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Chronic Inflammatory Disease Alters Adhesion Molecule Requirements for Acute Neutrophil Emigration in Mouse Skin

Joseph P. Mizgerd,* Daniel C. Bullard,† M. John Hicks,‡ Arthur L. Beaudet,§ and Claire M. Doerschuk*2

Mutant mice triply deficient in ICAM-1, E-selectin, and P-selectin did not develop the neutrophilic skin lesions that spontaneously arise in mutants doubly deficient in E-selectin and P-selectin. Thus, ICAM-1 is essential to skin disease resulting from endothelial selectin deficiency. During experimental dermatitis, acute neutrophil emigration was completely prevented in young mice deficient in both selectins (E/P and E/P/I mutants). However, older E/P mutants with spontaneous skin lesions displayed an endothelial selectin-independent pathway for acute neutrophil emigration. In contrast, emigration remained compromised in E/P/I mutants and CD18 mutants regardless of age or lesions. Experimentally induced chronic lesions elicited this pathway for acute emigration in young E/P mutants. Thus, an endothelial selectin-independent pathway for acute neutrophil emigration is induced in E/P mice by chronic inflammation at distant sites, and this pathway may contribute to skin disease resulting from endothelial selectin deficiency. The Journal of Immunology, 1999, 162: 5444–5448.

Materials and Methods

Mice

Mice with homozygous mutations resulting in deficiencies of E-selectin, P-selectin, ICAM-1, or combinations thereof were generated after homologous recombination and selective breeding (1, 7, 8). Homozygous mutations were confirmed using RT-PCR and Southern hybridizations. Among the younger (7–14 wk) mice, the genetic backgrounds of E/P/I and CD18 mutants were mixed 129/Sv × C57BL/6, and the other mutants were backcrossed to C57BL/6. All older (23–35 wk) mice were on the mixed background. Pilot studies revealed no difference in croton oil-induced dermatitis between wild-type (WT) mice of mixed background and C57BL/6 mice. In studies of the younger mice, no mice displayed skin lesions by gross examination. In studies using older mice, all CD18 mutants and no E/P/I mutants or WT mice displayed ulcerative skin lesions on their neck and cheeks, and E/P mutants displayed lesions as specified. Only mice with grossly healthy ears were included in dermatitis studies, and untreated ears from older and ulcerated mice did not show emigrated neutrophils in histologic sections. Mice were housed in barrier facilities. Experiments received institutional approval.

Croton oil-induced dermatitis

Acute dermatitis was induced by topical application of croton oil to the ear, as previously described (4). Mice were anesthetized by methoxyflurane inhalation, and 10 μl of 2% croton oil (Sigma, St. Louis, MO) in 4:1 acetone:olive oil were applied to each side of one ear. After 6 h, mice were killed by an overdose of halothane inhalation. The width of each ear was measured.
measured using spring-loaded calipers. Blood was withdrawn from the inferior vena cava. Leukocytes were counted with a hemacytometer after lysis of erythrocytes, and leukocyte differential counts were assessed in blood smears stained with LeukoStat (Fisher Scientific, Pittsburgh, PA). Ears were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for examination by light microscopy. Neutrophil emigration was assessed using morphometry, using a drawing tube to reflect a grid onto microscopically viewed histologic sections. For each ear, the volume fraction of emigrated neutrophils was assessed by point-counting within four 110-μm wide cross-sections separated by 1.5-mm intervals. Each point was assessed as falling on: 1) epidermis, dermis, or cartilage, and 2) an emigrated neutrophil or not an emigrated neutrophil. To limit the confounding effects of changes in dermal volume upon morphometric analyses, the volume fraction of emigrated neutrophils in each ear was standardized to that ear’s cartilage volume, which is not expected to change during acute (6 h) dermatitis, by dividing the volume fraction of ear composed of neutrophils by the volume fraction composed of cartilage. The relative volume of emigrated neutrophils in the dermis was expressed as “standardized volume fraction.” Edema was quantified as the percent increase in ear width in the croton oil-treated ear compared with the untreated ear for each mouse. The width of each ear was measured with spring-loaded calipers five times. Edema (% swelling) was calculated as: 100 × [(difference in ear widths)/width of the untreated ear]).

Chronic skin lesions were induced by applying 10% croton oil in 4:1 acetone:olive oil to the shaved backs of mice 15 days before acute dermatitis studies in the ear, as described above. This treatment resulted in focal skin lesions. These results suggest that ICAM-1 is essential to the spontaneous skin lesions observed in E/P mutant mice.

Adhesion molecule expression

The relative expressions by circulating neutrophils of LFA-1, very late Ag-4 (VLA-4), and L-selectin were measured using flow cytometry. Mice were killed by an overdose of halothane inhalation, and peripheral blood was drawn into a heparinized syringe from the inferior vena cava after hypotonic lysis of erythrocytes, leukocytes were stained with saturating concentrations of 1 of the following Ab pairs (PharMingen, San Diego, CA): FITC-conjugated M17/4 against murine CD11a and phycoerythrin (PE)-conjugated MEL-14 against murine CD62L, or FITC-conjugated R1–2 against murine CD49d and PE-conjugated RB6–8C5 against murine Gr-1. For the study of LFA-1 and L-selectin, neutrophils were identified by forward and right angle scatter. For the VLA-4 study, neutrophils were additionally identified as being within the neutrophil scatter gate and also positive for the Gr-1 Ag. Negative controls for each Ab consisted of leukocytes stained only with the other Ab from the Ab pair. Values for relative intensity of red and green fluorescence were collected for 5000 neutrophils from each mouse.

Statistics

Data were compared by one-way ANOVA. Circulating neutrophil counts were log-transformed before ANOVA. Individual groups of data were compared post hoc by the Scheffé test. There were five mice in each group for emigration experiments with younger mice. For older mice, there were six mice in the WT group and three mice each in the mutant groups (CD18, E/P, and E/P/I). For expression studies, each group consisted of three mice. All data were presented as mean ± SEM and considered significantly different when p < 0.05.

Results

No spontaneous skin lesions in E/P/I mutants

E/P/I mutant mice did not develop the spontaneous skin lesions that are typical of E/P mutants. By gross examination, no spontaneous skin lesions were observed in 193 E/P/I mutant mice, including 58 mice >18 wk of age and 14 mice >50 wk of age. In histologic sections, E/P/I skin did not appear different from WT skin. Whereas E/P and CD18 mutants developed lesions characterized by polymorphonuclear or mononuclear leukocytes, respectively, skin sections from the neck or face of E/P/I mutants up to 55 wk of age demonstrated no necrosis, bacterial aggregates, or inflammatory infiltrates. These results suggest that ICAM-1 is essential to the spontaneous skin lesions observed in E/P mutant mice.

Adhesion molecule requirements during acute dermatitis in young mice

Acute dermatitis was induced by the application of croton oil, which stimulates the production of proinflammatory cytokines and chemokines resulting in dermal neutrophil emigration and edema accumulation (9). The unirritated ears of WT mice contained few emigrated neutrophils (0.017 ± 0.011 standardized volume fraction), whereas the croton oil-irritated ears were rich with emigrated neutrophils (0.317 ± 0.050 standardized volume fraction). Emigrated neutrophils were present in the dermis; morphometric examination revealed no neutrophils in the cartilage or the epidermis. The unirritated ears were 260 ± 1 μm wide, whereas the irritated ears were 363 ± 14 μm wide, representing a 40% increase in tissue width due to edema accumulation. Histologic sections from croton oil-irritated ears demonstrated an expanded dermal compartment, with fluid-filled spaces separating layers of dermal connective tissue. No ulcers, abscesses, or acanthosis were observed.

Croton oil dermatitis was induced in young (7–14 wk of age) mutant mice. Deficiency of either E- or P-selectin alone had no significant effect on the number of neutrophils that emigrated within 6 h after application of croton oil (Fig. 1). Dermal neutrophil emigration was reduced by 61% in ICAM-1 mutants (Fig. 1). In E/P mutant mice, the standardized volume fraction of neutrophils within the dermis was only 0.017 ± 0.006 (Fig. 1), indicating that these mice had a complete defect in neutrophil emigration. E/P/I mutants also showed a complete defect in neutrophil emigration (Fig. 1).

Edema accumulation within 6 h after application of croton oil was not compromised in the ears of E-selectin, P-selectin, ICAM-1, or E/P mutants (Table I). Dermal edema was decreased by 41% in E/P/I mutants (Table I). Circulating neutrophils were

Table I. Circulating neutrophils and edema accumulation in younger (7–14 wk) WT mice and mice deficient in E-selectin (E), P-selectin (P), and/or ICAM-1 (I) during croton oil-induced dermatitis*

<table>
<thead>
<tr>
<th></th>
<th>Circulating Neutrophils</th>
<th>Edema Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.9 ± 0.4</td>
<td>39.6 ± 5.4</td>
</tr>
<tr>
<td>E</td>
<td>3.1 ± 0.8</td>
<td>40.6 ± 1.9</td>
</tr>
<tr>
<td>P</td>
<td>3.4 ± 0.6</td>
<td>40.0 ± 3.8</td>
</tr>
<tr>
<td>I</td>
<td>3.5 ± 0.7</td>
<td>35.2 ± 5.8</td>
</tr>
<tr>
<td>E/P</td>
<td>27.3 ± 3.9*</td>
<td>35.2 ± 5.7</td>
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<tr>
<td>E/P/I</td>
<td>14.4 ± 1.0*</td>
<td>23.3 ± 3.3*</td>
</tr>
</tbody>
</table>

* Blood was drawn from the inferior vena cava, and circulating neutrophils were expressed as millions/ml. Ear widths were measured with spring-loaded calipers, and edema accumulation was expressed as percent swelling compared with the untreated ear. Asterisks (*) represent significant differences from WT (p < 0.05).

FIGURE 1. Neutrophil emigration during acute irritant dermatitis in 7- to 14-wk-old mice deficient in endothelial adhesion molecules. WT mice and mice deficient in E-selectin (E), P-selectin (P), and/or ICAM-1 (I) were studied 6 h after topical application of 2% croton oil to the ear. Each bar represents the mean and SEM from five mice, and asterisks (*) represent significant differences from WT mice.
increased in E/P and E/P/I mutant mice with acute croton oil dermatitis (Table I), similar to observations of these mice in the absence of dermatitis (1).

Adhesion molecule requirements during acute dermatitis in older mice

The spontaneous skin lesions that develop in E/P mutants contain many neutrophils (1), even though the acute emigration of neutrophils in the skin was prevented in the present study. Thus, we examined the acute croton oil-induced emigration of neutrophils in the skin of the ear in older E/P mutants who demonstrated spontaneous skin lesions elsewhere on their face and neck. We also examined the acute emigration of neutrophils in older CD18 mutants, who demonstrated grossly similar spontaneous skin lesions, and in older WT and E/P/I mutant mice, who demonstrated no spontaneous dermal lesions.

In contrast to the younger E/P mutant mice, which demonstrated a complete defect in neutrophil emigration, the older E/P mutant mice showed no compromise in neutrophil emigration compared with WT mice (Fig. 2). Thus, the older E/P mutants have elicited an endothelial selectin-independent pathway that mediates acute (6 h) neutrophil emigration during irritant dermatitis.

As with the younger E/P/I mutant mice (Fig. 1), the older E/P/I mutants showed a complete abrogation of neutrophil emigration (Fig. 2) and a partial suppression of edema accumulation (Table II) compared with WT. Similarly, the older CD18 mutant mice demonstrated a defect in acute neutrophil emigration (Fig. 2), despite the presence of ulcerative skin lesions about the face and neck. The circulating neutrophil counts of mice with any of the targeted gene mutations were greater than WT and increased with age (Table II).

Table II. Circulating neutrophils and edema accumulation in older (23–35 wk) WT mice and mice deficient in CD18, E- and P-selectins (E/P), or E- and P-selectins and ICAM-1 (E/P/I) during croton oil-induced dermatitis

<table>
<thead>
<tr>
<th></th>
<th>Circulating Neutrophils</th>
<th>Edema Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.5 ± 0.5</td>
<td>54.3 ± 4.6</td>
</tr>
<tr>
<td>CD18</td>
<td>233 ± 107*</td>
<td>42.9 ± 15.1</td>
</tr>
<tr>
<td>E/P</td>
<td>357 ± 142*</td>
<td>66.3 ± 1.5</td>
</tr>
<tr>
<td>E/P/I</td>
<td>58 ± 33*</td>
<td>12.7 ± 1.3*</td>
</tr>
</tbody>
</table>

* Blood was drawn from the inferior vena cava, and circulating neutrophils were expressed as millions/ml. Ear widths were measured with spring-loaded calipers, and edema accumulation was expressed as percent swelling compared with the untreated ear. Asterisks (*) represent significant differences from WT (p < 0.05).

FIGURE 2. Neutrophil emigration during acute irritant dermatitis in older (23–35 wk) mice deficient in endothelial adhesion molecules. WT mice and mice deficient in CD18, E- and P-selectins (E/P), and E- and P-selectins and ICAM-1 (E/P/I) were studied 6 h after topical application of 2% croton oil to the ear. All CD18 and E/P mutants, but no E/P/I mutants or WT mice, displayed lesions about the face and neck. Each bar represents the mean and SEM from three to six mice, and asterisks (*) represent significant differences from WT mice.

FIGURE 3. Separating the effects of age and lesions in inducing the endothelial selectin-independent pathway for acute neutrophil emigration. A, Neutrophil emigration during acute irritant dermatitis in older E/P mutants who did not develop spontaneous skin lesions. Open bar represents five E/P mutants (27–36 wk) without spontaneous skin lesions; solid bar is data from Fig. 2. Asterisk (*) represents significant difference from older E/P mutants with spontaneous skin lesions. B, Neutrophil emigration during acute irritant dermatitis in young E/P mutants with experimentally induced chronic skin lesions. Open bar represents five E/P mutants (10 wk) with 15-day-old lesions induced by topical application of 10% croton oil to their backs; solid bar is data from Fig. 1. Asterisk (*) represents a significant difference from young E/P mutants without experimental skin lesions.

Roles of age and distant chronic lesions in endothelial selectin-independent emigration

To determine whether the endothelial selectin-independent pathway for emigration was particularly associated with age or with the occurrence of chronic skin lesions, acute croton oil dermatitis was studied in five older (27–36 wk) E/P mutants who, for unknown reasons, did not develop skin disease. Neutrophil emigration was prevented by deficiency of E- and P-selectins in older mice who did not develop spontaneous lesions (Fig. 3A), similar to younger mice, demonstrating that the aging of mice to 36 wk is not sufficient to evoke endothelial selectin-independent emigration during acute dermatitis. The circulating neutrophils were elevated in the older E/P mutants without spontaneous lesions (15.0 ± 2.6 ± 10⁶/ml), but less so than in the older E/P mutants with spontaneous lesions (Table II).

These observations suggest that distant chronic skin lesions can shift the adhesion molecule requirements for neutrophil emigration during acute dermatitis from endothelial selectin-dependent toward endothelial selectin-independent. To test this hypothesis, chronic experimental lesions were induced in young (8 wk of age) E/P mutant mice without spontaneous lesions by topical applying 10% croton oil to their shaved backs 15 days before acute (6 h) dermatitis studies in the ear. The dermis of the croton oil-treated ears of E/P mutants with experimentally induced lesions on their backs had nearly 5-fold more emigrated neutrophils compared with young E/P mutant mice without experimental lesions (Fig. 3B). The circulating neutrophil counts of young E/P mutants with experimental lesions (22.1 ± 3.0 ± 10⁶/ml) did not significantly differ from young E/P mutant mice without experimental lesions (Table I).
Adhesion molecule expression by neutrophils from younger and older E/P mutants

To determine whether age or chronic inflammatory lesions influenced adhesion molecule expression by circulating neutrophils, the relative expressions of CD11a/CD18 (LFA-1), CD49d/CD29 (VLA-4), and CD62L (L-selectin) by neutrophils from younger WT mice, younger E/P mutant mice without chronic lesions about the face and neck, and older E/P mutant mice with chronic lesions were assessed.

LFA-1 was expressed on peripheral blood neutrophils of WT mice (mean channel fluorescence of 24.0 ± 0.6, compared with background green fluorescence of 1.5). There were no significant differences among groups in the relative levels of LFA-1 expression (Fig. 4).

VLA-4 was expressed by peripheral blood neutrophils of WT mice (mean channel fluorescence of 3.6 ± 0.1, compared with background green fluorescence of 1.4). This increase in mean channel fluorescence was not due to a subpopulation of VLA-4 bright cells, but rather appeared to reflect a rightward shift of the entire cell population on the fluorescence histogram. Similar data have previously been reported for mice (10) and rats (11), suggesting that circulating neutrophils in rodents express low levels of VLA-4 under resting conditions. VLA-4 expression by neutrophils from younger or older E/P mutants did not significantly differ from WT (Fig. 4).

L-selectin was expressed by peripheral blood neutrophils from WT mice (mean channel fluorescence of 7.4 ± 1.3, compared with background red fluorescence of 0.1). Neutrophils in the peripheral blood from younger E/P mutant mice had lower levels of L-selectin (Fig. 4), consistent with previous observations (1, 12). This decrease in mean fluorescence was largely due to an increased number of neutrophils not expressing measurable levels of L-selectin; although 96 ± 1% of the neutrophils in WT blood expressed L-selectin, only 12 ± 4% of the neutrophils in the blood of younger E/P mutants were labeled with Abs against L-selectin. Similarly, the older E/P mutants had significantly less L-selectin expression than WT (Fig. 4). In older E/P mutants with chronic skin lesions, virtually none of the circulating neutrophils (1.8 ± 0.4%) expressed measurable levels of L-selectin.

Discussion

E/P mutants develop spontaneous skin lesions with abundant emigrated neutrophils. Although neutrophil emigration was compromised in the skin of young E/P mutants, emigration was not compromised during experimental dermatitis in older E/P mutants with spontaneous chronic skin lesions at sites distant from the ears. Furthermore, the experimental induction of distant chronic skin lesions in young E/P mutants resulted in increased neutrophil emigration during acute dermatitis. These data suggest that distant chronic inflammatory disease facilitates a novel endothelial selectin-independent pathway for acute neutrophil emigration. This endothelial selectin-independent pathway may contribute to the pathologic progression of the spontaneous neutrophilic skin lesions in E/P mutant mice.

Neutrophil emigration remained compromised in older CD18 mutants, who develop spontaneous lesions containing emigrated mononuclear leukocytes but not neutrophils. These data suggest that CD11/CD18 is essential to acute neutrophil emigration in the skin, regardless of spontaneous chronic lesions or age. CD11/CD18 complexes and their ligands, including ICAM-1, may thus be essential to the endothelial selectin-independent pathway for acute emigration. The absence of spontaneous skin disease in E/P mutants may be due to a requirement for ICAM-1 on endothelial or other cell types in the recruitment of endothelial selectin-independent pathways.

These data demonstrate the existence in mice of endothelial selectin-dependent and -independent pathways for acute neutrophil emigration in the skin. In the pulmonary circulation, where the regulation and mechanisms of neutrophil emigration differ from other organs (8, 13–15), acute neutrophil emigration during streptococcal infection is not inhibited in E/P mutant mice (16). Similarly, neutrophil adhesion and accumulation within the hepatic sinusoids and sinusoidal venules is not inhibited in E/P mutant mice after administration of bacterial LPS or f-met-leu-phe (17). However, acute (0–8 h) neutrophil emigration outside of the unique circulatory architectures of the lung and liver is generally dependent upon E- and/or P-selectins (1, 2, 5, 6, 18–20), similar to our observations in young mice with acute croton oil-induced dermatitis.

Several adhesion molecules, other than E- and P-selectins, can mediate rolling of neutrophils along the endothelial cell surface, and are therefore candidates for mediating endothelial selectin-independent acute neutrophil emigration. Endothelial selectin-independent rolling and emigration may be mediated by L-selectin, as the minimal rolling observed in acutely inflamed venules of E/P mutant mice is further reduced by Ab against L-selectin (12). However, L-selectin was virtually absent on neutrophils from older E/P mutant mice with chronic skin lesions, suggesting that this molecule is unlikely to be responsible for emigration under these circumstances. The integrin VLA-4 can mediate leukocyte rolling (21, 22) and neutrophil emigration (11), and thus, may mediate endothelial selectin-independent acute emigration in the skin. However, VLA-4 expression by circulating neutrophils was not altered by either the deficiency of both E- and P-selectins or by the appearance of chronic skin lesions, demonstrating that changes in the expression of VLA-4 did not contribute to this pathway. It remains possible that changes in VLA-4 conformation, or changes in the expression of VLA-4 ligands on endothelial cells, may yet contribute to this pathway.

Furthermore, it is conceivable that ICAM-1 and CD11/C18 mediate rolling during endothelial selectin-independent acute neutrophil emigration. Our results suggest that ICAM-1 and CD11/C18

![FIGURE 4. Adhesion molecule expression by circulating neutrophils from younger and older E/P mutant mice.](http://example.com/figure4.png)
CD18 are essential to emigration via this pathway. These molecules can mediate rolling under select conditions (23, 24), and ICAM-1 is essential to some leukocyte rolling, as residual trauma-induced rolling in venules of the cremaster muscle of P-selectin mutants is abolished by the additional deficiency of ICAM-1 (25). CD11a expression did not differ in mice that did or did not elicit the endothelial selectin-independent pathway, but other CD11/CD18 molecules, or regulation of CD11/CD18 by means other than expression, may contribute to this pathway. Perhaps more likely, ICAM-1 and CD18 may be required for steps in the emigration pathway other than rolling, such as firm adhesion, in both endothelial selectin-dependent and -independent acute neutrophil emigration. Finally, it must be considered that rolling may not be required for the dermal emigration of neutrophils under some circumstances.

Chronic inflammation may affect the pathways for neutrophil emigration at distant sites in several ways. Mediators elaborated in response to chronic inflammation may circulate and alter the regulation of acute inflammation elicited by local stimuli, for example, by influencing the expression of specific cytokines, chemokines, or adhesion molecules. In addition, neutrophils may be affected by transit through vascular beds perfusing chronically inflamed tissues in such a way that they respond differently to acute inflammation elicited by local stimuli.

The present findings have implications for antiinflammatory therapies based upon interruption of select functions. In particular, they suggest that chronic inflammatory disease may facilitate endothelial selectin-independent neutrophil emigration, even at distant sites of acute inflammation. Thus, patients with chronic inflammatory diseases, such as atopic dermatitis, arthritis, and asthma, may be poor candidates for endothelial selectin-based therapies. These observations suggest that therapies based on the inhibition of ICAM-1, in addition to endothelial selectins, may serve to decrease endothelial selectin-independent acute inflammation.

References