

Ionizing Radiation Mediates Expression of Cell Adhesion Molecules in Distinct Histological Patterns within the Lung¹

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Abstract

Inflammatory cell infiltration of the lung is a predominant histopathological change that occurs during radiation pneumonitis. Emigration of inflammatory cells from the circulation requires the interaction between cell adhesion molecules on the vascular endothelium and molecules on the surface of leukocytes. We studied the immunohistochemical pattern of expression of cell adhesion molecules in lungs from mice treated with thoracic irradiation. After X-irradiation, the endothelial leukocyte adhesion molecule 1 (ELAM-1; E-selectin) was primarily expressed in the pulmonary endothelium of larger vessels and minimally in the microvascular endothelium. Conversely, the intercellular adhesion molecule 1 (ICAM-1; CD54) was expressed in the pulmonary capillary endothelium and minimally in the endothelium of larger vessels. Radiation-mediated E-selectin expression was first observed at 6 h, whereas ICAM-1 expression initially increased at 24 h after irradiation. ICAM-1 and E-selectin expression persisted for several days. P-selectin is constitutively expressed in Weibel-Palade bodies in the endothelium, which moved to the vascular lumen within 30 min after irradiation. P-selectin was not detected in the pulmonary endothelium at 6 h after irradiation. The radiation dose required for increased cell adhesion molecule expression within the pulmonary vascular endothelium was 2 Gy, and expression increased in a dose-dependent manner. These data demonstrate that ICAM-1 and E-selectin expression is increased in the pulmonary endothelium following thoracic irradiation. The pattern of expression of E-selectin, P-selectin, and ICAM-1 is distinct from one another.

Introduction

Inflammatory cell infiltration of the lung is observed early during radiation-mediated lung injury (reviewed in Ref. 1). Most authors describe the presence of inflammatory cells within the alveolar space, alveolar septum, and perivascular space following irradiation (2–5). The histopathological findings observed during radiation pneumonitis consist of interstitial edema and profuse inflammatory cell infiltration associated with thickening of the alveolar septa (3, 6). A significant increase in the number of macrophages, granulocytes, and lymphocytes is found in bronchoalveolar lavage fluid from patients as well as mice receiving total-body irradiation (2, 7). It has been proposed that the inflammatory mediators released from leukocytes are involved in the pathogenesis of radiation pneumonitis (8). The present study addresses the mechanisms by which ionizing radiation mediates the inflammatory response in the lung.

Infiltration of leukocytes into inflamed tissue is a primary component in inflammation-mediated tissue injury (9–11). The circulatory and migratory properties of neutrophils allow rapid accumulation of these inflammatory cells at sites of injury and infection. Neutrophils

extravasate from the circulation in response to changes on the vascular endothelium that signal injury or infection. Several protein families present on the vascular endothelium provide molecular signals that regulate leukocyte adhesion and emigration are the CAMs.³ CAMs that are induced within irradiated endothelial cells include the ICAM-1 and endothelial leukocyte adhesion molecule 1 (E-selectin; Refs. 12 and 13). The role of these CAMs in the pathogenesis of tissue injury has been implicated in rodent lung, liver, and kidney models (14). The selectins E-selectin and P-selectin progressively reduce the velocity of leukocyte movement over the endothelium (9, 10, 15). Following the slowing of leukocytes within blood vessels, these inflammatory cells extravasate and migrate into the inflamed tissue (reviewed in Refs. 10 and 11). ICAM-1 is a proteoglycan in the immunoglobulin superfamily that mediates leukocyte emigration from the circulation (16, 17). Elimination of leukocyte binding to the selectins or ICAM-1 attenuates the inflammatory response (18, 19). Taken together, these findings implicate the role of CAMs in the radiation-mediated inflammatory response.

We have previously shown that increased expression of E-selectin and ICAM occurs after X-irradiation of endothelial cells in culture (12, 13). The objective of the present study was to determine whether thoracic irradiation alters the histological pattern of expression of P-selectin, E-selectin, and ICAM-1 within the irradiated lung. We treated mice with thoracic irradiation and stained lung sections with antibodies to P-selectin, E-selectin, and ICAM-1. X-irradiation induced the expression of E-selectin on the endothelium of larger vessels, whereas there was little expression in the pulmonary microvascular endothelium. ICAM-1 was expressed in the pulmonary capillary endothelium, but minimal expression was observed in the endothelium of larger vessels following thoracic irradiation. P-selectin was present in Weibel-Palade bodies in the endothelium before irradiation and migrated to the vascular lumen within 30 min after irradiation. These findings indicate that ionizing radiation alters the histological pattern of expression of the principle CAMs that regulate leukocyte emigration from the circulation.

Materials and Methods

Thoracic Irradiation and Immunohistochemical Staining. Twelve-week-old C3H mice (The Jackson Laboratory, Bar Harbor, MA) were treated with thoracic irradiation in the dose range and at the time intervals described in "Results." Lead shields protected the head and abdomen. Mice were euthanized by i.p. injection of barbiturate at 30 min, 6 h, and 1, 2, and 7 days after irradiation. Lungs were fixed in formalin and embedded in paraffin. Paraffin blocks were then sectioned (5- μ m thick) and placed on slides. Five- μ m sections of each lung were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry. Lung sections were baked at 60°C for 1 h, cleared in xylene, and hydrated through a descending alcohol series to distilled water. For E-selectin and CD45 immunostaining, the hydrated sections were incubated with Protease I (Ventana Biotech, Tucson, AZ) for 8 min at 42°C.

³ The abbreviations used are: CAM, cell adhesion molecule; ICAM-1, intercellular adhesion molecule 1.

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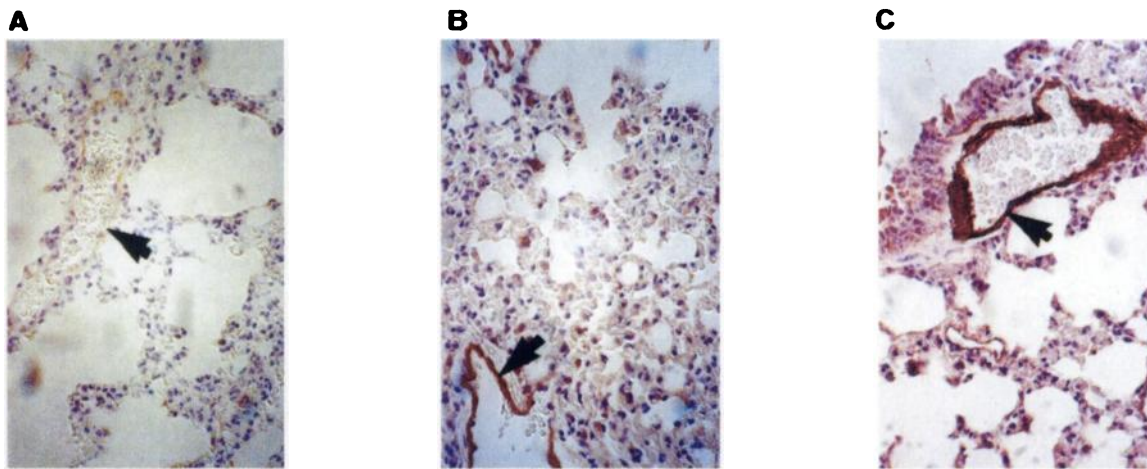


Fig. 1. E-selectin expression on the endothelium of pulmonary blood vessels. Shown is the immunohistochemical staining of E-selectin in lungs from mice treated with 10 Gy of thoracic irradiation. A, control (untreated) lung; B, 6 h after irradiation; C, 48 h after irradiation. Arrows, endothelium of larger pulmonary vessels.

For ICAM immunostaining, the hydrated sections were incubated with Protease II (Ventana Biotech) for 8 min at 42°C. After washing briefly in ddH₂O, endogenous activity was blocked by treatment of the sections with 3% hydrogen peroxide in methanol for 20 min. Two tissue sections from each case were then incubated overnight at 4°C at a titer of 2.5 µg/ml for P-selectin and ICAM and at 7.5 µg/ml for E-selectin [E-selectin (09521D), ICAM (01542D); Pharmingen, San Diego, CA]. One slide from each sample was treated in a similar fashion and incubated overnight in normal serum immunoglobulin (Ventana Medical Systems, Tucson, AZ). The immunohistochemical staining was performed on a Ventana Gen¹¹ system (Ventana Medical Systems). The Ventana Gen¹¹ uses an indirect streptavidin-biotin system conjugated with horseradish peroxidase for detecting the immunocomplex and diaminobenzidine as a substrate for localization. The Ventana Gen¹¹ uses a cartridge-delivered avidin-biotin blocking kit to block endogenous biotin. The immunostained sections were counterstained with hematoxylin, dehydrated through an ascending alcohol series, cleared, and coverslipped.

Quantitation of E-Selectin Expression Using Immunofluorescence. Lung sections of mice treated with thoracic irradiation were incubated with anti-E-selectin antibody as described above. Following incubation with biotinylated secondary antibody, blocking solution was added for 30 min in a humid chamber at 37°C. Avidin-CY3 (5 µg/ml) was added to 200 µl of blocking buffer and filtered through a 0.2-µm Millipore filter. Avidin-fluorochrome solution was added to tissue sections, coverslipped, and incubated for 30 min in a humid chamber at 37°C. Coverslips were removed and sections were washed using 4× SSC/0.1% Triton X-100 at 39°C. Slides were counterstained in 4',6-diamidino-2-phenylindole and rinsed with 2× SSC for 10 s. Slides were then coverslipped with

antifade and blotted. Fluorescence was then visualized using UV microscopy and NU200 software as we have described (21). Fluorescence intensity of pulmonary vessels was measured by use of NIH Image 1.58 software. 4',6-Diamidino-2-phenylindole staining of nuclei was used as a control to verify that fluorescence was measured in the same number of cells in each lung section. Fifty nuclei were framed and anti-E-selectin immunofluorescence was determined using NIH Image software. Fluorescence intensity was determined for each pixel within the framed cells and the number of fluorescent pixels were counted using NIH Image. The increase in the number of pixels showing fluorescence was determined. The mean and SE of anti-E-selectin immunofluorescence of three lungs was determined for each dose of thoracic irradiation.

Results

E-Selectin Expression in Pulmonary Vascular Endothelium of Mice Treated with Thoracic Irradiation. The study radiation-induced expression of CAMs in the pulmonary vessels of mice, we irradiated the thorax of C3H mice. Six, 24, 48, and 72 h after irradiation, lungs were fixed and embedded in paraffin. Slides containing lung sections were then incubated with rat anti-mouse E-selectin IgG2a (PharMingen). Lungs from irradiated mice showed E-selectin expression primarily in the endothelium of large vessels and minimally in the microvascular endothelium after X-irradiation (Fig. 1). E-selectin expression increased at 6 h and returned to baseline at 72 h after irradiation.

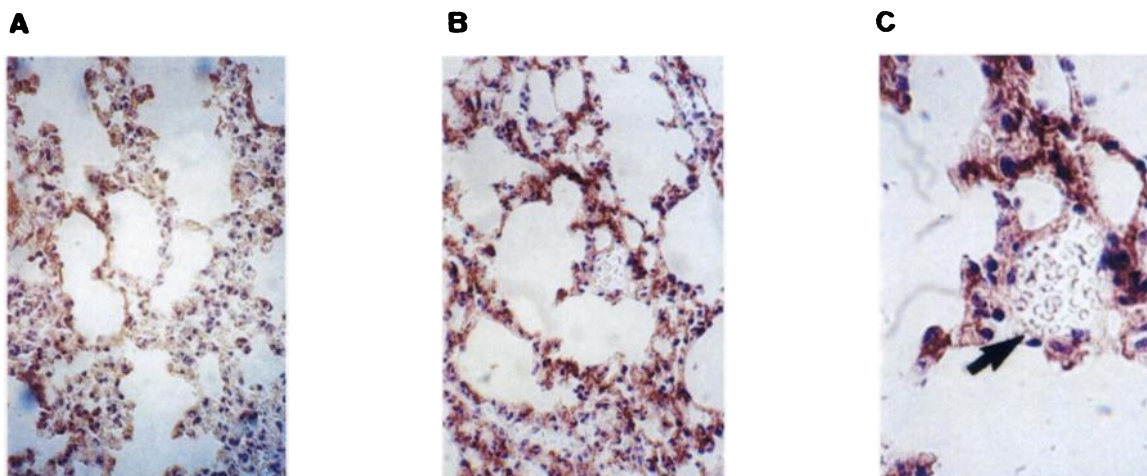


Fig. 2. ICAM-1 expression on the pulmonary vascular endothelium following thoracic irradiation (10 Gy). A, Lung sections from mice treated with sham irradiation. B, Lung sections from mice 48 h after treatment with thoracic irradiation. C, Photomicrograph of ×100 objective showing minimal ICAM-1 immunostaining in larger vessels (arrow).

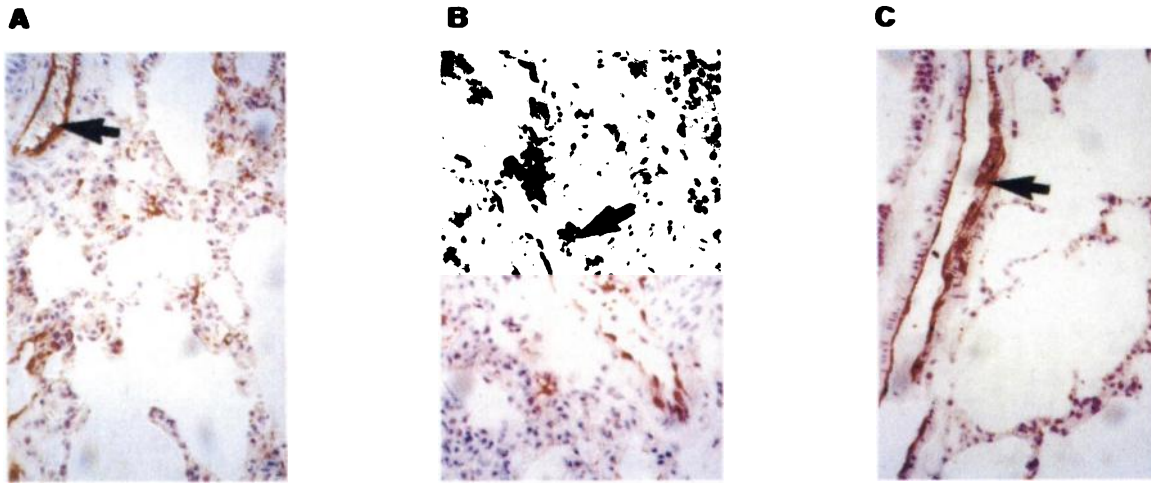


Fig. 3. Immunohistochemical staining for P-selectin expression after thoracic irradiation (10 Gy). Shown are lung sections obtained at 0 h (A), 30 min (B), and 24 h (C) after treatment with thoracic irradiation. Arrows, P-selectin immunostaining in the endothelium of larger pulmonary vessels.

ICAM-1 Expression in the Irradiated Lung. To determine the pattern of X-ray-induced ICAM-1 expression, we stained lung sections with rat anti-mouse ICAM-1. Low levels of ICAM-1 expression were found in the pulmonary vascular endothelium prior to irradiation (Fig. 2). ICAM-1 expression was increased at 24 h after irradiation and persisted for 7 days after irradiation. Radiation-induced ICAM-1 expression was increased in the pulmonary capillary endothelium, whereas there was little increase in ICAM-1 staining in the endothelium of larger vessels (Fig. 2A). Thus, the histological staining pattern for E-selectin (larger vessels) and ICAM-1 (microvascular endothelium) varied in the irradiated lung.

Histological Pattern of P-Selectin Expression in Pulmonary Vascular Endothelium following Thoracic Irradiation. Prior to thoracic irradiation, P-selectin was constitutively expressed within Weibel-Palade bodies in the endothelium. Figure 3 shows that P-selectin migrated to the vascular lumen within 30 min after irradiation. P-selectin was undetectable in the pulmonary endothelium at 6 h after irradiation. At 24 h after thoracic irradiation, P-selectin accumulated in the pulmonary vascular endothelium and returned to baseline levels.

Dose-dependent Increase and E-Selectin Expression in the Irradiated Lung. We have previously shown a dose-dependent increase in ICAM-1 expression in the irradiated lung (21). To determine whether E-selectin expression is induced following exposure to therapeutic doses of radiation, C3H mice were treated with increasing doses of thoracic irradiation, and lungs were sectioned and stained using the anti-E-selectin antibody (Fig. 4). Cy2-avidin was used for immunofluorescence after biotinylated secondary antibody because elastin in the pulmonary airways fluoresces at the same wavelength as

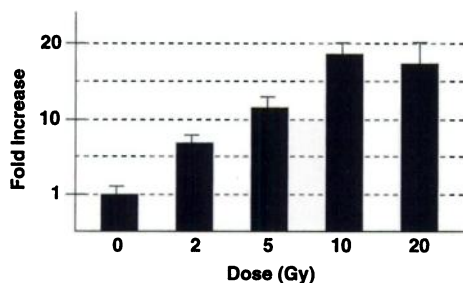


Fig. 4. Dose-dependent increase in E-selectin expression in the lungs from C3H mice. Intensity of E-selectin immunofluorescence at 6 h after exposure to the indicated dose of thoracic irradiation. Shown are the means of three experiments. Bars, SE.

fluorescein. Anti-E-selectin immunofluorescence in pulmonary blood vessels at 6 h after X-irradiation was calculated using NIH Image software. Mice were treated with 0, 2, and 10 Gy of thoracic irradiation. Fluorescence intensity of pulmonary vessels was measured in three experiments, and the mean and SE are shown in Fig. 4. E-selectin staining increased to 5-fold greater than that of control after 2 Gy, 12-fold in response to 5 Gy, and plateaued at 18-fold after 10 Gy or more. Anti-E-selectin immunofluorescence plateaus after a thoracic dose of 10 Gy.

Discussion

The purpose of this study was to characterize the radiation-mediated induction of CAMs *in vivo*. *E-selectin* and *ICAM-1* are radiation-inducible genes that are expressed in vascular endothelial cells (12, 13) and are associated with recruitment of inflammatory cells into sites of tissue injury (9, 10). The time course and dose-dependent increase in E-selectin expression on irradiated endothelial cells in culture are similar to those observed in the pulmonary vascular endothelial cells of mice treated with thoracic irradiation (12). The histological pattern of expression of E-selectin in the irradiated lung differed from that observed for ICAM-1 and P-selectin. ICAM-1 was primarily expressed in the endothelium of the pulmonary microvasculature, in contrast to E-selectin which was expressed primarily in the endothelium of larger vessels. P-selectin was expressed from the endothelium of larger vessels and was never expressed in the microvasculature. This contrasting pattern of expression may be associated with the function of each of these CAMs. The selectins slows the velocity of leukocytes (10), whereas ICAM-1 contributes to leukocyte extravasation from the microvasculature (9, 16, 17). This pattern is associated with the histological pattern of inflammatory cell infiltration into alveolar septa (3, 22).

We have previously shown a dose-dependent increase in ICAM-1 expression in X-irradiated pulmonary vascular endothelium (21). The present study demonstrates that E-selectin expression in the pulmonary vascular endothelium increases in dose- and time-dependent manners following thoracic irradiation. E-selectin expression increases after a dose as low as 2 Gy and plateaus when doses above 10 Gy are used. This threshold dose was similar to that for ICAM-1, but the fold-increase in immunofluorescence was greater for E-selectin than ICAM. This may be due to the higher basal expression of ICAM-1 in the lung as compared with E-selectin (21).

The radiation-mediated increase in E-selectin and ICAM-1 mRNA expression requires no *de novo* protein synthesis and is blocked by the transcription inhibitor actinomycin D (12, 13). The 587-bp segment of the 5' regulatory region of the E-selectin gene and the 1.2-kb segment of the 5' regulatory region of the *ICAM-1* gene are both sufficient to regulate activation of radiation-induced transcription (12, 13). We have also shown that deletion of the NF κ B-binding region within the E-selectin promoter eliminates radiation induction of the *CAM* gene (12). We noted that radiation induction of CAMs is distinct from that observed after cytokine stimulation in that radiation induction was limited to E-selectin and ICAM, but did not occur with VCAM-1 (13). Furthermore, production of tumor necrosis factor and interleukin 1 following irradiation of macrophages occurs 12–18 h after irradiation (23, 24), whereas *E-selectin* gene expression occurs 2 h after irradiation (12). Moreover, NF κ B binding to the E-selectin promoter occurs within 10 min after irradiation, indicating that this molecular response is rapid (12). Taken together, these data suggest that tumor necrosis factor and interleukin 1 are not necessary for radiation-mediated E-selectin induction in the vascular endothelium.

We have found that the histological pattern of E-selectin staining is distinct from that observed for P-selectin and ICAM-1. Because these adhesion molecules have been shown to participate in the pathogenesis of inflammatory cell-mediated tissue injury, we speculate that inhibition of leukocyte adhesion to the selectins or ICAM-1 may prevent radiation-induced inflammatory changes within the irradiated lung. Inhibition of radiation-induced CAM expression therefore represents a novel strategy to protect normal tissues during radiotherapy.

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