNs), are central mediators of microvascular endothelial injury in many acute pathologic processes [1-4]. PMNs have been identified as possibly contributing to the microvascular occlusion seen following burn injury both systemically and locally. Deitch et al. have demonstrated an increase in PMN activation when PMNs are exposed to burn blister fluid in vitro [5]. Nelson et al. have demonstrated an increase in the surface expression of CR3 expression (CD11b/CD18) on circulating PMNs following burn injury [6]. PMN-mediated injury is dependent in part on PMN adherence to the vascular endothelial cell (EC) surface and PMN–EC aggregation in the microvasculature. PMN–EC adherence results in the formation of a microenvironment between the PMN and the EC [7]. In this microenvironment, PMN-derived proteases and toxic oxygen products produced by both the EC and PMN can exist in high local concentrations. These highly reactive substances, partially protected from inactivation by circulating plasma anti-proteases and free radical scavengers, then produce endothelial cell injury resulting in intercellular gap formation, increases in microvascular permeability, edema, and thrombosis. PMN–PMN aggregation further compromises the microvascular circulation by plugging of capillaries and post-capillary venules, extending the zone of ischemia, and subsequent tissue loss.

Several receptor-counter receptor pairs of ligands on the EC and the PMN have been identified. One of the major sets appears to be the PMN CD11/CD18 complex and the intercellular adhesion complex (ICAM-1, CD54) on endothelial cells. The CD11/CD18 complex is a heterodimer composed of three distinct α chains CD11a, CD11b, and CD11c with a common β chain CD18. The CD11/CD18 complex, collectively labeled CDw18, is present constitutively on the surface of normal neutrophils and is upregulated in response to many stimuli. Monoclonal antibodies to portions of the CD11/CD18 complex have been developed which functionally inhibit
neutral adherence in vitro and in vivo [8–12]. Studies of isolated myocardial and intestinal ischemia–reperfusion injury have demonstrated protection by using anti CD18 antibodies to inhibit PMN–EC adherence [13, 14]. In addition, improved survival and reduced organ injury following hemorrhagic shock in rabbits and in nonhuman primates treated with anti-CD18 antibody have been reported [15]. The monoclonal antibody, R 15.7, is a murine-derived IgG2a which recognizes a functional epitope on CD18 [16, 17]. R 15.7 has been demonstrated both in vitro and in vivo to effectively block neutrophil adherence and emigration to a variety of stimuli including LPS, phorbol myristate acetate (PMA), N-formylmethionyl-leucyl-phenylalanine (FMLP), complement fragment, Interleukin-1 (IL-1), and tumor necrosis factor (TNF).

An alternate method of modulating PMN–EC interaction is through the use of antibodies directed against the endothelial components of the adherence complex, particularly ICAM-1. One such antibody, R 6.5, is a murine-derived IgG2a. Administration of R 6.5 has been reported to reduce in vivo PMN migration in models of airway inflammation in rabbits and has been effective in the prevention and treatment of acute renal rejection in nonhuman primates [17, 18].

We tested the hypothesis that inhibition of PMN-mediated microvascular injury by inhibition of PMN–EC adherence with the anti-CD18 monoclonal antibody, R 15.7, or the anti-ICAM monoclonal antibody, R 6.5, would reduce the microvascular damage in the marginal zones of stasis following thermal injury.

METHODS

A model of thermal injury was developed using New Zealand White rabbits (1.8–2.3 kg) of either sex. The animals’ backs were shaved and venous access was obtained by canulation of a peripheral ear vein with an angiocath (24 gauge). Catheter patency was maintained by twice daily flushes with 1.0 ml of heparin (10 U/ml). Blood samples were obtained by venipuncture of a peripheral ear vein. Leukocyte (WBC) counts were performed using a hemocytometer. Hematocrit (Hct) was determined with capillary microcentrifugation. Cutaneous blood flow (BF) measurement was performed using a laser doppler blood flow meter (Periflux-PF3, Perimed, Inc., Piscataway, NJ) and an integrating flow probe (PF 313, Perimed, Inc.) containing 7 efferent laser fibers and 14 afferent fibers which reflect capillary perfusion in a tissue volume of approximately 1200 mm² (Perimed, Inc.). Previous studies have shown good correlation between laser doppler blood flow measurement and standard radionabeled microsphere calculations of blood flow [19]. Leukocyte (WBC) counts and Hct were obtained at baseline at 24, 48, and 72 hr immediately post burn. Under general anesthesia (isoforane) two sets of three full-thickness burns separated by two 5 × 30-mm zones were produced by applying brass probes heated to 100°C to the animals’ backs for 30 sec. Diagrammatic depiction of the arrangement and dimension of the burns are shown in Fig. 1. Baseline blood flow was measured at designated burned sites, marginal zones, and shaved unburned skin sites and repeated 1, 2, 3, 4, 24, 48, and 72 hr postburn. There were five experimental groups; controls given saline alone, n = 12; animals given R 15.7 prior to burn injury (pre-R 15.7, n = 5); animals given R 15.7 30 min after burn injury (post-R 15.7, n = 6); animals given the anti ICAM-1 antibody, R 6.5, prior to burn (pre-R 6.5, n = 6); and animals given the R 6.5 30 min postburn injury (post-R 6.5, n = 6). The murine-derived antibodies R 15.7 and R 6.5 were produced and purified as previously described and administered in sterile saline in a concentration of 1.0 mg/ml [16, 17]. Both the R 15.7 and R 6.5 were given in a single dose by intravenous injection. The dose of R 15.7 was 1.0 mg/kg and the dose of R 6.5 was 2.0 mg/kg. These doses were selected based on previously reported efficacy in the studies cited above. All animals were given analgesic (buprenorphine 0.05 mg/kg iv every 12 hr) throughout the study period. Two animals in each group were anesthetized with ketamine (20 mg/kg iv) and biopsies of one burn site obtained at 24 hr postburn for histologic comparison of burn depth, edema, and leukocyte infiltration. Animals were evaluated twice daily for pain and suffering using a quantitative pain scale and were euthanized if pain or suffering was severe. At 72 hr postburn, the zones between the burn sites were evaluated for gross evidence of progres-
RESULTS

There was no significant difference in baseline weight, hematocrit, or leukocyte counts among the five groups. The total percentage of body surface area burn to each animal was <5% and did not result in detectable changes in behavior or feeding or significant changes in cutaneous blood flow at shaved unburned skin sites in any of the experimental groups. One animal in the pre-R 6.5 group was euthanized 24 hr postburn. Hematocrit remained unchanged in each group throughout the length of the study. Elevation in leukocyte counts were observed at 24 hr in the pre-R15.7 (18,000 ± 8300 cells/mm³), the post-R 15.7 groups (37,800 ± 8300 cells/mm³), the pre-R 6.5 (18,300 ± 4900 cells/mm³), and the post-R 6.5 (24,100 ± 6100 cells/mm³) groups compared to the controls (8500 ± 2300 cells/mm³). WBC counts in the antibody-treated groups returned to the levels of the control animals by 48–72 hr postburn.

Baseline cutaneous blood flow in absolute perfusion units (PU) as measured with the laser doppler blood flow meter is presented (Fig. 2) for burn sites, the marginal zones, and the shaved unburned skin sites in each of the experimental groups. There were some differences in baseline cutaneous blood flow between experimental groups with the pre-R 15.7 group having lower baseline levels compared to controls; however, within each group, cutaneous blood flow measurements did not vary. To account for intergroup variations in baseline cutaneous blood flow, serial changes are compared as percentages of baseline blood flow.

Serial changes in blood flow in the shaved unburned skin sites presented (Fig. 3) as a percentage of baseline showed no difference among groups at each evaluation point.

Blood flow in burn contact sites expressed as a percentage of baseline are shown in Fig. 4. All groups demonstrated immediate and persistent decreases in perfusion to less than 20% of baseline blood flow which persisted through Day 3 postburn. The consistent reduction in blood flow at the burn contact sites observed among all groups indicates the equivalence of the burn injury produced in each set of animals. On gross and histologic examination the burn contact sites of all five groups were clearly full-thickness injuries.

Serial blood flow measurements in the marginal zones of stasis for controls and the pre- and postadministration of anti-CD18 antibody, R 15.7 are shown in Fig. 5. The control animals developed decreases in blood flow in the initial postburn period which persisted throughout the 72-hr period of observation. The pre-R 15.7 group also developed decreases in perfusion in the zone.
of stasis initially but showed gradual recovery over 24, 48, and 72 hr, which was significantly ($P < 0.05$) higher than the blood flow seen in the control group. The post-R15.7 group showed even greater improvements in blood flow over the course of study, with values significantly higher than controls at all time points. Both the pre-R 15.7 and post-R 15.7 groups had perfusion near baseline at the conclusion of the 72-hr observation period. The serial blood flow measurements in the marginal zones of stasis for the pre-R 6.5 and the post-R 6.5 groups are presented in Fig. 6 along with the controls again presented for comparison. The pre-R 6.5 groups had relative blood flow values significantly greater than controls at 1, 4, 24, 48, and 72 hr postburn. The post-R 6.5 group had relative blood flow values significantly above control at 4, 24, 48, and 72 hr. Just as in the R 15.7 groups, both the R 6.5 groups had relative blood flow values near or above baseline levels at 72 hr.

The visual evidence of burn extension in the marginal zones of ischemia to the point of confluence with the probe contact sites was observed in 18 of 44 zones in the control animals, 5 of 20 (20%) zones in the pre-R 15.7 group, 1 of 20 (5%) zones in the post-R15.7 group, 1 of 20 (5%) zones in the pre-R 6.5 group, and 1 of 20 (5%) zones in the post-R 6.5 group. There was no evidence of infection in any of the groups.

**DISCUSSION**

The administration of antibodies to inhibit PMN-EC resulted in improved microvascular perfusion in the zone of stasis during the 3-day observation period. The microvascular system, especially the microvascular endothelium, has become a focal point in understanding acute injury processes. Endothelial injury by PMNs is thought to occur as a result of the release by PMNs of proteases and toxic oxygen products, each capable of injuring the endothelium and surrounding matrix, leading to increases in permeability. When these substances are released in the circulation, they are inactivated by circulating antiproteases and free radical scavengers. When the PMN is adherent to the endothelium, a microenvironment is formed between the endothelial cell and the PMN where the PMN-derived proteases and toxic oxygen products in high relative concentrations can act synergistically on the endothelial surface protected from inactivation [10, 11]. Endothelial injury occurs, intercellular gaps form, permeability increases, and PMN emigrate through to the extravascular matrix. The mechanism by which R 15.7 exerts beneficial effects in this model of burn injury is presumed to be by inhibition of
PMN–EC adherence or PMN–PMN aggregation. R 15.7 blocks increased PMN adherence in response to TNF, IL-1, C5a, and LTB4 in vitro and TNF, IL-1, and LPS in vivo. These substances have been implicated in many of the physiologic changes following thermal injury. The increased PMN adherence caused by these cytokines or other factors following thermal injury may be the cause of increased PMN-mediated microvascular injury and the increase in vascular permeability following thermal injury. The ability of R 15.7 to inhibit the increase in PMN adherence from such stimuli, thus preventing endothelial injury, may explain the increased marginal zone blood flow in animals treated with R 15.7. The leukocytosis observed in the R 15.7 and the R 6.5 groups at 24 hr postburn is consistent with observations in other animal models and may represent the release into circulation of the marginated pool of leukocytes [14]. The effectiveness of the anti-ICAM-1 antibody, R 6.5, was similar to that observed for the anti-CD18 antibody, R 15.7, and suggests that PMN–EC adherence is an important mechanism in the pathogenesis of microvascular destruction following thermal injury. Precise definition of the stimuli responsible for the increased PMN–endothelial adherence after thermal injury remains to be determined.

Unexpectedly, the post-treated groups had results as good or better than pretreated animals with both R 6.5 and particularly with R 15.7. One potential explanation for this observation is that in the early postburn period the proinflammatory response which allows recruitment of leukocytes and changes in vascular autoregulation is desirable and indeed potentially beneficial; however, as they progress unchecked or escape down regulation, subsequent microvascular injury and burn progression/extension result. If this is the case, then a window of opportunity may exist following thermal injury during which modulation of this process is possible.

While there was no evidence of burn wound infection, the limited duration of this study, 3 days, is not adequate to address the infectious sequelae of leukocyte inhibition. Clearly, concern over potentially increasing susceptibility to infection when inhibiting leukocytes needs to be addressed. In the clinically relevant situation of leukocyte adherence deficiency, soft tissue infection is a common problem. This was recently addressed by Sharar et al. using a rabbit model to test susceptibility to Staphylococcus aureus inoculation [20]. It was reported that inhibition of leukocyte adherence with the anti-CD18 monoclonal antibody MAb 60.3, resulted in substantial increases in incidence and severity of infection compared to saline controls despite the use of antibiotics. This may be related to the inhibition of leukocyte–complement interaction as CD11b/CD18 CR3 is inactivated by MAb 60.3. The R 15.7 antibody would be expected to have similar effects. R 6.5, on the other hand, does not directly effect PMN function and may not be associated with an increased risk of infection.

In this study, we have demonstrated that inhibition of PMN adherence with the anti-CD18 antibody R 15.7 and the anti-CAM-1 antibody R 6.5, prior to and 30 min following burn injury, improves microvascular perfusion in the marginal zone of stasis following thermal injury and prevents burn extension/progression in the marginal zone of stasis. This supports the role of PMNs as central mediators of the microvascular injury responsible for extension of burn size in the zone of stasis. Further study of the timing of postburn administration of these antibodies to determine the window of opportunity may help determine the potential usefulness of this approach.

REFERENCES


