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Role of Leukocyte Adhesion Molecules in Lung and Dermal Vascular Injury after Thermal Trauma of Skin

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Acute second degree thermal injury of rat skin involving 25 to 30% total body surface of anesthetized rats results at 4 hours in evidence of vascular injury both locally (in skin) and remotely (involving lung). The neutrophil dependency for both types of injury has now been established. Monoclonal antibodies to various adhesion molecules have been used to define the requirements for these molecules in the development of vascular injury. In dermal vascular injury, a requirement for Mac-1 (CD11b/CD18) but not for leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) has been established. In this model requirements have also been demonstrated for intercellular adhesion molecule-1 (ICAM-1) and E- and L-selectin but not for very late arising antigen-4 (VLA-4) or P-selectin. With respect to lung vascular injury, dual requirements for both leukocyte function-associated antigen-1 and Mac-1 were found as well as for ICAM-1 and E- and L-selectin but not for VLA-4 and P-selectin. In the lung, there was a close correlation between neutrophil content of the tissue (as assessed by myeloperoxidase) and the effects of protective interventions (directed against blocking of adhesion molecules). These data emphasize the roles of β2 integrins, selectins (L and E), and ICAM-1 in events that lead to neutrophil-mediated vascular injury of dermis and lung after thermal trauma to skin. (Am J Pathol 1994, 144:1008–1015)

Second degree thermal injury of skin involving 28% of the total body surface area in rats leads to complement activation and release of a number of mediators resulting in local and remote inflammatory tissue injury. Locally there is an immediate and persistent reduction (by approximately 70%) in dermal blood flow after thermal injury.[1] The development of edema in skin 1 hour after thermal trauma is complement dependent but unaffected by prior neutrophil depletion.[1,2] Concurrent with the diminution in dermal blood flow is local and systemic activation of complement.[3] Anaphylatoxin generation occurs in parallel with histamine release.[2,3] Under these conditions, plasma levels of xanthine oxidase are elevated.[2,4] Generation of O_2^-, H_2O_2 [and perhaps the hydroxyl radical ($HO^\cdot$)] appear to be related to the ultimate development of endothelial cell damage in skin and in lung 4 hours after thermal trauma.[1,3,5] Edema in lung and skin is attenuated by treatment of animals with superoxide dismutase, catalase, scavengers of $HO^\cdot$, allopurinol, and the iron chelator, deferoxamine.[1,2] The requirement for neutrophils in the second phase of permeability change in the burn wound (3 to 4 hours postburn) has not been determined, although lung injury, which occurs secondary to thermal injury of skin, is known to be complement- and neutrophil-dependent and oxygen radical mediated.[3,5] These studies were designed to assess

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whether dermal vascular edema developing 4 hours after thermal injury is neutrophil dependent and the extent to which the resultant dermal and lung vascular injury occurring 4 hours after thermal trauma requires engagement of leukocyte α4 and β2 integrins, intercellular adhesion molecule-1 (ICAM-1), and the E-, P-, and L-selectins.

Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma Co. (St. Louis, MO).

Monoclonal Antibodies

All murine monoclonal antibodies were of the IgG1 subclass except as noted. A monoclonal antibody against rat CD18 (CL-26) was produced according to previously described techniques.[6] Because the intact form of this antibody caused neutropenia in rats, $F(ab')_2$ preparations were obtained (Immunopure $F(ab')_2$ preparation kit; (Pierce, Rockford, IL). A total dose of 100 µg was injected intravenously in three equally divided dosages at 2.5, 3, and 3.5 hours after thermal trauma. In the experiments where dermal vascular injury was investigated at 1 hour, 100 µg $F(ab')_2$ CL-26 was injected intravenously 5 minutes before thermal trauma. Monoclonal antibody reactive with rat CD11a (WT.1) was generated according to recently published methods and has been demonstrated to recognize a leukocyte epitope of 160 to 170 kd. It was of the IgG2a subclass and did not react with the β-subunit of leukocyte function-associated antigen-1 (LFA-1) (95 to 100 kd). This antibody has been shown to inhibit binding of stimulated lymphocytes to cultured rat high endothelial cells.[7] In these studies, 200 µg of intact antibody (which did not induce neutropenia) was injected intravenously just before thermal injury. A new monoclonal antibody to rat CD11b was generated by immunization of Balb/C mice with rat neutrophils and subsequent fusion of splenocytes from these mice with NS-1 myeloma cells. This antibody has been shown to attenuate neutrophil adhesion *in vitro* and reduce neutrophil-mediated lung injury *in vivo*.[8] When used, 200 µg was intravenously injected at the time of thermal injury.

Antibody to the rat homologue of ICAM-1 (IA29) was produced as described in a recent publication.[9] This antibody was shown to inhibit aggregation of phorbol myristate acetate-induced T cell blasts and to specifically recognize a homologue on high endothelium in lymph nodes and Peyer's patches and blood vascular endothelial cells. For use *in vivo*, $F(ab')_2$ fragments were used (200 µg in three divided dosages at 2.5, 3, and 3.5 hours after thermal injury).

Monoclonal antibody to rat VLA-4 (TA-2) was generated according to recently described methods and was found to inhibit lymphocyte migration into sites of cutaneous and joint inflammation *in vivo*.[10,11] In these studies, 800 µg was injected intravenously just before thermal injury according to a previously established protocol.[10] Antibody against human E-selectin (CL-3) with reactivity to rat E-selectin was generated according to previously published methods.[12] CL-3 did not demonstrate binding to rat monocytes, lymphocytes, platelets, or neutrophils by flow cytometry. The 135 µg $F(ab')_2$ preparations of CL-3 were injected intravenously in three equally divided dosages at 2.5, 3, and 3.5 hours after thermal injury. For all the above studies, positive control animals were given similar dosages of IgG1 (MOPC-21) or $F(ab')_2$ preparations of the same IgG1 on the same dosing schedules.

Antibody against rat L-selectin (HRL-1) was generated in hamsters according to previously published methods and has been shown to inhibit neutrophil binding to activated endothelium.[13] When used, 200 µg $F(ab')_2$ was infused in equally divided dosages at 2.5, 3, and 3.5 hours after thermal injury. This dose schedule did not produce neutropenia (data not shown). Murine monoclonal antibody to human P-selectin (PB 1.3) was used as the intact IgG1 molecule and injected intravenously in a total dose of 200 µg given in three equally divided dosages at 2.5, 3, and 3.5 hours after thermal trauma. This antibody has recently been shown to block P-selectin-dependent lung inflammatory reactions in rats.[14] For the studies, two different monoclonal antibodies were used that were directed against human P-selectin and demonstrated cross-reactivity with a rat endothelial epitope. PB1.3, which is protective in the rat model of cobra venom factor (CVF)-induced lung injury, and PBN1.6 which is not protective.[14]

Neutrophil Depletion

Neutrophil depletion in rats was induced by the intraperitoneal injection of 600 mg cyclophosphamide/kg body weight. Ninety-six hours later, peripheral venous blood was obtained from the tail vein and analyzed for total neutrophil counts. Mean total neutrophil counts were reduced to a mean value of 250 ± 190 cells/mm^3 blood in treated animals.

Animal Model of Thermal Injury

Adult male (300 to 350 g) specific pathogen-free Long-Evans rats (Charles Rivers Laboratories, Portage, MI) were used in these studies. Ketamine (100 mg/kg body weight) (Parke-Davis, Morris Plains, NJ) and xylazine (13 mg/kg body weight) (Miles Laboratories, Shawnee Mission, KS) were used for continuous sedation and anesthesia (for 4 hours). After induction of anesthesia, the dorsal skin over the lumbosacral and flank areas was exposed to 70 C water for 30 seconds. The total body surface area burned was 25 to 30%. ^{51}Cr-labeled red blood cells (RBCs) (prepared according to recently published methods) and ^{125}I-labeled bovine serum albumin were then intravenously injected as markers of hemorrhage and vascular permeability, respectively.[12] Animals were killed at 4 hours (as indicated) and 1 ml of blood obtained from the posterior vena cava at the time of death. The pulmonary vasculature was flushed with 10 ml saline and standard-sized full-thickness biopsies of burned skin were obtained according to recently published methods.[1] Hemorrhage indices were calculated by assaying the amount of ^{51}Cr radioactivity present in lungs and skin sites compared with that remaining in 1 ml blood. Permeability indices were calculated by quantifying the amount of radioactivity (^{125}I-bovine serum albumin) in lungs or skin sites compared with the amount remaining in 1 ml blood.

Lung Myeloperoxidase (MPO) Content

A standard reference curve was established by measuring MPO in lungs that had been injected with known numbers of neutrophils. Lungs from animals involved in these studies were then homogenized and sonicated according to previously described methods.[14] MPO activity in supernatant fluids was measured by the change in optical density (at 460 nm) resulting from decomposition of H_2O_2 in the presence of o-dianisidine.

Statistical Analysis

All values were expressed as mean ± SE unless otherwise indicated. Statistical significance was defined as $P < 0.05$. The data were analyzed using one-way analyses of variance and differences between individual group means and positive controls and were also analyzed using either paired or unpaired Student's *t*-tests.

Results

Protective Effects of Neutrophil Depletion in Skin and Lungs

Neutropenia was induced with cyclophosphamide as described above. ^{125}I-bovine serum albumin extravasation was used as the measure of tissue injury in lung and skin 4 hours after thermal trauma. The results of neutropenia on the development of increased vascular permeability in lung and skin are shown in Figure 1. Protection was calculated after subtracting the negative control values from the values obtained from positive controls and treated positive controls. Neutrophil depletion was associated with a 44% attenuation ($P < 0.01$) of the increase in lung vascular permeability after thermal injury (neutropenic rats showed a mean permeability value of 0.29 ± 0.002 compared with a value of 0.42 ± 0.02 in the nonneutropenic rats). Similarly, the mean permeability index in the thermally injured skin at 4 hours was reduced by 37% ($P < 0.001$) to a mean of 0.55 ± 0.02 in neutropenic animals compared with the mean skin permeability index of 0.82 ± 0.03 among positive controls. Thus, the full development of burn wound edema and lung microvascular injury at 4 hours after thermal trauma to skin is neutrophil dependent in rats. Based on the knowledge that skin

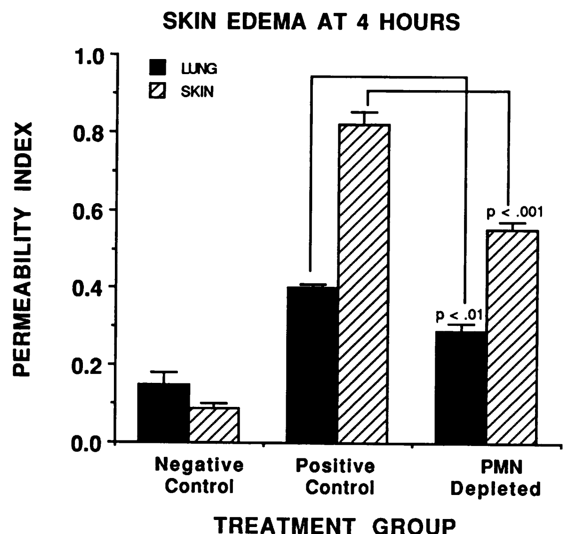

Figure 1. *Protective effects of neutrophil depletion on increases in lung and skin dermal vascular permeability 4 hours after thermal trauma of skin. Neutrophil depletion was associated with 44 and 37% reductions in vascular permeability in lung and skin, respectively, when compared with positive controls with intact blood neutrophils. The* n = *4 for each group in the lung studies and for skin studies* n = *24 for the positive controls and 12 for the other groups. By one-way analysis of variance,* P < *0.001.* P *values for additional Student's* t*-tests comparing treatment groups to positive control groups are shown.*

and lung vascular injury was neutrophil dependent, the role of leukocyte adhesion molecules was evaluated, as described below.

Protective Effects Against Dermal Vascular Injury by Blocking of Adhesion Molecules

Protection against increased vascular permeability and hemorrhage in skin sites 4 hours after thermal injury were evaluated by the use of blocking antibodies to adhesion molecules. The amount of ^{125}I-bovine serum albumin radioactivity in skin sites was compared with the amount present in 1 ml of blood to yield a permeability index. A hemorrhage index was also calculated using ^{51}Cr-labeled rat erythrocytes. For calculation of degree of protection of a given intervention, the appropriate mean negative control value was first subtracted from individual values of relevant positive control treatment groups. Protection was calculated by determining the ratio of the treated to the untreated positive controls. These data are presented in Figure 2. The mean negative control skin permeability index was 0.09 ± 0.01; this was increased markedly to 0.76 ± 0.03 in positive controls. Treatment with anti-CD18 was associated with a 44% (P < 0.001) reduction in dermal vascular permeability. The mean negative control hemorrhage index of 0.003 ± 0.001 was increased to 0.07 ± 0.003 in positive controls; treatment with anti-CD18 was associated with a 60% (P = 0.001) reduction in skin hemorrhage compared with positive controls. Treatment with antibody to CD11a was not associated with a significant reduction in dermal vascular permeability or hemorrhage compared with positive controls (8 and 20% reductions, P NS). In contrast, animals treated with antibody to CD11b demonstrated a 42% reduction in dermal vascular permeability (P < 0.001) and a 60% reduction (P = 0.001) in dermal vascular hemorrhage.

Animals treated with antibody to ICAM-1 had a mean dermal vascular permeability index that was 40% (P < 0.001) lower than the mean positive control permeability index; in the same group, dermal vascular hemorrhage was reduced by 57% (P = 0.002). Treatment with anti-VLA-4 was not associated with any reduction in dermal vascular permeability (−1% reduction, P NS) or hemorrhage (−10% reduction, P NS). Similarly, animals treated with antibody to P-selectin demonstrated dermal vascular permeability values that were 3% decreased below positive control values, although this difference was not statistically significant; hemorrhage values were only 10% lower than positive controls in the same group (P NS). Infusion of antibody to E-selectin was associated with significant attenuation of dermal vascular injury; treated animals had a mean permeability index that was 47% (P < 0.001) lower than the mean positive control values, whereas mean dermal vascular hemorrhage values were 60% (P = 0.003) lower than the mean positive control hemorrhage index. Finally, animals treated with antibody to L-selectin had permeability and hemorrhage values that were 40% (P < 0.001) and 42% (P < 0.001) lower than positive control values. Thus, dermal vascular injury 4 hours after thermal trauma requires Mac-1 and ICAM-1 (but not LFA-1 or VLA-4) and L- and E-selectins but not P-selectin.

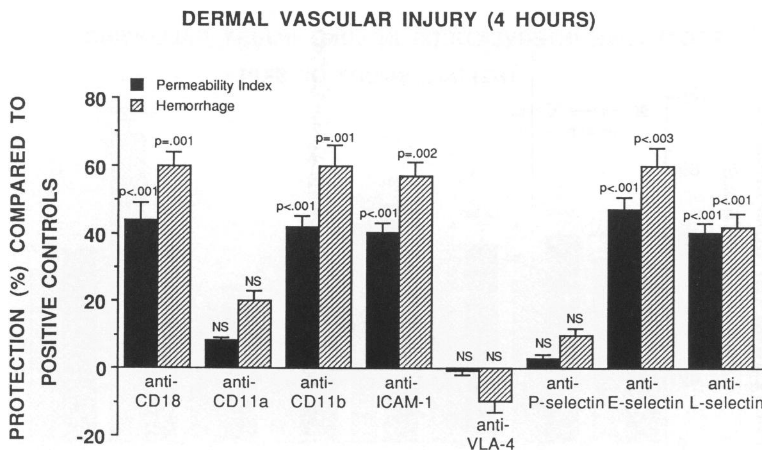

Figure 2. *Effects of blocking adhesion molecules on dermal vascular injury 4 hours after thermal trauma to skin as measured by leakage of ^{125}I-bovine serum albumin and extravasation of ^{51}Cr-RBC. For each vertical bar, n = 12. Details of treatment are contained within the text.*

Protective Effects of Monoclonal Antibodies Against Adhesion Molecules on Lung Injury Developing After Thermal Trauma of Skin

Animals were subjected to thermal injury as described above and were killed 4 hours later and lung vascular permeability and hemorrhage indices determined as described for skin earlier. The data are expressed as percentage of protection and are shown in Figure 3. The mean negative control lung vascular permeability index of 0.16 ± 0.01 rose in positive controls to 0.39 ± 0.02. The mean negative control hemorrhage index, which was 0.04 ± 0.003, also rose significantly to 0.26 ± 0.02 in positive controls. The mean permeability and hemorrhage indices in anti-CD18-treated animals were 48% (P = 0.008) and 53% (P = 0.004) lower, respectively, compared with values in positive controls. Similarly, animals treated with anti-CD11a had 60% (P = 0.001) and 47% (P = 0.012) reductions, respectively, in lung vascular permeability and hemorrhage, respectively.

Animals treated with anti-CD11b also demonstrated 57% (P = 0.003) reduction in lung vascular permeability and 53% (P = 0.006) reduction in lung hemorrhage. The mean permeability and hemorrhage indices in anti-ICAM-treated animals were reduced by 69% (P = 0.001) and 63% (P = 0.003), respectively. Although anti-VLA-4 was not protective in the skin, in animals treated with anti-VLA-4 there was an associated reduction of 30% (P = 0.043) in lung vascular permeability but there was no associated significant reduction in lung hemorrhage when compared with positive controls (21% reduction, P NS). In the case of anti-P-selectin, mean lung vascular permeability values were 39% reduced (P = 0.029); however, lung hemorrhage was not significantly reduced after treatment with this antibody (32%, P NS). Animals treated with anti-E-selectin had mean permeability values that were 57% (P = 0.013) lower than positive controls and mean hemorrhage indices that were 89% (P < 0.001) lower than positive controls. Finally, infusion of anti-L-selectin was associated with 56% (P < 0.001) and 50% (P = 0.001) reductions in lung vascular permeability and hemorrhage, respectively, compared with positive controls. Thus, lung injury that occurs 4 hours after thermal injury of rat skin is LFA-1 and Mac-1 dependent, ICAM-1 dependent, and E- and L-selectin dependent. Interventions designed to block VLA-4 or P-selectin did not show consistent, protective effects in this model.

Lung Tissue Extraction of MPO

Lungs from injured animals treated with irrelevant antibodies (MOPC-21) or with specific monoclonal antibodies directed against the adhesion molecules were extracted for MPO activity as a measure of tissue accumulation of neutrophils. The mean negative control MPO content in lung was 0.11 ± 0.03, whereas in the positive control lungs the mean MPO content was 0.52 ± 0.03. Agents that were significantly protective against lung vascular permeability and hemorrhage (Figure 3) were also protective against tissue neutrophil accumulation (Figure 4). Anti-CD18-treated animals had a mean MPO content that was 81% (P = 0.003) lower than positive controls. Anti-CD11a- and anti-CD11b-treated animals demonstrated MPO values that were 59% (P = 0.012) and 61% (P = 0.013) lower than positive controls, respectively. Treatment with anti-ICAM-1 resulted in a 77% reduction (P < 0.001) in MPO content, whereas anti-VLA-4 did not significantly reduce MPO content and treatment with anti-P-selectin caused a barely statistically significant

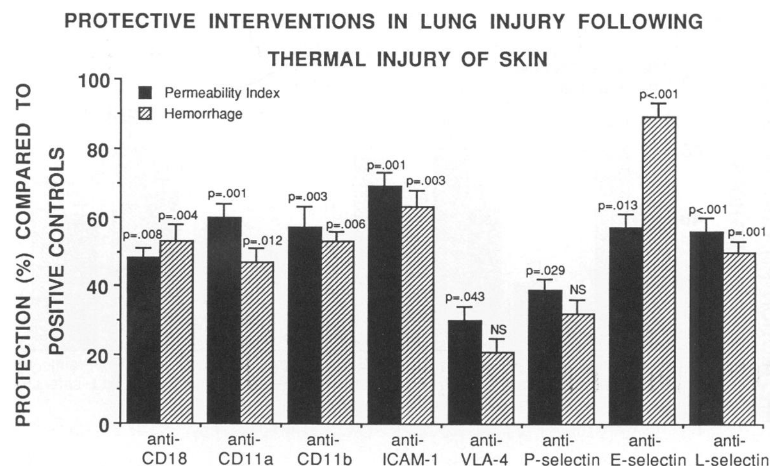

Figure 3. *Effects of blocking of adhesion molecules on lung injury, as assessed by leakage of ^{125}I-bovine serum albumin and extravasation of ^{51}Cr-RBC, 4 hours after thermal trauma to skin. For each vertical bar,* n = *4.*

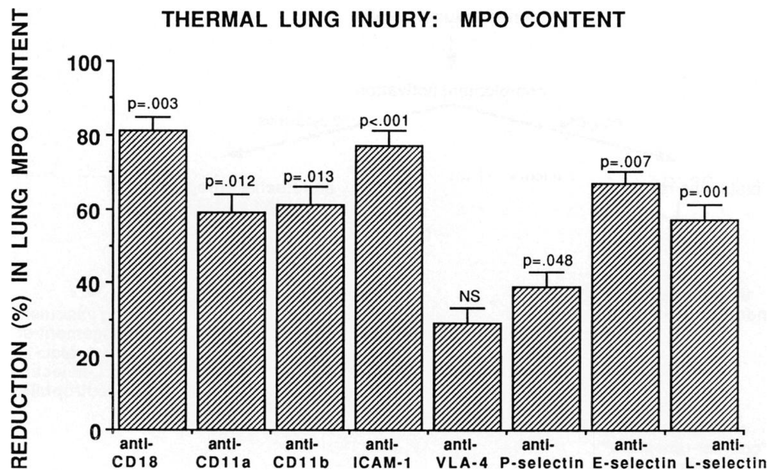

Figure 4. *Effects of antiadhesion molecule interventions on MPO content in lungs companion to experiments described in Figure 3. For each vertical bar, n = 4.*

reduction (39%, $P = 0.048$) in MPO content. Treatment with antibody to E-selectin or L-selectin caused significant reductions in MPO content (67%, $P = 0.007$ and 57%, $P = 0.001$, respectively). Thus, the protective effects of antiadhesion molecule interventions in lung injury (Figure 3) very closely parallel the reductions in MPO content in lung (Figure 4).

Discussion

These studies indicate that vascular injury (as defined by increased permeability and hemorrhage) in rat skin 4 hours after thermal trauma of skin is neutrophil dependent and requires participation of the Mac-1 (but not the LFA-1) β2 integrin complex, ICAM-1, E-selectin, and L-selectin without consistent evidence for a role of P-selectin or VLA-4. Remote lung injury assessed at 4 hours requires both LFA-1 and Mac-1, ICAM-1, E-selectin, and L-selectin and no consistent evidence for the role of VLA-4 or P-selectin. Thus, the β2 integrins involved in these neutrophil-dependent reactions appear to be Mac-1 in the case of dermal vascular injury and both LFA-1 and Mac-1 in the case of lung vascular injury. Given the body of evidence broadly incriminating the role of β2 integrins in the inflammatory response,[15–19] their roles in injury to dermal and lung vascular beds after thermal trauma of skin is not surprising.

Up-regulation of adhesion-promoting molecules allows for increased neutrophil adherence in vitro.[20–25] The demonstration that injury in the skin and lung at 4 hours is neutrophil dependent is consistent with the conclusion regarding the engagement of these adhesion molecules. This is similar to the findings in neutrophil recruitment into lung and subsequent lung injury after deposition of IgG immune complexes in which a requirement for the up-regulation of E-selectin has been demonstrated.[12] In the local (dermal) and systemic (lung) vascular complications after thermal trauma to skin, mediators that are responsible for up-regulation of ICAM-1, the β2 integrins or E-selectin are not currently known but could conceivably be interleukin-1 and/or tumor necrosis factor-α. The requirements for cytokines in these complications that follow thermal trauma have not been established. The requirements for β2 integrins in the skin and in the lung 4 hours after dermal vascular injury once again point out what appear to be intrinsic differences in various beds from the same animal. In IgG and IgA immune complex-induced lung injury, the β2 integrin requirements included LFA-1 but not Mac-1 in the former and a predominant requirement for Mac-1 and a lesser but statistically significant role for LFA-1 in the latter.[26] The data in this study suggest that although lung injury secondary to thermal trauma of skin requires both LFA-1 and Mac-1, in the skin only Mac-1 is required. Requirements for β2 integrins are consistent with the role of neutrophils for the full development of vascular injury in both organs. Why there should be differences in the requirement for LFA-1 between skin and lung is not apparent at present.

As defined either by complement depletion procedures (using serial injections of cobra venom factor)[3] or by complement blockade (using soluble human recombinant receptor 1),[27] vascular injury in both skin and lung 4 hours after thermal injury of skin is complement dependent. It is also known that the injury is allopurinol sensitive,[1] suggesting a complex series of mediator pathways. A possible sequence of pathophysiological events is provided in Figure 5. In this context, thermal trauma activates complement, which locally leads to anaphylatoxin generation. This could cause histamine release and local unlimited

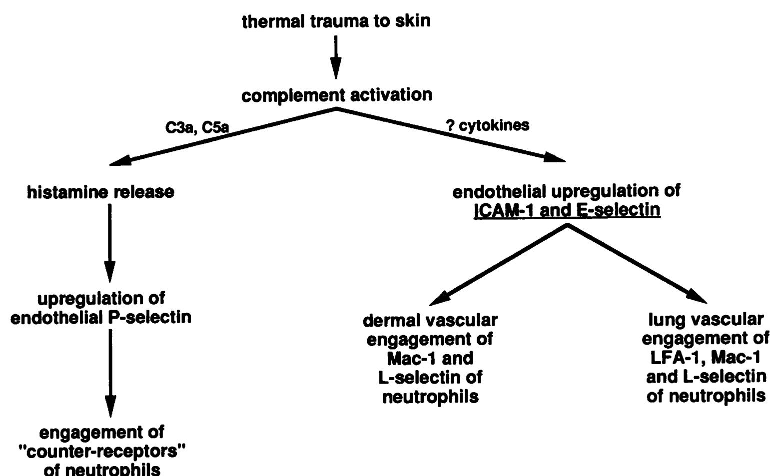

Figure 5. *Proposed sequence of events involving inflammatory mediators and adhesion molecules in microvascular injury of skin and lung after thermal trauma to skin.*

up-regulation of endothelial P-selectin, which could then result in interaction with the neutrophil acceptor oligosaccharides such as sialyl Lewis^x. Together with neutrophil L-selectin, which would react with an unidentified acceptor on the endothelial cell, engagement of these selectins would lead to the initial reversible adhesive interactions that morphologically are described as the "rolling phenomenon." Simultaneously, ICAM-1 and E-selectin up-regulation would be occurring in the endothelial cells in both the dermal and the pulmonary vascular beds, perhaps as a result of cytokine generation and this production of complement activation products. The combined result would be interactions of these adhesion molecules with their complementary adhesion-promoting molecules on the leukocyte (oligosaccharides in the case of E-selectin and β2 integrins, CD11a/CD18 and CD11b/CD18, in the case of ICAM-1 and 2). E-selectin, like L- and P-selectin, may also participate in the "rolling phenomenon." It is also possible that C5a and other chemotactic peptides (eg, interleukin-8) might alter (increase) the affinity of LFA-1, thus increasing its functional expression.[28] The sequential engagement of these molecules would cause cessation of neutrophil movement along the endothelial surface and transmigration, which would be under the influence of chemotactic factor (*versus* chemotactic cytokine and C5a). The ultimate endothelial and tissue damaging event would be the release from neutrophils of toxic oxygen products and proteases that would bring about compromise of vascular integrity, as shown by increased permeability and hemorrhage.

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