



## Expression and Functional Significance of Adhesion Molecules on Cultured Endothelial Cells in Response to Ionizing Radiation

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### ABSTRACT

**Objective:** Upregulation of adhesion molecules on endothelial cells following irradiation has been shown, but the functional significance of this upregulation in various endothelial cell lines is not clear. We have developed an *in vitro* flow model to study the functional consequences of the radiation-induced upregulation of E-selectin and intercellular adhesion molecule (ICAM-1).

**Methods:** Human dermal microvascular endothelial cells (HDMEC), human umbilical vein endothelial cells (HUVEC), or transformed human microvascular endothelial cells (HMEC-1) were grown in 35-mm dishes and irradiated with a single dose of 10 Gy. HL-60 (human promyelocytic leukemia) cells were perfused over the irradiated endothelial cells in a parallel plate flow chamber at shear stress ranging from 0.5 to 2.0 dynes/cm<sup>2</sup>. Flow cytometry was used to quantify the expression of E-selectin and ICAM-1 on the various endothelial cells.

**Results:** Flow cytometric analysis revealed an upregulation of ICAM-1 expression on all three cell types postirradiation (post-IR), and an upregulation of E-selectin expression only on HDMEC post-IR. E-selectin expression was detected on control HDMEC, but at a lower level than that detected on post-IR HDMEC. Flow assays revealed a significant increase in the number of rolling and firmly adherent HL-60 cells on post-IR HDMEC at shear stress  $\leq 1.5$  dynes/cm<sup>2</sup>; pretreatment of control and irradiated HDMEC with antibodies to E-selectin and ICAM-1 significantly diminished the number of rolling and firmly adherent HL-60 cells, respectively. No rolling or firm adhesion of HL-60 cells was observed on HUVEC or HMEC-1 monolayers post-IR.

**Conclusion:** These findings suggest that ICAM-1 is upregulated on irradiated HDMEC, HUVEC, and HMEC-1. E-selectin is upregulated to a functional level only on irradiated HDMEC, and not on irradiated HUVEC or HMEC-1. **Microcirculation (2001) 8, 355–364.**

KEY WORDS: endothelium, E-selectin, flow chamber, ICAM-1, leukocyte adhesion

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### INTRODUCTION

Ionizing radiation is a major therapy for the treatment of a variety of diseases including cancer, arteriovenous malformations, macular degeneration, and intimal hyperplasia. A limiting factor in the use of radiation therapy is the damaging effect the radiotherapy has on the normal tissue. Elucidation of a

detailed molecular and functional understanding of tissue response to radiation will increase the efficacy and applicability of radiation therapy, and perhaps lead to the development of novel treatments for disease.

Alteration in the structure and function of the microvasculature in response to radiation is believed to be a key factor in radiation-induced organ damage. A significant cause of these alterations is the induction of an inflammatory reaction involving leukocyte infiltration of the irradiated organs. A variety of studies focused on granulocyte adhesion to the endothelium in general (i.e., in response to stimuli other than radiation) have revealed that leukocyte recruitment and emigration involves a well-orchestrated adhesion cascade. The adhesion cascade begins when the granulocyte attaches to the endothelium and begins to roll in the direction of fluid flow at a low velocity. As the granulocyte rolls, it becomes activated, subsequently firmly adheres to the endothelium, and migrates across the endothelium into the extravascular space. These adhesion events are mediated, in part, by molecular interactions that occur between glycoproteins (ligands) present on the surface of the granulocytes and cognate glycoproteins present on the endothelium. A variety of studies have revealed that the endothelial cell adhesion molecule E-selectin (CD62E, ELAM-1) (5,6) can interact with SLe<sup>x</sup>-type glycan presenting granulocyte ligands to mediate the attachment and rolling steps of the adhesion cascade (1,7,14,28). The downstream steps of the cascade involve the interaction of endothelial-expressed intercellular adhesion molecule (ICAM-1) (4,8) with granulocyte-expressed CD18 integrins (30,40).

*In vivo* observations suggest that both E-selectin and ICAM-1 are involved in radiation-induced inflammation. Immunohistochemical staining has revealed the presence of E-selectin and ICAM-1 on lung tissue in response to radiation (21), and intravital microscopy has revealed an increase in the number of rolling, firmly adherent and emigrated leukocytes within the microvasculature postirradiation (post-IR) (33). Administration of monoclonal antibodies (mAbs) to the CD18 integrins or ICAM-1 attenuates the number of adherent leukocytes post-IR (33), and radiation-induced leukocyte infiltration has been reported to be abrogated in mice lacking ICAM-1 (20). A recent clinical study (23) reported that both ICAM-1 and E-selectin were significantly upregulated in oral mucosa of head and neck cancer patients treated with radiotherapy (30–60 Gy in 2-Gy daily fractions). Other adhesion molecules such as platelet-endothelial cell adhesion molecule

(PECAM-1), vascular cell adhesion molecule (VCAM-1), and P-selectin may also be upregulated by ionizing radiation (18,37).

To further the understanding of radiation-induced leukocyte adhesion, several *in vitro* studies have focused on the expression of E-selectin and ICAM-1 on cultured endothelial cells in response to irradiation. Upregulation of ICAM-1 on human umbilical vein endothelial cells (HUVEC) in response to irradiation has been reported to occur as early as 24 hours post-IR (17), and to continue up to at least 10 days post-IR (12). Upregulation of mRNA levels of ICAM-1 on dermal microvascular endothelial cells has also been reported, having a time course of 24 hours post-IR (24). However, there is disagreement over the time course of the upregulation of ICAM-1 on irradiated endothelial cells, with upregulation being reported from 24 hours (17) to 48 hours (12,37) post-IR.

The literature concerning the expression of E-selectin post-IR on cultured endothelium is inconsistent. Reports from Hallahan's group (17,19) clearly indicate upregulation of E-selectin on HUVEC 4 hours post-IR. In support of this observation, Hallahan and colleagues (19) reported an increase in HL-60 cell adhesion, under semistatic conditions, to 4-hour post-IR HUVEC. This increase in adhesion was attenuated by mAbs to E-selectin expression on HUVEC 4 hours post-IR (12). The expression of E-selectin on dermal microvascular endothelial cells in response to UV radiation has been studied by Heckman and colleagues (24). This group found that mRNA levels of E-selectin were upregulated at 24 hours post-IR (24).

Thus, the expression of E-selectin and the time course of ICAM-1 upregulation post-IR on cultured endothelium is unclear. Although determining the expression of adhesion molecules is important, it is equally important to determine the functional consequence of the presence of these molecules. As the primary role for E-selectin *in vivo* is to mediate the attachment and rolling step of the adhesion cascade (1,26,28,40,41) and that of ICAM-1 is to mediate subsequent events (e.g., firm adhesion) (27), an important issue is whether the level of irradiation-induced upregulation of E-selectin and ICAM-1 is sufficient to support significant attachment, rolling, and firm adhesion of leukocytes under fluid flow conditions. For these reasons, we studied the upregulation of E-selectin and ICAM-1 on cultured endothelium, and the adhesion of a leukocytic cell line to cultured endothelium post-IR under *in vitro* flow conditions that mimic, in part, flow conditions present *in vivo*.

## MATERIALS AND METHODS

### Reagents

M199, RPMI-1640, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, DETA-trypsin solution, trypsin neutralizing solution, and Dulbecco phosphate buffer solution (DPBS) were obtained from Biowhittaker (Walkersville, MD). MCDB131 was purchased from Life Technologies (Rockville, MD); endothelial growth factor from Biomedical Technologies (Boston, MA); human serum from Irvine Scientific (Santa Ana, CA); and gelatin, heparin, dibutyl cyclic AMP, formaldehyde, bovine serum albumin (BSA), and hydrocortisone from Sigma Chemical (St. Louis, MO). Interleukin-1 $\beta$  (IL-1 $\beta$ ) was obtained from Calbiochem (La Jolla, CA). Murine mAb BBIG-E4 (anti-E-selectin, IgG1), murine mAb BBIG-I1 (anti-ICAM-1, IgG1), mouse ICAM-3.3 (anti-ICAM-3, IgG1), and murine IgG1 were obtained from R&D systems (Minneapolis, MN). Murine mAb TS1/22 (anti-LFA-1, IgG1) and murine mAb TS1/18 (anti- $\beta_2$  chain, IgG1) were obtained from Endogen (Woburn, MA). Murine mAb CSLEX (anti-SLe<sup>x</sup>, IgM) was obtained from Becton Dickinson (San Jose, CA). Murine PSL-275 mAb (anti-PSGL-1) was a generous gift from Dr. Raymond T. Camphausen of Genetics Institute (Cambridge, MA). Murine mAb R6.5 (anti-ICAM-1, IgG2a) was a generous gift from Boehringer Ingelheim (Ridgefield, CT). FITC-labeled goat antimouse IgG (heavy- and light-chain specific) F(ab')<sub>2</sub> fragment and goat antimouse IgM F(ab')<sub>2</sub> fragment were purchased from Caltag Laboratories (La Jolla, CA).

### Cell Culture

HUVEC was isolated using the methods of Gimbrone and colleagues (13) and Wagner and colleagues (43) as modified by Sporn and colleagues (39). HUVEC were maintained in M199 supplemented with 10% FBS, 2 mM L-glutamine, 100  $\mu$ g/mL heparin, 50  $\mu$ g/mL endothelial growth factor, and 100 U/mL penicillin/streptomycin. HUVEC were grown on tissue culture plastic pretreated with 0.2% gelatin. HUVEC were subcultured at a ratio of 1:3 and used at passage 3–6.

Primary human dermal microvascular endothelial cells (HDMEC) were isolated from human neonatal foreskins as described previously (42). HDMEC were maintained in MCDB131 supplemented with 10% human serum, 10% FBS, 2 mM L-glutamine, 5  $\times 10^{-4}$  M dibutyl cyclic AMP, 1  $\mu$ g/mL hydrocortisone acetate, and 100 U/mL of penicillin/streptomycin. The cells were grown on tissue culture plastic

pretreated with 0.2% gelatin. HDMEC were subcultured at a ratio of 1:3 and used at passage 4–7.

Transformed human microvascular endothelial cells (HMEC-1) (2) were obtained from CDC (Centers for Disease Control, Atlanta, GA). These are essentially HDMEC transformed with SV40 Ag. HMEC-1 were maintained in MCDB131 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin. HMEC-1 were grown on tissue culture plastic pretreated with 0.2% gelatin. HMEC-1 were subcultured at a ratio of 1:3 and used at passage 21–26.

HL-60 cells were obtained from ATCC (American Type Cell Culture, Manassas, VA) and maintained in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL of penicillin/streptomycin, as previously described.

### Irradiation of Endothelial Cells

Prior to irradiation, confluent endothelial cells in T25 cm<sup>2</sup> flasks were replenished with fresh media. HDMEC media was replaced with media lacking cyclic AMP, because cyclic AMP has been found to suppress the expression of E-selectin (32,35). Cells were irradiated with a single dose of radiation (10 Gy gamma). The gamma radiation was delivered from a Mark-I cesium source irradiator at a dose rate of 4.2 Gy per minute. The negative control endothelial cells were taken to the radiation facility but were not irradiated. IL-1 $\beta$  (10 U/mL) activated endothelial cells served as positive controls. Irradiated endothelial cells were incubated at 37 °C for 5, 24, 48, and 72 hours post-IR, and subsequently analyzed for the expression of E-selectin or ICAM-1 using flow cytometry.

### Flow Cytometric Analysis of Endothelial Cells

At the respective time points post-IR (5, 24, 48, or 72 hours), endothelial cells were harvested using a 1% BSA, 0.01% EDTA-trypsin solution. Harvested endothelial cells were washed in DPBS containing 1% BSA and incubated with mAb BBIG-E4 (anti-E-selectin), mAb BBIG-I1 (anti-ICAM-1), mAb ICAM-3.3 (anti-ICAM-3), or murine IgG1 at a concentration of 20  $\mu$ g/mL for 30 minutes at 4 °C. Following the incubation, endothelial cells were washed, incubated for 30 minutes at 4 °C with FITC labeled goat antimouse IgG, and washed and fixed in 2% formaldehyde. All antibodies were diluted in DPBS containing 1% BSA. The percentage of fluorescent cells in each case was obtained by subtracting the percentage of the fluorescent cells for the isotype-

matched control antibody (IgG) from the percentage of the fluorescent cells for the specific antibody.

### Flow Cytometric Analysis of HL-60 Cells

HL-60 cells, which grow in suspension, were washed in RPMI-1640 containing 5% FBS. Following the wash, the HL-60 cells were incubated for 30 minutes at 4 °C in RPMI-1640 containing 5% FBS and 20 µg/mL mAb CSLEX (anti-SLe<sup>x</sup>), mAb PSL-275 (anti-PSGL-1), mAb TS1/22 (anti-LFA-1), or mAb TS1/18 (anti-β<sub>2</sub> chain). Following the incubation, the HL-60 cells were washed, incubated for 30 minutes at 4 °C with FITC-labeled goat antimouse IgG or IgM as appropriate, and washed and fixed in 2% formaldehyde. The fluorescence of the endothelial and HL-60 cells was analyzed on a flow cytometer (Epics-Profile II, Beckman Coulter Inc., Fullerton, CA) within 7 days of sample preparation. The results indicated that PSGL-1, LFA-1, the β<sub>2</sub>-integrin, and SLe<sup>x</sup> were present on HL-60 cells (data not shown).

### In Vitro Flow Chamber Assays

A parallel plate flow chamber (Glycotech, Rockville, MD) was used to probe the functional consequence of the presence of adhesion molecules on the irradiated endothelial cells. The procedure for the adhesion assay has been described previously (9). In brief, endothelial cells were grown to confluence on 35-mm tissue culture dishes. The dishes were placed in the flow chamber and the endothelial cell monolayer rinsed. A suspension of HL-60 cells, 5 × 10<sup>6</sup>/mL, in HL-60 cell growth media (RPMI-1640, 10% FBS and 100 U/mL of penicillin/streptomycin) was drawn through the flow chamber at various flow rates. The height and width of the flow chamber, the viscosity of the media, and the volumetric flow rate determine the shear stress at the HL-60 cell-endothelial cell interface. Adhesion data is given as a function of this shear stress.

After 3 minutes of flow, the number of rolling and adherent HL-60 cells was quantified using Metamorph imaging software (Universal Imaging Corporation, West Chester, PA). Any cell that remained stationary for 30 or more seconds was scored as an adherent cell. Experiments were carried out at 37 °C. In some experiments, HDMEC were incubated with an mAb to either ICAM-1 (mAb R6.5, saturating concentration 10 µg/mL) or E-selectin (mAb BBIG-E4, saturating concentration of 10 µg/mL) for 30 minutes before the flow experiments; the same concentration of mAb was maintained in the perfusion buffer in these experiments.

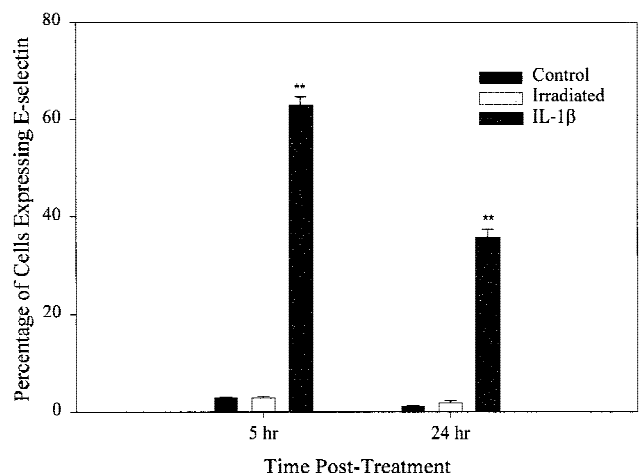
### Statistical Analysis

One-way analysis of variance (ANOVA) was used to detect significant differences among the groups (control, irradiated, and IL-1β activated) for the upregulation of adhesion molecules. A multiple comparison method (Fisher's least significant difference, LSD) was used to discriminate between the means. Differences between the means were considered statistically significant if  $p < 0.05$ .

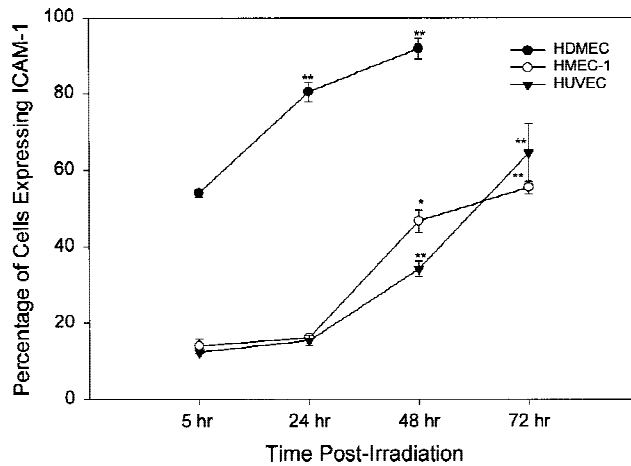
## RESULTS

### E-selectin was not Upregulated on HUVEC Post-IR, and Post-IR HUVEC did not Support Adhesion of HL-60 Cells under Flow Conditions

HUVEC were treated with 10 Gy radiation. Five hours, 24 hours, and 48 hours later, the HUVEC were analyzed for the presence of E-selectin via flow cytometry. As shown in Fig. 1, E-selectin was not upregulated on HUVEC at 5 hours, 24 hours, or 48 hours (data not shown) post-IR. As a positive control for this study, we investigated the expression of E-selectin on HUVEC in response to treatment with IL-1β. Four-hour treatment of HUVEC with IL-1β has been shown to elicit upregulation of E-selectin (4). As shown in Fig. 1, E-selectin was upregulated on HUVEC after 5-hour and 24-hour treatment with IL-1β, indicating that the HUVEC used for this study had the ability to express E-selectin. In contrast to the lack of expression of E-selectin in response to radiation on HUVEC ICAM-1 was upregulated on HUVEC at 48 hours and 72 hours post-IR (Fig. 2). This finding, which is consistent with that reported in the literature (12), demonstrates that the



**Figure 1.** Using flow cytometric analysis, E-selectin was not found to be upregulated on irradiated HUVEC [ $n = 3$ , mean  $\pm$  SEM, (\*\*) $p < 0.01$ ].



**Figure 2.** Using flow cytometric analysis, ICAM-1 was found to be upregulated on irradiated HDMEC at 24 hours post-IR, and on HUVEC on HMEC-1 at 48 hours post-IR [ $n = 3$ , mean  $\pm$  SEM, (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ ].

HUVEC were exposed to radiation and that the radiation was able to alter the adhesion molecule profile on the HUVEC.

E-selectin has been shown to support the attachment and rolling of leukocytes and leukocytic cell lines under fluid flow conditions (1,28,34,41). To further investigate the apparent lack of expression of E-selectin on HUVEC in response to radiation, we studied the adhesion of HL-60 cells to 5-hour post-IR HUVEC under *in vitro* flow conditions that mimic, in part, flow conditions present *in vivo*. As shown in Table 1, HL-60 cells did not roll or adhere to 5-hour post-IR HUVEC at shear stresses ranging from 2.0 dynes/cm<sup>2</sup> down to 0.5 dynes/cm<sup>2</sup>. No rolling was observed 24 hours and 48 hours post-IR (data not shown). Note that these stresses are at the middle to lower end of physiologically relevant microvascular shear stresses (15). As a positive control for this study, we investigated the adhesion of HL-60 cells to IL-1 $\beta$ -activated HUVEC. As shown in

Table 1, 5-hour IL-1 $\beta$ -activated HUVEC supported significant levels of attachment, rolling, and adhesion of HL-60 cells at all shear stresses tested. These findings, combined with the results from the flow cytometric analysis, strongly suggest that E-selectin is not expressed to a functional level on HUVEC post-IR.

**E-selectin was not Upregulated on a Transformed Microvascular Endothelial Cell Line Post-IR, and the Post-IR Transformed Microvascular Endothelial Cell Line did not Support Adhesion of HL-60 Cells under Flow**

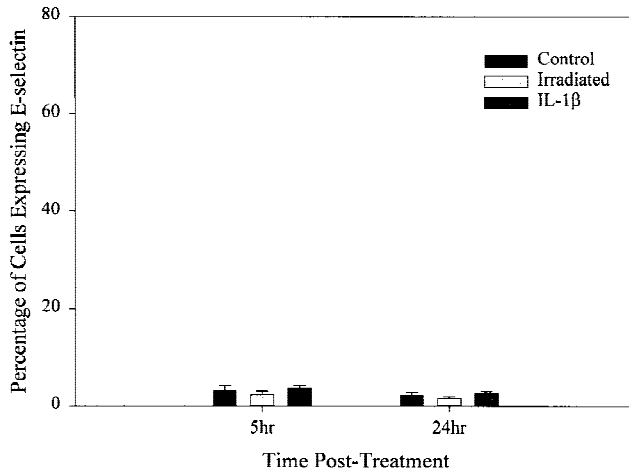
We are primarily interested in leukocyte-endothelial adhesion events that occur in the microvasculature. As microvascular endothelial cells may respond differently than large-vein endothelial cells (i.e., HUVEC), we next sought to study the response of microvascular endothelial cells to radiation. We first used an immortalized microvascular endothelial cell line, HMEC-1. HMEC-1 were treated with 10 Gy radiation. Five hours, 24 hours, and 48 hours later, HMEC-1 were analyzed for the presence of E-selectin via flow cytometry. As shown in Fig. 3, E-selectin was not upregulated on HMEC-1 at 5 hours, 24 hours and 48 hours (data not shown) post-IR. As a positive control for this study, we investigated the expression of E-selectin on HMEC-1 in response to treatment with IL-1 $\beta$ . As shown in Fig. 3, E-selectin was not upregulated on HMEC-1 after 5 hours, 24 hours, and 48 hours (data not shown) of treatment with IL-1 $\beta$ , indicating that the HMEC-1 used for this study may not have the ability to express E-selectin. Interestingly, ICAM-1 was upregulated at 48 hours and 72 hours post-IR on HMEC-1, demonstrating that HMEC-1 were exposed to radiation and that the radiation was able to alter the adhesion molecule profile on HMEC-1 (Fig. 2).

To further investigate the apparent lack of expression of E-selectin on HMEC-1 in response to radia-

**Table 1.** HL-60 cells rolling and adhering on 5-hour posttreatment HUVEC

Shear stress (dynes/cm <sup>2</sup> )	Control (number of cells)		Irradiated (10 Gy) (number of cells)		IL-1 $\beta$ -activated (number of cells)	
	Rolling	Adhering	Rolling	Adhering	Rolling	Adhering
0.5	0	0	0	0	85.2 $\pm$ 2.0**	80.0 $\pm$ 3.2**
1.0	0	0	0	0	70.0 $\pm$ 1.5**	61.0 $\pm$ 4.3**
1.5	0	0	0	0	42.3 $\pm$ 1.7**	37.0 $\pm$ 3.0**
2.0	0	0	0	0	34.7 $\pm$ 0.8**	17.3 $\pm$ 2.0**

$n = 5$ , mean  $\pm$  SEM, (\*\*)  $p < 0.01$  for differences between control and treatment.



**Figure 3.** Using flow cytometric analysis, E-selectin was not found to be upregulated on irradiated HMEC-1 or after treatment with IL-1 $\beta$  ( $n = 3$ , mean  $\pm$  SEM).

tion and IL-1 $\beta$ , we studied the adhesion of HL-60 cells to 5-hour post-IR HMEC-1 and 5-hour IL-1 $\beta$ -treated HMEC-1 under *in vitro* flow conditions. HL-60 cells did not roll or adhere to 5-hour, 24-hour, and 48-hour post-IR or to 5-hour, 24-hour, or 48-hour IL-1 $\beta$ -treated HMEC-1 at shear stresses ranging from 2.0 dynes/cm<sup>2</sup> down to 0.5 dynes/cm<sup>2</sup> (data not shown). These findings, combined with the results from flow cytometry, strongly suggest that E-selectin is not expressed to a functional level on HMEC-1 post-IR or on IL-1 $\beta$ -treated HMEC-1.

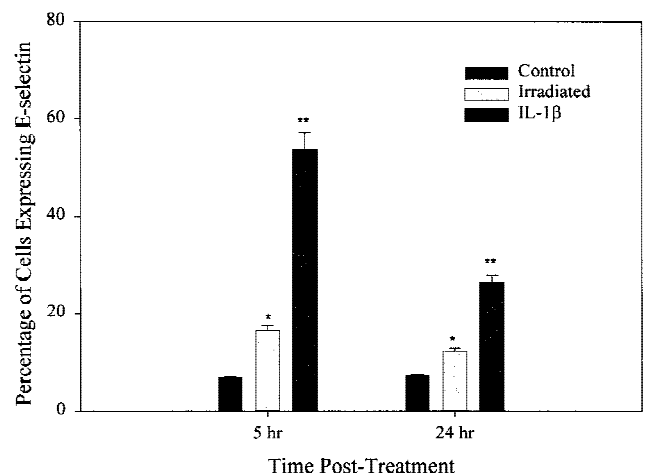
**The Expression of E-selectin on Dermal Microvascular Endothelial Cells was Significantly Increased by Treatment with Radiation, and Post-IR Dermal Microvascular Endothelial Cells Supported an Increased Level of HL-60 Cell Adhesion under Shear Flow**

As the HMEC-1 endothelial cell line did not express E-selectin in response to IL-1 $\beta$ , HMEC-1 cells may not be a good model for investigation of the expression of E-selectin on microvascular endothelial cells in response to radiation. Thus, we next used untransformed, low passage, microvascular endothelial cells isolated from human foreskin, HDMEC. HDMEC were treated with 10 Gy radiation. Five hours, 24 hours, and 48 hours later, the HDMEC were analyzed for the presence of E-selectin via flow cytometry. Five hours and 24 hours post-IR, the percentage of HDMEC expressing E-selectin was significantly increased (see Fig. 4). At 48 hours post-IR, E-selectin expression returned to baseline levels (data not shown). Although there is a significant increase in E-selectin in response to radiation, it is only ~25% that seen in response to IL-1 $\beta$ . HDMEC ap-

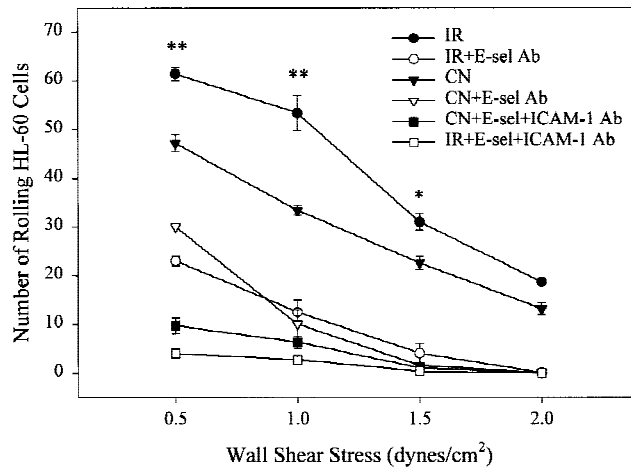
pear to express a low basal level of E-selectin which is significantly different from the background. Note that ICAM-1 was upregulated on HDMEC at 24, 48, and 72 hours post-IR, but not at 5 hours post-IR (Fig. 3). There was not a basal level of ICAM-3 on HDMEC, and their levels were not upregulated on irradiated HDMEC (data not shown).

To investigate the functional consequence of this increase in E-selectin expression, we studied the adhesion of HL-60 cells to 5-hour and 24-hour post-IR HDMEC. As shown in Fig. 5, 5-hour post-IR HDMEC supported higher levels of rolling HL-60 cells (which was statistically significant at shear values of 1.5 dyne/cm<sup>2</sup> or lower) compared to the level supported by nonirradiated HDMEC. This higher level of rolling appeared to lead to a higher level of firm adhesion of HL-60 cells on HDMEC, which again was statistically significant at shear values of 1.5 dyne/cm<sup>2</sup> or lower (Fig. 6). [Note that we did not see an upregulation of ICAM-1 on 5-hour post-IR HDMEC (not shown)]. The rolling velocity of HL-60 cells on irradiated HDMEC was significantly ( $p < 0.001$ ) reduced by 70% to 80% at all shear values. At 24 hours post-IR, a significant increase in HL-60 cell rolling and firm adhesion was observed only at shear stress values of 1 dyne/cm<sup>2</sup> or lower (data not shown). Taken together, the data strongly suggest that there is an increase in E-selectin expression on HDMEC in response to radiation, and a parallel increase in the ability of HDMEC to support HL-60 cell adhesion under flow.

Pretreatment of 5-hour post-IR HDMEC or nonirradiated HDMEC with an antibody to E-selectin (BBIG-E4) significantly ( $p < 0.01$ ) diminished the



**Figure 4.** Using flow cytometric analysis, E-selectin was found to be modestly upregulated on irradiated HDMEC [ $n = 3$ , mean  $\pm$  SEM, (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ ].



**Figure 5.** Under shear flow conditions, there was a significant increase in rolling of HL-60 cells on 5-hour post-irradiated (IR) HDMEC compared to nonirradiated (CN) HDMEC. Rolling of HL-60 cells on both nonirradiated and irradiated HDMEC was significantly ( $p < 0.01$ ) inhibited after incubation with an antibody to E-selectin (+E-sel Ab) [ $n = 3-5$ , mean  $\pm$  SEM, (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ ]. Rolling of HL-60 cells on both nonirradiated and irradiated HDMEC was completely inhibited after simultaneous incubation with antibodies to both E-selectin and ICAM-1 (+E-sel+ICAM-1 Ab).

number of rolling HL-60 cells (Fig. 5). Pretreatment of 5-hour post-IR HDMEC or nonirradiated HDMEC with an antibody to ICAM-1 (mAb R6.5) significantly ( $p < 0.01$ ) diminished the number of firmly adherent HL-60 cells (Fig. 6). At 0.5 dynes/cm<sup>2</sup>, firm adhesion of HL-60 cells was not completely inhibited by pretreatment of post-IR HDMEC with an antibody to ICAM-1 (mAb R6.5). The latter finding might be explained by the fact that the increased expression of E-selectin on irradiated HDMEC could be involved in the firm adhesion at very low shear values (0.5 dynes/cm<sup>2</sup>). In fact, simultaneous pretreatment of HDMEC with antibodies to both ICAM-1 (mAb R6.5) and E-selectin (BBIG-E4) completely diminished the number of rolling and firmly adherent HL-60 cells (see Figs. 5 and 6).

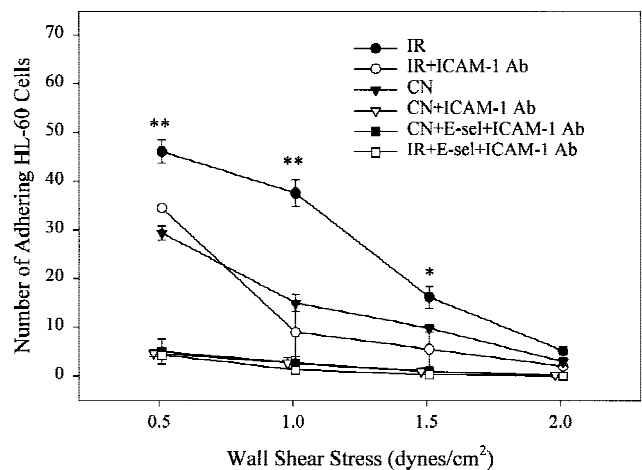
As stated above, although there was an increase in HL-60 cell adhesion to irradiated HDMEC, the significant increase occurred only at the lower shear stresses ( $\leq 1.5$  dyne/cm<sup>2</sup>), and the percentage increase ranged from 50% to 100%. In contrast, the percentage increase in HL-60 cell adhesion in response to IL-1 $\beta$  ranged from 250% to 400%, and occurred throughout the entire range of shear stresses tested (data not shown). Flow cytometric analysis indicated that E-selectin was significantly upregulated on HDMEC following a lower dose of

irradiation (5 or 2 Gy), but this increase was not paralleled with an increase in rolling or firm adhesion of HL-60 cells to irradiated HDMEC (data not shown).

## DISCUSSION

The effects of ionizing radiation were studied on endothelial cells derived from large vessels (HUVEC), small vessels (HDMEC), and transformed dermal microvascular endothelial cell line (HMEC-1). Expression of E-selectin and ICAM-1 was quantified by flow cytometry. The functional significance of the upregulation of adhesion molecules was investigated using an *in vitro* flow chamber at shear stress values comparable to those observed in the postcapillary venules.

These findings suggest that ionizing radiation does not upregulate E-selectin to a functional level on HUVEC and HMEC-1, but does increase the ability of HDMEC to support HL-60 cell adhesion under shear flow conditions. These findings suggest that the upregulation of adhesion molecules may be functionally significant only at low-flow microcirculatory



**Figure 6.** Under shear flow conditions, there was a significant increase in the firm adhesion of HL-60 cells to 5-hour post-irradiated (IR) HDMEC compared to nonirradiated (CN) HDMEC. Firm adhesion of HL-60 cells to both nonirradiated and irradiated HDMEC was significantly ( $p < 0.01$ ) inhibited after incubation with an antibody to ICAM-1 (+ICAM-1 Ab) [ $n = 3-5$ , mean  $\pm$  SEM, (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ ]. Firm adhesion of HL-60 cells on both nonirradiated and irradiated HDMEC was completely inhibited after simultaneous incubation with antibodies to both E-selectin and ICAM-1 (+E-sel+ICAM-1 Ab).

conditions. Rolling and adhesion of HL-60 cells in both control and irradiated HDMEC was inhibited by pretreatment of the HDMEC with antibodies to E-selectin and ICAM-1, respectively.

HUVEC did not express E-selectin following ionizing radiation. Previously published studies from Hallahan and colleagues suggest the presence of (16,17,19,21,22), and [those from Gaugler and colleagues suggest that absence of (12), E-selectin expression following ionizing radiation exposure on HUVEC]. Our data agrees with the published work of Gaugler and colleagues (12) that E-selectin is not upregulated on HUVEC following ionizing radiation exposure. Consistent with the absence of E-selectin on postirradiated HUVEC, no rolling of HL-60 cells was observed on postirradiated HUVEC. As a positive control, HL-60 cells rolled and adhered on IL-1 $\beta$ -activated HUVEC.

Gaugler and colleagues (12) proposed that the difference between their findings and those of Hallahan and colleagues (16,17,19,21,22) could be based on the radiation source (x-ray vs. gamma) and the radiation dose rate. However, we have found that the radiation source and the radiation dose rate (4.2 Gy/min for gamma radiation and 2.0 Gy/min for x-ray) did not cause significant differences in the upregulation of adhesion molecules. For example, there were no significant differences in the level of ICAM-1 upregulation at 24 hours and 48 hours post-IR between x-ray and gamma-irradiated HUVEC (36).

HMEC-1 also did not express E-selectin following ionizing radiation. This is in agreement with previously published results using UV radiation (38). Interestingly, even after activation with IL-1 $\beta$ , E-selectin was not upregulated on these cells. Consistent with the absence of E-selectin, HL-60 cells did not roll on HMEC-1. HMEC-1 is the immortalized transformed progeny of HDMEC, and our findings suggest that this immortalized cell line may not be a good model for investigation of the expression of E-selectin on microvascular endothelial cells in response to radiation exposure. HMEC-1 constitutively express PECAM-1 (CD31), lymphocyte function antigen (LFA-3), and major histocompatibility complex (MHC) class I, but not CD36, neural cell adhesion molecule (NCAM), and MHC class II (44). In addition, very modest levels of E-selectin can be induced on HMEC-1 with both tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 $\alpha$  activation at 100 U/mL (29,44), which is 10 times the concentration of IL-1 $\beta$  used in our study.

E-selectin expression was upregulated post-IR on

HDMEC. This upregulation was paralleled with a significant increase in rolling of HL-60 cells under shear flow conditions. There has been only one previous study of the expression of E-selectin following ionizing radiation on HDMEC (24). The latter study indicated that E-selectin mRNA was strongly induced at 24 hours post-IR. The same group of investigators showed E-selectin expression at the cell surface was upregulated 6 hours post-UV radiation (25). In agreement with these findings our results also indicate that E-selectin was upregulated on HDMEC at 5 hours and 24 hours post-IR.

Several investigators have reported that ICAM-1 is upregulated on HUVEC following irradiation (3,11,12,20,21,24), but there is disagreement over the time course of this upregulation. In our study, we observed upregulation of ICAM-1 in all three endothelial cell types. On HUVEC, ICAM-1 was upregulated 48 hours post-IR which is in disagreement with the observations of Hallahan and colleagues (17) that ICAM-1 is upregulated as early as 24 hours post-IR on HUVEC. However, we observed the upregulation of ICAM-1 at the same time point (48 hours) as that observed by Gaugler and colleagues (12) and Quarmby and colleagues (37).

On HDMEC, ICAM-1 was upregulated as early as 24 hours post-IR. This is in agreement with the ICAM-1 mRNA levels of upregulation observed in a previous study (24). On HMEC-1, ICAM-1 was upregulated at 48 hours post-IR. This is in contrast to previously published results where ICAM-1 was reported to be upregulated by 24 hours post-IR using UV radiation (38), and to the results of Hallahan and colleagues who reported a threefold increase in ICAM-1 expression on irradiated HMEC 24 hours post-IR (17).

Ionizing radiation therapy causes vascular lesions and damage in normal tissues, but the microvascular system is by far the most sensitive to radiation (10). Interestingly, our results indicate that the adhesion molecule profile on microvascular endothelial cells (HDMEC) are more susceptible to radiation damage than that of large-vessel endothelial cells (HUVEC). At the microvascular level, adhesion molecules other than E-selectin and ICAM-1 may also be upregulated by ionizing radiation (18,37), and may play key roles in the ensuing adhesion cascade. For example, *in vivo*-increased P-selectin expression has been shown to mediate residual rolling after E-selectin blockade (31). Additional studies are needed to establish the role of various adhesion molecules in different parts of the vasculature and in different organs.

In conclusion, our study suggests that ionizing radiation does not upregulate E-selectin to a functional level on HUVEC and HMEC-1, but increases the ability of HDMEC to support HL-60 cell adhesion under shear flow conditions.

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