Neutrophil Activation and Tissue Neutrophil Sequestration in a Rat Model of Thermal Injury

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Neutrophil (PMN) deposition in tissues (leuko-sequestration) after shock may produce local tissue injury from proteases and oxygen intermediaries which are released from sequestered PMNs. We quantified leukosequestration in tissues in burned rats using two methods of analysis: 1), measurement of lung myeloperoxidase (MPO); 2), measurement of radio-labeled PMNs and erythrocytes deposited in multiple tissues. After tracheostomy and venous cannulation, rats received 17% TBSA full-thickness contact burns and were resuscitated with 20 cc intraperitoneal saline. Lung PMNs were estimated by measuring MPO in lung tissue. PMN influx into lung, liver, spleen, gut, skin, muscle, kidney, and brain was determined by removing (preburn) and differentially radio-labeled PMNs (111In) and erythrocytes (51Cr), reinfusing cells 4.5 hr postburn, and measuring tissue radioactivity 5 hr postburn. Tissue edema was measured by determining extravasation of 125I-labeled albumin in tissues. Peripheral blood PMNs were analyzed for intracellular H2O2 content utilizing a fluorescent dye which reacts with H2O2 coupled with analysis of cell fluorescence by flow cytometry. MPO was elevated in lungs 8 hr postburn (P < 0.05). PMN influx into lung tissues was confirmed by histologic examination. Radioisotope studies demonstrated significant (P < 0.05) leukosequestration into lung, gut, kidney, skin, and brain tissues at 5 hr postburn. Respiratory burst activity of peripheral blood PMNs was increased 5 hr postburn (P < 0.05). Flow cytometric analysis indicated that peripheral blood PMNs were capable of producing markedly increased H2O2 levels 5 hr postburn. Tissue edema, manifested by radio-labeled albumin influx, was not seen in any tissues. Since others have shown that sequestration of metabolically active PMNs may induce remote tissue injury, therapies which block postburn leukosequestration may be able to improve clinical outcomes by limiting remote tissue injury.

INTRODUCTION

In the United States, the gradual and increasingly widespread adoption of burn wound excision and aggressive wound coverage in most burn centers [1–3] has dramatically decreased the incidence of burn wound sepsis which previously was the major cause of mortality in burn patients [4]. The major source of mortality following burn injury has now become pulmonary failure and pneumonia [5]. Pulmonary complications are usually manifested as acute respiratory distress syndrome (ARDS) and pneumonia.

There is evidence that the development of ARDS following injury, sepsis, and shock is closely related to accumulation of neutrophils (PMNs) in the lungs, followed by local release of toxic mediators including proteases and high energy oxygen metabolites [6–11]. Priming, adherence, and activation of PMNs may be mediated by endotoxin [10, 12, 13] in association with release of multiple cytokines, which may elicit both systemic and local effects.

Our previous experiments verified that 32% TBSA burn injury in mice resulted in transient leukosequestration in the lungs [14, 15]. We have proceeded to study PMN activation and leukosequestration in multiple tissues in a rat burn injury model, since the use of the larger animal facilitates more detailed studies of local and systemic effects of injury and shock.

MATERIALS AND METHODS

Animals and burn injury. Rats were maintained in accordance with the guidelines of the U.C.S.D. Animal Research Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The radiolabeling studies, performed in Sweden, were approved by the Göteborg Ethical Review Committee on Animal Experiments. Male Wistar rats, weighing 200–300 g, were anesthetized by intraperitoneal (i.p.) injection with Nembutal (Abbott Labs, N. Chicago, IL), 2 mg/100 g body weight (b.w.), and Diazepam (Elkins and Sinn, Cherry Hill, NJ), 2.5 mg/100 g b.w. The animals were housed in a temperature-controlled facility and were allowed 24 hr access to food and water. Wound sepsis which previously was the major cause of mortality in burn patients [4]. The major source of mortality following burn injury has now become pulmonary failure and pneumonia [5]. Pulmonary complications are usually manifested as acute respiratory distress syndrome (ARDS) and pneumonia.

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NJ), 0.4 mg/100 g b.w. Animals were shaved and depilated using Nair (Carter-Wallace Inc., New York, NY). The animals then underwent tracheostomy and were allowed to breathe spontaneously through the tracheostomy tube (3/8-in, polyethylene tube, 2 mm ID). Femoral or internal jugular vein was then cannulated by cutdown with polyethylene tubing (PE 50). Line patency was maintained with .2 cc of a 10 U/cc heparin in .9% NaCl solution. Animals were maintained normothermic by being placed on a warm (37°C) heating pad.

A convex, body-conforming stainless steel template (approximately 8 cm x 5.8 cm x .4 cm) was heated to 250°C using a thermostor for calibration, and then applied to the depilated dorsum of the anesthetized animal for 7 sec. The template was resuscitated with 20 cc of .9% NaCl i.p. immediately postburn. The template produced a burn covering approximately 17% of the body surface area. Slight changes in template size were utilized for various b.w.s to produce burn sizes of uniform percentage total body surface areas; surface area was calculated from a published formula for small animals [16].

Duration of the experiments was up to 24 hr postburn. Animals were euthanized and viscera were immediately procured and assayed at appropriate time points. For the radiolabeled studies, rats were kept anesthetized for 5 hr so that the animals could not remove intravenous catheters. For the analysis of lung myeloperoxidase (MPO), animals were permitted to awaken postburn and they were then killed at indicated time points.

Measurement of tissue MPO content. At various time points after burn injury, animals were killed by cervical dislocation. The chest cavity was opened and the lungs were flushed with normal saline via cardiac puncture of the still-heating heart. Lungs were harvested, weighed, and snap frozen at -70°C for subsequent batch processing. MPO was assayed by measuring the H2O2-dependent oxidation of o-dianisidine. Lung tissue samples were homogenized and resuspended in 45% saline. After centrifugation the supernatant was discarded and the pellet resuspended in 1 ml/50 mg tissue of .05 M potassium phosphate buffer, pH 6.0, containing .55 mixed trimethyl-lammonium bromide (Sigma, St. Louis, MO). This was followed by a three freeze-thaw cycle and additional sonication. After centrifugation at 4°C, .033 ml of the supernatant was mixed with .967 ml of .05 M potassium phosphate buffer, pH 6.0, containing .167 mg/ml of o-dianisidine hydrochloride (Sigma, St. Louis, MO) and 4 µl/ml of 3% H2O2, and absorbance was immediately measured at 450 nm (DU-7 Spectrophotometer, Beckmann Instruments, Fullerton, CA). A standard curve was created using human MPO (Sigma, St. Louis, MO). MPO is a heat-stable enzyme, and by heating the tissue samples certain factors which can interfere with the assay are eliminated [17]. There were four to six animals in each control and experimental group.

Measurement of radiolabeled PMN and erythrocyte content in tissues. Leukocyte sequestration in various tissues during burn shock was studied using radiolabeled autologous PMNs and erythrocytes. Immediately before the burn injury was created, 1 ml of venous blood was drawn from the indwelling catheter into a heparinized tube for preparation of the cells. The PMN fraction was obtained by centrifuging diluted whole blood on a two-step, Percoll (Pharmacia Biotech, Sollentuna, Sweden) density gradient [18]. The PMN fraction was retrieved and labeled with 111In as described (19): 1 ml of the cell suspension (10−3 x 106 cells) was mixed with 1-5 µl of 111In (indium oxide) solution (37 MBq/ml, Amersham International plc, Amersham, UK) and incubated for 30 min at room temperature. Cells were washed twice and resuspended in 1 ml of phosphate-buffered saline (PBS). Radioactivity was measured in a gamma counter before reinjection via the venous catheter. Erythrocyte labeling was performed on the leukocyte-free fraction obtained at the separation procedure described above. Cells were suspended in 1 ml of Hank's Balanced Salt Solution and incubated with 20 µl of 51Cr sodium chromate solution (37 MBq/ml, Amersham) for 30 min at room temperature. Cells were washed twice, pooled with the PMNs and injected 4.5 hr after the burn was performed.

A 1-ml blood sample was drawn from the venous catheter 5 hr after the burn and immediately before the rats were sacrificed by rapid installation of 5 ml glutaraldehyde (2.5%) in .05 M sodium cacodylate) through the tracheostomy. The procedure insured that the blood in the pulmonary circulation is immobilized [20]. The radioactivities (disintegrations per minute (dpm)) of the blood samples, the excised lungs, kidneys, liver, spleen, and sections of liver, intestine, skeletal muscle, nonburned skin, and brain were measured. Tissue-specific leukocyte sequestration was calculated using the leukocyte transit time factor as a measure. The transit factors were calculated according to the relation: Transit factor = (Regional 111In cm dpm X .Cr dpm/ml venous blood) / Regional 51Cr activity X .Cr activity/ml venous blood) [20]. There were five animals in each of the control and burn groups.

Measurement of tissue edema. Tissue edema was measured by injection of 1-10 µl of 111In-labeled human albumin (37 MBq/ml, Amersham) together with the radiolabeled cells. The ratio of albumin to erythrocytes was calculated in the same manner as for the leukocytes, and used as a measure of tissue edema in the various tissues and organs.

Measurement of mixed venous blood gases. Animals were anesthetized and a catheter placed by cutdown into either the femoral or jugular vein. The animals were then burned and permitted to awaken at 12:30. At appropriate time points, venous blood samples were withdrawn into a heparinized tube and blood gas analysis was performed immediately utilizing a Corning 180P Blood Gas Analyzer (Corning Scientific, Medfield, MA) to determine the base deficit. Five to eight animals were utilized for each time point.

Histologic preparation of lung tissue. Representative samples of lungs of control and burned animals were taken. Tissues were fixed in formalin, embedded in paraffin, and then stained with hematoxylin and eosin.

Measurement of PMN respiratory burst. We used a modification of the technique developed to quantify intracellular content of H2O2 [21] with certain modifications to utilize flow cytometry [22-24]. The dye which was utilized, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), crosses the cell membrane and changes its fluorescence emission spectrum after reacting with intracellular H2O2.

Blood was drawn from the vena cava of anesthetized rats and placed in a heparinized tube. A 100-µl aliquot of blood was diluted with 850 µl of PBS and 8 µl of 5 mM DCFH-DA (Molecular Probes, Eugene, OR) was added. Following 15 min of incubation, 30 µl of LDS-751 (1.67 µg/ml) (Exciton, Dayton, OH) was added just prior to flow cytometric analysis of cell fluorescence. For F-met-leu-phe (FMLP)-induced reactive oxygen species measurement, DCFH-DA labeled samples were incubated with 25 µl of 20 mM FMLP (Sigma) at 37°C for 30 min. Samples were analyzed at 0 min and 30 min on a Becton-Dickinson FACStar dual-channel flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The instrument was set up to measure linear forward scatter, which is a measure of particle size, light scatter, which is a measure of granularity, and green fluorescence at 535 nm (corresponding to DCFH) and red fluorescence (LDS-751) at 620 nm. LDS-751 was used to label the nuclei of living cells and permit their identification in whole blood without interference from erythrocytes. Nucleated (red fluorescent) cells were analyzed for PMN mean channel fluorescence at 535 nm. There were five animals in each group.

Statistical analysis of data. MPO data are expressed as units per 100 mg of lung tissue. For the MPO, base-deficit and respiratory burst experiments, statistical comparisons between experimental groups were performed by multiple analysis of variance (when there were more than 2 groups) and the t-test (Instat software, San Diego, CA); with significance assumed at P < 0.05. For the radiolabeled studies, the results obtained for the various organs in the control and burned groups of animals were compared using the Mann-Whitney U-test for non-parametric data, with the chosen level of significance at P < 0.05, two tailed distribution. All data are shown with the mean and standard error of the mean indicated.

RESULTS

Following burn injury the animals developed pronounced metabolic acidosis as determined by blood gas analysis, which persisted through at least 12 hr post-
burn (Fig. 1). At 18 and 24 hr postburn, the base deficit values were unchanged from those of control animals.

Mortality after burn injury ranged from 20% to 50%. No mortalities occurred in the unburned groups. The mortalities were high and indicated that the burn injury was extremely deep. Previous studies in our laboratory showed that increasing the amount of resuscitation fluid did not improve animal survival. Tissues from animals which expired spontaneously were not utilized for obtaining any of the data in these studies.

Figure 2 shows the results of MPO determinations in lung tissue at various time periods after burn injury. MPO values were increased from control animal levels at the 8- through 12-hr time points postburn.

PMN sequestration as determined by the leukocyte transit factor showed significantly higher values in burned animals for the lungs, kidneys, liver, and nonburned skin than for the control group (Fig. 3). No statistical difference was observed for skeletal muscle or spleen, although numerically the values were markedly increased in the burned animals in these two tissues.

Histologic slides of the lung tissues were evaluated and showed marked accumulation of leukocytes at 5 hr postburn.

Analysis of the PMN respiratory burst (Fig. 4) showed that intracellular H$_2$O$_2$ was significantly increased in rats 5 hr postburn, compared to control rats.

**DISCUSSION**

Polymorphonuclear leukocytes (PMNs) play an important role in host defenses by releasing oxygen radicals and lysosomal enzymes to kill engulfed microorganisms. Although PMNs are important for killing microorganisms, the accumulation of PMNs at sites of tissue injury coupled with excessive release of proteases and oxygen metabolites may produce tissue injury which can lead to organ dysfunction and organ failure [6-13, 25]. The hydroxyl radical has also been shown to activate complement, which leads to further undesirable inflammatory sequelae following shock [26]. These events may be related to the actions of multiple inflammatory mediators following thermal injury, which may have effects at both local and distant sites [25, 27-29].

It has become clear that events associated with distant tissue ischemia and reperfusion can induce local tissue injury. These effects can be mediated by the induced expression of multiple cell adhesion molecules on the surfaces of PMNs and endothelial cells [7, 13].
These molecules include integrins, selections, and intracellular adhesion molecules [30, 31]. It is now believed that the ischemic/reperfused gastrointestinal tract may serve as a priming bed for circulating PMNs, where upregulation of surface receptors via the release of various inflammatory mediators appears to occur [32-34]. Most PMN emigration occurs in specialized regions of the vascular tree, i.e. in postcapillary venules, where leukocytes are normally only loosely tethered to the vessel wall as they roll along the surface on the endothelium. PMN recruitment to sites of inflammation occurs in several discrete steps involving rolling of cells on activated endothelium, activation of PMNs, adhesion of PMNs to endothelium, and migration of PMNs into surrounding tissues [35]. Although PMN adherence appears to be influenced by the CD18/CD11 integrin, as well as endothelial selectins [13, 36], PMN extravasation and superoxide release appear more specifically governed by the CD11b (Mac-1, Mol, CR3) subunit. Thus the primed state of the PMN may be a decisive factor in the production of tissue injury [37]. Evidence suggests that trauma including burns may upregulate the baseline and stimulus-mediated production of toxic oxygen intermediates by PMNs [11, 31, 38]. Thus, recruitment of PMNs into tissue sites followed by release of toxic mediators may induce tissue injury.

MPO is a constituent enzyme of azurophilic neutrophil granules, constituting 5% of PMN dry weight [39, 40]. Assessment of tissue MPO activity provides an indirect assessment of PMN numbers, which correlates well with morphometric evaluations [41-43] and with radiolabeling in vitro. Thus, there are theoretical concerns with utilizing MPO values to estimate tissue PMN content, primarily related to the possible release of MPO by activated PMNs into the plasma, prior to or during their adherence to endothelium. Another concern relates to the relative insensitivity of the MPO assay. Background levels of tissue MPO are at the barely detectable range of the assay, and therefore the actual increase in tissue MPO content may be far greater than reflected in the experiments.

While removal of leukocytes from the animal followed by their radiolabeling in vitro may permit more sensitive measurement of leukocyte adherence to endothelium and subsequent extravasation into tissues, the removal, manipulation, and reintroduction of leukocytes could theoretically result in their activation and subsequent exaggerated responses in tissues. Thus the MPO results serve as a backup method of analysis of pulmonary leukosequestration in these studies. While the spectrophotometric assay which was utilized here also measures the activity of erythrocyte catalases and peroxidases and of MPO in PMNs in residual blood in the lung tissue, blood peroxidase contributes only about 7% of total measured lung peroxidase activity in blood-perfused lung [41]. By using the improved assay for MPO, [17] erythrocyte enzymes and other interfering proteins were eliminated by heat inactivation since MPO is highly stable to exposure to higher temperatures.

While PMN adherence to endothelium is an important component of normal host defenses [35, 45], reports have demonstrated that PMN adherence to endothelium may be dissociated from PMN-induced tissue injury [33, 46] which occurs primarily via release of toxic oxygen intermediates and proteases into the local tissue environment [7-10, 32, 47]. This event may require activation of PMNs by a second stimulus [48]. Although PMN adherence appears to facilitate endothelial injury by creating a local protective environment for the cytotoxic agents which are released by stimulated PMNs, PMN adherence may also occur and be unrelated to release of injurious mediators.

Our studies demonstrate that leukosequestration into multiple tissues occurs during acute burn shock in this rat burn model. Absence of leukocyte sequestration in the gut has been previously reported following burn injury by others in the rat [49], and our own laboratory found no gut leukosequestration in burned mice [15]. Leukosequestration may be a result of upregulation of adhesion receptors by capillary endothelium. The differential relationship between integrins and leukocyte adherence to lung and skin endothelium has been reported following burn injury [31], but we found similar levels of leukosequestration in those two tissues. This could suggest that multiple mechanisms could contribute to the events. Leukosequestration in some tissue beds could be secondary to severe vasoconstriction during acute burn shock, with microvascular stasis and resultant opportunistic leukocyte adherence to endothelium. This process could be accentuated by the known process of PMN aggregation, a process which has been shown to be primarily a CD11b-dependent event [50], and aggregation between PMNs and platelets, an event which appears primarily dependent on P-selectin [51]. In addition, results from filter studies performed by some of the investigators contributing to this paper indicate that non-viscous properties of leukocytes (elasticity and adhesiveness) may become important determinants for the passage of leukocytes through narrow flow channels at low driving pressures [52]. The importance of rheological properties of leukocytes for their pulmonary margination at low flow was shown in previous experiments on isolated, artificially perfused rat lungs [18]. Flow-dependent capillary retardation of leukocytes has also been shown in isolated, perfused skeletal muscle [53].

Further investigations into the mechanisms related to leukosequestration may yield therapies which will protect the tissues from injury, particularly the lungs, during acute burn shock. Protection may also be desirable during extensive surgical procedures following burn injury, since our studies in mice suggest that early burn wound excision may exacerbate pulmonary leukosequestration [15]. Such therapies could include the use of blocking agents for various intracellular adhesion molecules or selectins [31, 54].

The analysis of PMN respiratory burst activity dem-
observed that burn injury significantly upregulated baseline and FMLP-stimulated intracellular H₂O₂ content. Our studies with radiolabeled albumin demonstrated that at this early time point (5 hr) after burn injury, tissue edema could not be demonstrated. Although Mulligan et al. [31] demonstrated edema in tissues following burn injury in rats, a much larger burn injury was utilized in their report (25 to 30% total body surface area) than in our studies. In addition, our failure to determine edema formation may relate to the experimental model which we utilized; the radiolabeled albumin was injected only 30 min prior to sacrifice of the animals. Future experiments will evaluate edema formation over a longer time line.

Although PMNs are key participants in the development of tissue injury in a variety of acute inflammatory diseases as well as following trauma, the mere presence of tissue PMNs does not prove tissue injury. However, the increased respiratory burst activity of peripheral blood PMNs in burned rats, as demonstrated in our studies, suggests that leukosequestration of metabolically active PMNs is occurring in the burned animals. Further studies are underway to study the relative roles of tissue leukosequestration and PMN activation leading to tissue damage following thermal injury.

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