Cell Adhesion Molecules Mediate Radiation-induced Leukocyte Adhesion to the Vascular Endothelium

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ABSTRACT

The predominant early histological changes in irradiated tissues are edema and leukocyte infiltration. Cell adhesion molecules (CAMs) are required for the extravasation of leukocytes from the circulation. To study the role of CAMs in the pathogenesis of radiation-mediated inflammation, we quantified the expression of P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on the irradiated human endothelial cells. We found that E-selectin and ICAM-1 expression increased after irradiation, whereas there was no increased expression of other cytokine-inducible adhesion molecules (P-selectin or vascular cell adhesion molecule-1). We found a dose- and time-dependent increase in radiation-induced expression of both E-selectin and ICAM-1. Furthermore, the threshold dose for ICAM-1 synthesis was 5 Gy of X-rays. Northern blot analysis of RNA from irradiated endothelial cells demonstrated that ICAM-1 is expressed at 3–6 h following irradiation. No de novo protein synthesis was required for increased ICAM-1 mRNA expression. The 1.1-kb segment of the 5' untranslated region of the ICAM-1 gene was sufficient for X-ray induction of chloramphenicol acetyltransferase reporter gene expression. We measured whether ICAM-1 mediates adhesion of leukocyte to the irradiated endothelium and found that leukocyte adhesion occurred concurrently with ICAM-1 induction. Radiation-mediated leukocyte adhesion was prevented by anti-ICAM-1 blocking antibodies. These data indicate that ICAM-1 participates in the inflammatory response to ionizing radiation. Moreover, radiation induction of these CAMs occurs in the absence of tumor necrosis factor and interleukin 1 production.

INTRODUCTION

An inflammatory-like response, in part, contributes to the acute and subacute sequelae of radiation therapy. For example, histological changes in radiation-mediated pneumonitis and pericarditis have inflammatory components. Studies of radiation-induced tissue pathology noted neutrophil infiltration of irradiated tissues as the initial histological change prior to organ damage (1–4), and neutrophils are known to bind to irradiated endothelial cells in vitro (5, 6). Normal tissues respond to irradiation with increased adhesion of leukocytes to the endothelium (7). For example, increased adherence of neutrophils to endothelial cells occurs during acute pulmonary radiation injury (3). Margination of neutrophils in the vasculature and infiltration of the perivascular region are observed after irradiation or reactive oxygen species (reviewed in Refs. 4, 6, 8, and 9). Our objective of this study was to determine the mechanisms by which ionizing radiation mediates leukocyte adhesion to the vascular endothelium. Cytokines are proteins released by irradiated tissues and are implicated in the acute phase response to ionizing radiation (10, 11).

Examples of radiation-induced cytokines include TNFα, IL-1, and stem cell factor (11–16). These cytokines mediate inflammation by inducing synthesis of CAM within the vascular endothelium (reviewed in Ref. 17). IL-1 and TNF bind to receptors on leukocytes and endothelial cells to activate the inflammatory response (18). For example, TNF stimulates the expression of cell adhesion molecules on endothelial cells, which mediate emigration of leukocytes from the circulation. CAMs expressed on the surface of vascular endothelial cells include the selectin family (E-selectin and P-selectin) and the immunoglobulin superfamily (e.g., ICAM-1). These CAMs interact with their respective counter-receptors on leukocytes to initiate inflammatory cell extravasation (reviewed in Ref. 17). E-selectin and P-selectin are not expressed on the surface of unstimulated endothelial cells. These selectins initiate the adhesion of leukocytes to the endothelium after stimulation by cytokines. Following this primary adhesion of neutrophils to the vascular endothelium, inflammatory cells extravasate and migrate into the inflamed tissue (reviewed in Refs. 17 and 19). Extravasation of leukocytes from the vasculature requires ICAM-1, which is expressed on the surface of endothelial cells in response to inflammatory cytokines (20–22). ICAM-1 interacts with counter-receptors on the surface of leukocytes to mediate emigration of leukocytes (23). Neutrophils then migrate into the tissue region of highest adhesiveness, which is regulated by ICAM-1 expression (17). Monoclonal antibodies directed against ICAM-1 inhibit transendothelial migration of neutrophils (21, 22), indicating that ICAM-1 is required for this migration.

We studied the expression of CAMs in irradiated human endothelial cells. We have shown previously that E-selectin is induced by X-irradiation of endothelial cells, and that E-selectin gene expression is regulated through the activation of NFκB (24). Furthermore, the ICAM-1 mRNA level is increased in the brains and dermis of irradiated mice (11, 25). In the present study, we found that ICAM-1 protein is expressed on irradiated vascular endothelial cells. There was no increase in the other cytokine-inducible CAMs, VCAM-1, or P-selectin. Furthermore, radiation induction of E-selectin and ICAM-1 occurs immediately and without de novo protein synthesis. These findings indicate that radiation-induced expression of E-selectin and ICAM-1 is independent of radiation-induced cytokine production. We found that adherence of leukocytes to these irradiated endothelial cells was prevented by anti-ICAM-1 antibody. The importance of these findings is that ICAM-1 mediates emigration of neutrophils from the vasculature and migration of these inflammatory cells into tissue. ICAM-1 blocking agents, therefore, represent a novel means of preventing the inflammatory-like response of the vascular endothelium to radiation therapy.

MATERIALS AND METHODS

Endothelial Cell Cultures. HUVEC cultures were prepared from fresh (<24 h of age) human umbilical veins transported to the laboratory in sterile buffer at 4°C, as we have described (24). The veins were cannulated, filled...
with 0.2% collagenase, and incubated at 37°C for 15 min. Cells were flushed, and complete medium was added, followed by centrifugation at 2000 rpm for 5 min. The cell pellet was resuspended and maintained in M199 with 10% FCS, 10% human serum, and penicillin/streptomycin/Ampicillin B solution (Sigma Chemical Co.) on gelatin-coated (0.2%) tissue culture dishes at 37°C in 5% CO₂. The purity of endothelial cell cultures was verified by staining for von Willebrand factor. Confluent cells were harvested with 0.1% collagenase, 0.01% EDTA, and subcultured at a ratio of 1:3. HUVECs were used at third passage; this reduced the number of passenger cells and allowed for uniform expression of CAMs.

The 3B-11 endothelial cell line was derived from a solid tumor in a nude mouse injected with SV4T4-10. These murine endothelial cells were cloned by limited dilution (26, 27). This cell line expresses the cell surface MHC class I antigen, H2K, VCAM-1, ICAM-1, and SP40-T antigen. 3B-11 cells were maintained in 90% DMEM with high glucose (4.5 g/l), 10% heat-inactivated fetal bovine serum, and penicillin/streptomycin/Ampicillin B solution. 3B-11 cells were grown to 90% confluence for all experiments.

Quantification of Cell Adhesion Molecules in Irradiated Endothelial Cells. Endothelial cells were irradiated with a GE Maxitron X-ray generator, as described previously (14). Cells were removed from flasks with 0.1% collagenase, 0.01% EDTA, and 0.25% BSA and pelleted in polystyrene tubes. Cells were then incubated with primary IgGl antibody (mouse antihuman E-selectin, P-selectin, VCAM-1, and ICAM-1; R&D Systems, Inc., Minneapolis, MN, and Becton Dickinson, San Jose, CA) for 20 min at 4°C. The cells were then rinsed with isotonic PBS, pelleted, and incubated with FITC-conjugated secondary antibody (goat antimouse IgGl) for 20 min at 4°C. The fluorescein-labeled cells were rinsed in PBS and fixed in PBS containing 0.01% paraformaldehyde. Nonspecific binding was evaluated with the use of FITC-conjugated secondary antibody alone and with a lymphocyte-specific first-step antibody, anti-CD₁₄, which does not bind to endothelial cells.

Fluorescence-activated cell sorting analysis was used for quantification of receptor expression of E-selectin on HUVECs. The Becton Dickinson FAC-Scan was used with Lysis II software. Forward and side scatter fluorescence data identified 10,000 viable endothelial cells in each experimental group for unlabeled cells, nonspecific antibody-labeled cells, and anti-CAM antibody-labeled cells. Fluorescence data were then accumulated on each group of 10,000 cells at 530 nm; the wavelength was emitted by FITC after treatment with X-rays or IL-1 (10 ng/ml; R&D Systems). These fluorescence data were expressed as histograms of events versus log fluorescence and analyzed in comparison to the autofluorescence of unlabeled cells as well as the fluorescence of baseline or anti-CAM-labeled cells as appropriate. The percentage of cells expressing adhesion molecules was determined by quantification of the number of cells demonstrating an increase in log fluorescence beyond that of untreated control cells. Experiments were performed three to four times and the mean and SE were calculated. Endothelial cell viability was determined at the time of antibody incubation (4 h) by use of trypan blue dye exclusion.

Northern Blot Analysis of Irradiated HUVECs. HUVECs were exposed to 10 Gy (GE Maxitron X-ray generator) as described previously (14). RNA was extracted with the single-step guanidinium thiocyanate-phenol-chloroform method (28) following irradiation. RNA from nonirradiated cells and treated cells were size-fractionated by 1% agarose formaldehyde electrophoresis. Cycloheximide, 5 µg/ml, and actinomycin D, 0.5 µg/ml, were added to HUVECs 1 h before irradiation. Ethidium bromide staining of the RNA demonstrated equal loading of each lane. RNA gels were then transferred to a nylon membrane (Genescreen Plus; DuPont NEN). Northern blots were hybridized to a 23S-labeled 830-bp segment from the NarI and Asp-cut human ICAM-1 cDNA (29, 30). We quantified 7S RNA (31) to verify equal loading of RNA into lanes followed by autoradiography for 3 days at −85°C with intensifying screens. RNA levels were quantified by densitometry as we have described (32).

Leukocyte Adhesion Studies. HL-60 and U937 cells express the counter-receptor for ICAM-1 binding and, therefore, serve as standards for assessing leukocyte adhesion to stimulated endothelial cells (5, 33, 34). 3B-11 cells were grown to 90% confluence and irradiated with 10 Gy. Twenty-four h later, U937 cells were added at a density of 10⁶/dish with rocking for 30 min at 36°C. Cultures were then rinsed three times with PBS and rocking. Cell cultures were scrapped, and leukocytes were counted by hemocytometer. Cell counts were performed in three separate experiments, and the mean and SE were determined. HUVECs were grown to 90% confluence and irradiated with 2–10 Gy.

HL-60 cells were labeled with 51Cr according to methods described previously (5, 33, 34). 51Cr-labeled HL-60 cells (10⁵) were added 4 h after irradiation and incubated with rocking for 30 min. Cultures were then washed three times, and adherent HL-60 cells were quantified by scintillation counting for 51Cr. IL-1 (10 ng/ml)-stimulated HUVECs served as a positive control, whereas cells exposed to identical conditions without irradiation or cytokines served as negative control.

During antibody-blocking studies, HUVECs were irradiated as described above and treated with ICAM-1 blocking antibody (Becton Dickinson; 1:50) at 20 h after irradiation. Cultures were then washed, and HL-60 cells were added 30 min after drug or antibodies. Cell adhesion was quantified by cell counting. 51Cr quantification, and light microscopy. Experiments were repeated three to four times, and the mean and SE were determined. Statistical significance was determined by χ² analysis.

Analysis of Transcriptional Regulation. The ICAM-1 promoter fragment (−1162/+1) was cloned upstream of the CAT coding region of the pCAT promoter (Promega) after removal of the SV40 promoter by BglIII/Stul digestion., which created the pBS-CAT plasmid (35). The pBS-CAT plasmid was co-transfected with a plasmid containing a cytomegalovirus promoter linked to the LacZ gene (1 µg) and 12 µg of carrier DNA into HUVECs by use of lipofection. The medium was changed to optiMEM, and cells were transfected with Lipofectin reagent (Life Technologies, Inc.) for 8 h, followed by rinsing, the addition of complete medium, and incubation overnight. Transfectants were incubated for 16 h after transfection, followed by treatment with 10 Gy (1 Gy/min; GE Maxitron) of ionizing radiation or with IL-1 (10 ng/ml). The cells were harvested by scraping at 36 h and lysed. β-Galactosidase levels were quantified to normalized transfection efficiencies. Reporter gene expression was measured, as we have described previously (24, 31, 36). Experiments were repeated three to four times, and the mean and SE were determined. Statistical significance was determined by χ² analysis.

RESULTS

Adhesion Molecule Expression in Irradiated Endothelial Cells. To examine the effects of ionizing radiation on the expression of CAMs, we exposed 3B-11 cells and HUVECs to doses of 1, 2, 5, 10, and 20 Gy. Cells were then incubated with antibodies to E-selectin, ICAM-1, and VCAM-1 at 1, 2, 4, 8, 16, and 24 h. ICAM-1 was expressed on the surface of 30% of untreated control HUVECs and 25% of untreated 3B-11 cells. Increased expression of ICAM-1 was not observed before 20 h following treatment with X-rays or cytokines. As shown in Fig. 1, the percentage of cells expressing ICAM-1 increased 2–3-fold (P < 0.05) following treatment with 5 to 20 Gy at 24 h after irradiation. Moreover, the percentage of cells demonstrating E-selectin synthesis increased 20-fold at 4 h following exposure of HUVEC to 2 Gy (P < 0.001). Expression of VCAM-1 and P-selectin proteins was not increased by ionizing radiation (Fig. 2), whereas expression was induced by IL-1 and TNF.

E-selectin expression began to increase at 2 h, peaked at 4–6 h, and gradually returned to baseline at 20 h (Fig. 2). In contrast, ICAM-1 expression remained at baseline levels until 16 h after irradiation, and peak expression occurred at 24 to 36 h following irradiation. HUVECs were then irradiated with doses ranging from 0.5 to 50 Gy and assayed at 4 or 24 h for study of the dose dependence of the X-ray-mediated expression of cell adhesion molecules. E-selectin expression increased at 4 h after exposure to 0.5 Gy and increased in a dose-dependent manner up to 20 Gy, where a plateau was reached. E-selectin expression returned to baseline within 20 h after irradiation. In contrast, ICAM-1 expression was not increased at X-ray doses below 5 Gy, but demonstrable increases occurred at 24 h after treatment with higher doses. ICAM-1 expression persisted at 48 h after irradiation (Fig. 2). These data indicate that E-selectin is induced transiently after low doses of irradiation, whereas ICAM-1 induction requires higher radiation doses and is sustained.
TNF and IL-1 Are Not Induced in Irradiated Endothelial Cells. ICAM-1 induction by TNF and IL-1 occurs in a time-dependent manner that is similar to that observed in Fig. 2 (17). Because TNF and IL-1 are known radiation-inducible cytokines, we quantified these proteins in the medium and in endothelial cells following irradiation. We found no detectable levels of TNF or IL-1 in cells before or after irradiation. IL-1 did induce TNF expression in endothelial cells. Therefore, endothelial cells have the capacity to synthesize TNF but not in response to X-rays. These findings are supported by the observation that TNF and IL-1 induce VCAM-1, whereas radiation does not activate expression of this adhesion molecule.

Radiation-mediated ICAM-1 mRNA Expression. To study the mechanism of radiation-mediated ICAM-1 expression, we irradiated HUVECs with 10 Gy, and RNA was extracted at 3, 6, 8, 10, and 12 h. Northern blot hybridization to human ICAM-1 cDNA probe revealed a 2.5-fold increased ICAM-1 mRNA expression at 3–8 h (Fig. 3). This increased expression returned to baseline at 10–12 h. To determine whether protein synthesis is required before radiation-mediated ICAM-1 mRNA expression, we treated HUVECs with the protein synthesis inhibitor cycloheximide for 1 h before irradiation. Total cellular RNA was then extracted at 6 h, and Northern blot analysis was used for quantifying mRNA expression (Fig. 3). When endothelial cells were treated with cycloheximide, expression of ICAM-1 increased. When cells were treated with cycloheximide followed by irradiation, superinduction of ICAM-1 expression was observed. This indicates that protein-synthesis inhibition did not prevent X-ray induction of ICAM-1 mRNA expression. Inhibition of transcription by actinomycin D prevented X-ray induction of ICAM.

The promoter segment of the ICAM-1 gene was linked to the CAT reporter gene and transfected into HUVECs. Northern blot analysis revealed a 3.1-fold increase in CAT expression (P < 0.05) following irradiation of transfected endothelial cells (Fig. 4). These data, taken together with the inhibition of mRNA expression by actinomycin D, suggest that ICAM-1 transcription is induced by ionizing radiation.
Fig. 3. Northern blot analysis of ICAM-1 gene expression in irradiated HUVEC. HUVECs were irradiated, and total cellular RNA was isolated at
the indicated times after irradiation. Northern blots were hybridized to the
ICAM-1 cDNA and 7S. Cycloheximide (CYC) and actinomycin D (ACT)
were added 1 h before irradiation. Positive controls are endothelial cells
treated with TNF or IL-1.

Fig. 4. Transcriptional activation of the ICAM-1 promoter by ionizing radiation. The
1.1-kb segment of the 5' untranslated region of the ICAM-1 gene was cloned to the CAT
reporter gene and transfected into HUVECs by use of liposomes. Transfectants were
irradiated, and CAT enzyme activity is shown as the means (bars, SE) of three experi-
ments.

**Leukocyte Adhesion to Irradiated Human Endothelial Cells.**
HL-60 cells and U937 cells serve as standards for assaying leukocyte
adhesion to stimulated endothelial cells (5, 33, 34). To determine
whether leukocytes adhered to irradiated endothelial cells, we added
U937 cells to 3B-11 cultures at 24 h after x-irradiation or treatment
with IL-1. U937 cell counts demonstrated a 4-fold increase in cell
adhesion to x-irradiated 3B-11 endothelial cells as compared to un-
treated 3B-11 cells (Fig. 5). HUVEC cells were grown to 90%
confluence and irradiated with 10 Gy. 51Cr-labeled HL-60 cells (10^6)
added 20 h after irradiation were quantified by scraping and scintil-
lation counting. A 3.5-fold increase in HL-60 binding occurred
(P < 0.01) after irradiation (Fig. 5). IL-1 stimulated HUVECs served
as a positive control and demonstrated a 5-fold increase in HL-60 cell
binding. HUVECs exposed to identical conditions, but without irra-
diation or cytokine stimulation, served as negative control.

**Effects of anti-ICAM-1 Blocking Antibody on Leukocyte Adhesion to Irradiated Endothelial Cells.** HUVECs were irradiated as
described above and treated with the anti-ICAM-1 blocking antibody
(Becton Dickinson; 1:50) at 20 h after irradiation. Cultures were then
washed, and HL-60 cells were added 30 min after antibodies. We
found that the anti-ICAM-1 blocking antibody eliminated radiation-
mediated leukocyte adhesion to the irradiated endothelial cells (Fig.
6). Untreated HUVECs incubated with anti-ICAM-1 antibody dem-
onstrated no decrease in HL-60 cell binding. X-irradiated HUVECs
incubated with the anti-ICAM-1 antibody demonstrated a reduction in
HL-60 cell binding that reflected baseline adhesion (P < 0.005).
However, the anti-ELAM-1 blocking antibody did not prevent radia-
tion-mediated leukocyte adhesion at 24 h. IL-1-treated HUVECs
incubated with the anti-ICAM-1 antibody demonstrated an attenuation
of HL-60 cell binding.

**DISCUSSION**

Inflammatory mediators include cytokines, leukocytes, and the
adhesion molecules that mediate leukocyte infiltration of the in-
flamed tissue (reviewed in Ref. 17). We and others have studied the
role of cytokines in the pathogenesis of radiation injury (13, 14, 37).
These studies have shown that ionizing radiation induces the expres-
sion of cytokines such as TNF and IL-1. These cytokines stimulate
endothelial cells, resulting in leukocyte adhesion and extravasation.
Adhesion molecules must be expressed on the surface of the vascular
endothelium before leukocyte extravasation can occur (20–22). Our
purpose in the present study was to determine whether adhesion
molecules are induced by x-irradiation of human endothelial cells. We
found no IL-1 or TNF produced by endothelial cells following irra-
diation. These results are supported by the finding that TNF and IL-1
antibody (U), anti-ICAM-1 blocking antibody (C), or anti-ELAM-1 antibody (E) for 30
endothelial cells. HUVECs were treated with X-rays (10 Gy), IL-1 (5 ng/ml), or sham
irradiation (control). Twenty-four h after treatment, HUVECs were treated with no
antibody (O), anti-ICAM-1 blocking antibody (C), or anti-ELAM-1 antibody (E) for 30
min with rocking at 24 h after irradiation. HL-60 cells labeled with 51Cr were added to
treated HUVECs. Cultures were then washed, and adherent cells were counted by
scintillation counting. Shown are the means (bars, SE) of three experiments.

Several findings support the hypothesis that CAM induction by
ionizing radiation is independent of cytokine production: (a) CAM
expression occurs in vitro in the absence of leukocytes or cytokines;
(b) CAM induction is more rapid (2–3 h) than cytokine induction (6
h; Refs. 14, 32, and 41); (c) cytokine-inducible CAMs are not induced
by X-rays, whereas they are induced by TNF or IL-1; (d) we found no
detectable TNF or IL-1 in the irradiated endothelial cells; (e) inhibi-
tion of protein synthesis does not prevent the increase in expression of
either E-selectin or ICAM-1 genes after irradiation. Taken together,
these findings indicate that CAM expression in the irradiated vascular
endothelium does not require the induction of cytokines.

The mechanisms by which X-rays induce adhesion molecules on
the endothelium have been studied previously (24). We have found
that the transcription factor NFKB is activated following irradiation
of endothelial cells. It has been reported that ICAM-1 mRNA is depend-
ent on NFKB activation (42, 43). NFKB is also activated by inhibition
of protein synthesis (44). We found that inhibition of protein synthesis
increases ICAM-1 expression and results in superinduction by X-rays.
Superinduction also results from protein synthesis inhibition com-
bined with cytokine or phorbol ester treatment (45). Furthermore,
others have shown that glucocorticoids attenuate ICAM-1 expression
in the irradiated mouse brain (11). The mechanism of steroid inhibi-
tion of adhesion molecules involves the ligand-activated glucocorti-
coid receptor, which represses RelA-mediated activation of the
ICAM-1 Fkbp site (46). Transcriptional repression of NFBk, medi-
at by glucocorticoid, is not caused by binding of glucocorticoid
receptor to the ICAM-1 Fkbp element but by a physical interaction
between the glucocorticoid receptor and RelA proteins (46). Several
studies have shown that NFBk participates in X-ray-mediated tran-
scriptional regulation (24, 44, 47, 48). Taken together, these findings
indicate that radiation-activated NFBk may regulate CAM expression
through a cytokine-independent pathway.

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