Irradiation Induces Upregulation of CD31 in Human Endothelial Cells

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Abstract—Radiation-induced vascular injury is believed to be a major factor contributing to parenchymal atrophy, fibrosis and necrosis in normal tissue after radiotherapy. In this study irradiation of human umbilical vein endothelial cells (HUVECs) significantly increased adherence of U-937 cells in a time-dependent manner. Given the potential multifunctional role of CD31 in the vasculature we have examined the possible effects of irradiation on levels of CD31 expression in HUVECs. Irradiation upregulated CD31 expression on HUVECs, independently of initial plating density and radiation-induced changes such as cell number, cell cycle stage, or cell size. CD31 mRNA levels were raised in irradiated HUVECs relative to controls. Both CD31 mRNA and surface protein showed similar changes, suggesting that the increase in mRNA in irradiated HUVECs is responsible for the elevation in cell surface protein. A semi-quantitative study of tissue specimens from patients who had received radiotherapy indicated that CD31 staining in the blood vessels from irradiated tissues was increased compared with controls. Endothelial CD31 is important in the transmigration of leukocytes. We have demonstrated that the incorporation of monoclonal antibody to CD31 significantly inhibited the transmigration of human peripheral blood leukocytes through a monolayer of irradiated HUVECs. Taken together these data strongly suggest that irradiation induces a marked increase in CD31 expression on endothelial cells as part of a general response to irradiation. Its upregulation may play an important role in the development of radiation-induced normal tissue damage and thus is a possible target for therapeutic intervention. (Arterioscler Thromb Vasc Biol. 1999;19:588-597.)

Key Words: CD31 ■ irradiation injury ■ endothelial cells ■ fibrosis

A serious complication of radiotherapy in the treatment of patients with cancer is the late onset of necrosis and fibrosis in normal tissues that can lead to marked morbidity and even death. The blood vessels in irradiated normal tissues often undergo adverse changes that can compromise their functions, and because these changes precede the development of fibrosis, it has been hypothesized that vascular damage could be an important factor in the pathogenesis of late radiation damage. Endothelial cell dysfunction after irradiation can be recognized by morphological changes such as cell swelling, increased leukocyte-endothelial cell adherence and infiltration of leukocytes into tissues, platelet adherence to the vascular wall, a loss in vascular wall integrity resulting from reduced endothelial cell numbers, and the formation of tortuous vessels. Recent experimental evidence suggests that radiation-induced expression of the membrane glycoproteins E-selectin and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells is involved in the mechanisms mediating increased leukocyte-endothelial cell interactions after irradiation. Platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31) is a 130-kDa membrane glycoprotein belonging to the immunoglobulin superfamily and is constitutively expressed on the surface of endothelial cells, platelets and circulating leukocytes. Mounting evidence implicates its involvement in a number of cell adhesion processes that take place in the vasculature, such as leukocyte adhesion and transmigration, the adherence of platelets to the vascular wall at sites of endothelial cell damage, and endothelial cell–cell adherence and migration, a process required for neovascularization that is essential for tissue growth and repair. Anti-CD31 monoclonal antibodies (Mabs) have also been shown to have a protective effect in a myocardial ischemia and reperfusion injury in animal models, raising the possibility of their potential therapeutic use in other inflammatory disorders. CD31-ligand interactions may participate in the activation of integrin receptors on leukocytes during adherence to endothelial cells and recent studies suggest that the cytoplasmic domain of CD31 could be involved in the signal transduction process mediating this phenomenon. Alternative splicing of the cytoplasmic domain has been shown to occur and may be a mechanism for regulating the homophilic and heterophilic ligand interactions mediated by the molecule.

Given the effects of irradiation on the vasculature and the role of CD31 in endothelial cell adhesion, we hypothesized...
that irradiation might alter the expression of endothelial cell CD31. We showed that irradiation of tissue-cultured human umbilical vein endothelial cells (HUVECs) led to increased adherence of U-937 cells in a time-dependent manner. Therefore, the effect of irradiation on the expression of CD31 by HUVECs using indirect immunofluorescence and flow cytometry was examined. In addition, the effect of changes in cell size, cell numbers and the proportion of cells in each phase of the cell cycle after irradiation on CD31 expression were evaluated. Furthermore, CD31 mRNA expression was compared and the presence of alternatively spliced variants of the cytoplasmic domain was investigated using reverse transcription polymerase chain reaction (RT-PCR). Because CD31 is important in leukocyte transmigration, an in vitro model was used to compare the relative migration of peripheral blood leukocytes through irradiated and nonirradiated endothelial cell monolayers. The occurrence of CD31 was examined in irradiated human tissues to further relate these findings to in vivo events.

Materials and Methods

Isolation and Irradiation of HUVECs

HUVECs were obtained by collagenase digestion (Type IV, Sigma) of human umbilical veins using a modification of the method of Jaffe et al. and characterized as reported previously. HUVECs from 3 to 4 cords were pooled and used at passage 4 when >99% of cells were positive for the endothelial-specific marker, von Willebrand factor, as determined by an indirect immunofluorescence staining procedure.

HUVECs were cultured to passage 3 and then subcultured into T25 flasks at either 2 x 10^5 cells/cm^2 or 4 x 10^5 cells/cm^2. The cultures were incubated for an additional 48 hours, the medium changed and test cultures irradiated with a single dose of gamma radiation (3.3 Gy/min) using a ^60^Co source. The culture medium consisted of 40% (vol/vol) medium 199 (Gibco), 40% (vol/vol) fetal bovine serum (Techgen), 10% (vol/vol) medium 199 (Gibco), and 20% (vol/vol) fetal bovine serum (Techgen), 10 μg/mL endothelial cell growth supplement (Sigma), 4 μg/mL hydrocortisone (Sigma) (excluded after plating), and 0.0024 μM hirudin (CP Pharmaceuticals). The cells were incubated for 6, 24, 48, 72, and 96 hours and assayed according to the procedures outlined below.

Cell Adherence Studies

U-937 cells were maintained at cell concentrations between 2 x 10^5 cells/mL and 9 x 10^5 cells/mL in RPMI 1640 medium, 10% (vol/vol) fetal bovine serum (FCS), and 300 μg/mL glutamine and radiolabeled by incubating with 1 μCi/mL [3H]thymidine (Amersham Pharmacia Biotech) for 24 hours following by washing in PBS. HUVECs were subcultured into 48-well plates (Costar) at 4 x 10^5 cells/well in 0.5 mL of medium and incubated for 48 hours. The medium was changed and then test plates irradiated with 10 Gy using a Pantak X-ray machine. Irradiated and control plates were incubated for 6, 24, 48, and 72 hours, washed with PBS and 4 x 10^5 radiolabeled U-937 cells in 0.5 mL RPMI medium added to wells containing HUVECs. After 30 minutes incubation, wells were rinsed twice with PBS to remove nonadherent U-937 cells and the contents of each well lysed by adding 0.5 mL NaOH for 15 minutes. The radioactivity (cpm) of the lysates was measured by scintillation counting allowing the degree of U-937 adherence to be compared between wells.

Immunocytochemistry and Flow Cytometry

HUVEC suspensions were obtained by trypsinization [0.05% vol/vol trypsin EDTA (Gibco) in PBS (Oxoid)]. The cell surface expression of CD31 was determined by incubation with Mab 5.6E (1:100, Immunotech) using indirect membrane immunofluorescence staining at 4°C. The binding of Mab 5.6E was detected by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse polyclonal antibody (1:40, DAKO). HUVECs were washed twice with PBS and centrifuged at 250g for 5 minutes at 4°C. All antibodies were diluted with PBS. The cells were fixed by the addition of 2% (vol/vol) buffered formalin. The degree of FITC staining (and hence the level of CD31) was quantified with a fluorescence-activated cell sorter (FACS) (Becton Dickenson). The forward scatter of each cell analyzed for FITC staining was measured simultaneously to determine the relative cell size.

Cell Numbers

HUVECs were harvested by trypsinization and cells counted using a Coulter counter (Coulter Electronics).

Cell Cycle Studies

A modification of the method of Jensen et al. was used. HUVEC cultures were trypsinized, fixed with 70% ethanol in PBS, washed with 2.5 μL PBS at 4°C, and successively centrifuged, resuspended by vortexing, pelleted and then subjected to proteolysis and DNA denaturation by adding 0.7 mL of 0.2 mg/mL pepsin (Sigma) in 2 mol/L HCl (BDH), vortex mixed and incubated at 37°C for 30 minutes. After the addition of 1.8 mL of 1 mol/L Tris (Sigma) and 2 washes with PBS, pelleted nuclei were resuspended in 50 μg/mL propidium iodide solution (Sigma) containing 5 mg/mL RNAse (Sigma) and kept on ice for at least 15 minutes before analysis. All centrifugations were carried out at 600g for 10 minutes at 4°C. The profiles of propidium iodide-stained nuclei were obtained by flow cytometry and analyzed with WinMDI and PC/lysis software to determine the proportion of nuclei from cells in G1/G0 (nondividing or resting), S (synthesising DNA) and G2/M phases (ready for mitosis). The percentage of cells in the S phase is obtained by counting the number of cells between G1/G0 (first peak) and G2/M (second peak).

Northern Blot Analysis of HUVEC RNA

For Northern blotting, HUVEC monolayers were lysed in situ by the addition of RNAlon (Biogene). RNA was precipitated from the lysate according to the manufacturer’s protocol and dissolved in sterile distilled water. After electrophoresis in a 1% (wt/vol) agarose gel under denaturing conditions, RNA was transferred onto GeneScreen Plus membranes (Dupont) according to manufacturer’s protocol, and hybridized at 65°C with a 32P-labeled probe corresponding to bases 700 to 2556 of CD31 cDNA generously provided by Dr D. Simmonds, Oxford). The probe was radiolabeled by random priming using a kit supplied by Boehringer Mannheim. The hybridization buffer comprised 1 mol/L NaCl (BDH), 10% (wt/vol) dextran sulfate (Biogene), 1 mmol/L EDTA (BDH), 1% (wt/vol) SDS (Sigma), 10 mmol/L HEPES (Sigma) at pH 7.0, 10 μg/mL salmon sperm DNA, and 20 μg/mL sodium ribonuclease (Fluka). Membranes were hybridized overnight and washed initially in 2X SSC, then 2X SSC, 0.1% (wt/vol) SDS for 30 minutes and twice with 0.2X SSC, 0.1% SDS for 30 minutes. All washes were carried out at 65°C. The extent of probe hybridization was revealed by autoradiography and the use of a phosphorimager (Molecular Dynamics). The blots were stripped by boiling in 0.1% SDS and subsequently hybridized with a 32P-labeled probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a kind gift from Dr R. Clark, Manchester), for use as a loading control.

RT-PCR

Total RNA was extracted from control and irradiated HUVECs as described above. For cDNA synthesis, 10 μg of RNA was denatured at 65°C for 5 minutes, chilled on ice and added to a mixture containing 200 IU/mL Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco), 1 X first strand reaction buffer (Gibco), 0.01 mmol/L dT (Gibco), 1 mmol/L dNTPs (Promega) and 0.05 μU/mL RNase Guard (Pharmacia) to a final volume of 20 μL. The reaction was primed with 0.05 μg/μL of oligo(dT) (Pharmacia) in order to selectively convert the Poly A+ mRNA into cDNA. After 65 minutes incubation at 37°C, the samples were heated to 80°C for 10 minutes to inactivate the reverse transcriptase and used for PCR analysis.

For PCR, 1 μL cDNA was added to a reaction mixture with a final volume of 50 μL containing 1.25 U Taq polymerase (Promega) plus...
1× reaction buffer as supplied by the manufacturer, with 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs (Promega), 0.1 μg forward primer (5′-CAA CGA GAA AAT GTG AGA-3′) and 0.1 μg antisense primer (5′-GGA GCC TTC CGT TCT AGA GT-3′). The sequences of the forward and antisense primers were identical to those detailed by Kirschbaum et al.²⁹ in their study documenting alternative splicing of CD31 in the cytoplasmic domain. The thermocycling sequence was as follows: 60 seconds at 94°C, 60 seconds at 56°C and 90 seconds at 72°C for 35 cycles followed by 60 seconds at 56°C and 10 minutes at 72°C. To visualize the products of these reactions, 15 μL of each sample was subjected to electrophoresis on a 1% agarose gel containing ethidium bromide with 1× Tris-borate-EDTA running buffer [0.089 mol/L Tris (BDH), 0.088 mol/L boric acid (BDH) and 0.004% vol/vol of a 0.5 mol/L EDTA solution, pH 8.0].

Southern Blot Analysis of PCR Products
The PCR products were run on an agarose gel as described above and transferred onto Genescreen Plus membranes (Dupont) as described in the manufacturer’s protocol. The membranes were fixed with UV light, baked at 80°C for 2 hours, re-wetted with 2× SSC and hybridized with a 32P-labeled probe for CD31 (prepared as described in the methods for Northern blotting) in a hybridization buffer containing 1× SSC, 1% SDS and 10 μg/mL salmon sperm DNA (Sigma) at 65°C for 18 to 24 hours. The membranes were rinsed initially with 2× SSC, followed by 2× SSC, 0.1% SDS for 30 minutes then 2 further washes in 0.2× SSC, 0.1% SDS for 30 minutes. They were analyzed using a phosphorimager and autoradiography.

Transendothelial Migration Assay
The transendothelial migration of leukocytes was quantified in irradiated and nonirradiated HUVEC monolayers cultured on native type 1 collagen gels in 48-well plates.³⁵ Leukocytes for this study were obtained aseptically from healthy donors using lymphoprep following the manufacturer’s instructions (Nycoderm). Leukocytes were washed in serum-free HUVEC medium, centrifuged (900g for 10 minutes) and resuspended in fresh serum-free medium (8×10⁴ cells/mL). Tropocollagen was extracted from rat tail tendons in 0.5 mol/L acetic acid, dialyzed for 3 days against distilled water, then centrifuged at 10 000g overnight at 4°C. Collagen gels were subsequently prepared by adding 1 mL of 10× M199 and 0.5 mL of 4.4% (wt/vol) sodium bicarbonate to 8.5 mL of collagen solution (2 mg/mL). The mixture was kept on ice, quickly mixed, then added to 48-well plates at 0.25 mL per well and transferred to a 37°C incubator. After a few minutes, when the gels had set, they were seeded with HUVECs (5×10⁴ per well), incubated for 24 hours and irradiated with 5 Gy (for details see Cell Adherence Studies). After 72 hours further incubation, the medium was removed and replaced with 0.25 mL of fresh serum-free HUVEC medium. A 0.25-mL aliquot of serum-free HUVEC medium containing 8×10⁴ leukocytes per milliliter was added to each HUVEC monolayer making the final volume 0.5 mL. Irradiated and nonirradiated HUVECs were also co-incubated with leukocytes and a 1:100 dilution of 5.6E anti-CD31 Mab. After 2, 6 and 24 hours incubation, HUVEC monolayers were washed in PBS and fixed for 10 minutes in 2% (vol/vol) buffered formalin. The collagen gels were viewed at different depths by phase-contrast microscopy and the number of transmigrated leukocytes counted in 4 fields. The total number of leukocytes that had transmigrated through nonirradiated and irradiated HUVEC monolayers was calculated and statistically compared using paired Student’s t test.

Immunohistochemical Localization of CD31 in Normal and Irradiated Tissues
Formalin-fixed, paraffin-embedded tissues (n=21) were skin specimens from patients who had received radiotherapy and showed evidence of radiation-induced tissue injury.⁶ Controls consisted of nonirradiated normal skin tissues taken from the same patients. Five micrometer-thick sections of both irradiated and control tissues were stained with Mab CD31 (J.C70, DAKO) following a previously published procedure.⁷ The stained preparations were semiquantitatively scored as (+), (++), or (++++) depending on the intensity of staining by endothelial cells of blood vessels. These were assigned arbitrary numerical values of 2, 4, and 8, respectively. The scores from irradiated tissues were compared with the controls using a Mann-Whitney U-test.

Results
Unless otherwise stated, the results from control and irradiated HUVECs have been statistically compared using paired Student’s t test. Where appropriate, regression analysis has also been performed. A P value of ≤0.05 was considered significant for all analyses.

The Effect of Irradiation on U-937 Cell Adherence to HUVECs
HUVECs were irradiated with 10 Gy and assayed for U-937 adherence at 6, 24, 48, and 72 hours after irradiation. The percentage differences in cpm values exhibited by irradiated HUVECs over controls addition of radiolabeled U-937 cells were calculated from 3 experiments. Cell adherence showed a time-dependent increase after irradiation (P<0.01 using linear regression; Figure 1).

The Effect of Irradiation on the Expression of CD31 by HUVEC
Two plating densities were used to represent different degrees of confluence. Data shown are a representative experiment or pooled from 3 experiments. Irradiated HUVECs assayed 48 hours after exposure exhibited significant dose-dependent increases in CD31 expression compared with the control (Figure 2A). Profiles of cell number against fluorescence (channel number), generated for each sample analyzed by FACS, also illustrated dramatic changes in CD31 expression with time (Figure 2B). At 6 hours, the profiles were virtually identical but at later time points, the irradiated population began to exhibit skewing (ie, became more intensely positive) compared with controls and CD31 expression was highest by 72 hours after irradiation. The changes in CD31 expression appeared uniform; ie, were not confined merely to a small subpopulation of cells. Analysis of the corresponding geometric mean fluorescence values from 3 experiments confirmed that at both plating densities, cell surface CD31

Figure 1. The adherence of radiolabeled U-937 cells to irradiated HUVECs. Irradiated (10 Gy) and nonirradiated HUVECs incubated for 6, 24, 48, and 72 hours when 4×10⁵ ³H-thymidine-labeled U-937 cells were added to each well and incubated for 30 minutes. HUVEC monolayers were rinsed with PBS to remove nonadherent cells and lysed with NaOH. The percentage increase in cpm demonstrated by irradiated HUVECs over controls was calculated from 3 experiments and plotted as mean±SE. Linear regression analysis of the data confirmed that the observed time-dependent percentage increases exhibited by the cpm values of irradiated HUVECs over controls (indicated by *) were statistically significant and reflected elevated U-937 cell adherence after irradiation.
expression on irradiated HUVECs had increased significantly, 48 hours onward, suggesting that irradiation induced similar increases in CD31 expression with time at both plating densities (Figure 3). These trends were confirmed by linear regression analysis: slope values for percentage difference against time for the low and high density cells were $120$ ($P$, 0.0001) and $116$ ($P$, 0.0001), respectively.

The Effect of Radiation on HUVEC Numbers, Cell Size, and the Proportion of Cells in Each Phase of the Cell Cycle

In addition to measuring membrane changes in CD31 on HUVEC after irradiation, HUVEC numbers, size and the percentage of cells in each phase of the cell cycle at each time point were examined to determine if there was any link to alterations in CD31 levels.

**Cell Numbers**

At both plating densities, the number of cells present in control and irradiated cultures (calculated from 3 experiments) differed significantly at 24, 48, 72, and 96 hours. In irradiated cultures, cell number decreased over time at both plating densities. In contrast, in controls, cell numbers exhibited a significant increase over time at the lower plating density and at the higher plating density showed a similar trend. The percentage differences in irradiated cell numbers over controls exhibited similar changes over time at both plating densities (Figure 4). These data suggested that CD31

**Figure 2.** Increased expression of CD31 on irradiated HUVECs as determined by indirect immunofluorescence and flow cytometry. A, Geometric mean fluorescence values (representing 3 experiments), corresponding to anti-CD31 antibody-stained HUVECs seeded at the higher density and assayed 48 hours after irradiation, showed a significant dose-dependent increase in CD31 expression taking place on irradiated HUVECs compared with the control (* indicates $P$=0.05; paired Student’s $t$ test for controls versus irradiated); a similar pattern was also observed at the lower seeding density (not shown). B, Profiles generated from the analysis of anti-CD31 antibody-stained HUVECs. Cells were seeded at high density, harvested after 6, 24, 48, 72, and 96 hours after 5-Gy irradiation and stained using Mab 5.6E (CD31) by an indirect immunofluorescence technique. Each profile shows the degree of fluorescence for approximately 10 000 control (solid profile) and 10 000 irradiated (silhouetted profile) HUVECs from a representative experiment. With successive time points, irradiated HUVECs exhibited a marked increased presence of CD31 (ie, accumulation at higher fluorescence levels relative to controls). This change was also typical of irradiated HUVECs seeded at low density (not shown).

**Figure 3.** Increase in CD31 expression on irradiated HUVECs over controls with time. The geometric mean fluorescence values, obtained from anti-CD31 antibody-stained irradiated HUVECs incubated for 6, 24, 48, 72, and 96 hours, were expressed as percentage increase over controls and pooled from 3 experiments. The values for both plating densities showed a similar increase with time, (* indicates fluorescence values of irradiated HUVECs were significantly increased over controls, $P$=0.05; paired Student’s $t$ test).

**Figure 4.** Effect of radiation on cell numbers. Numbers of HUVECs present in cultures exposed to 5Gy and controls were determined after 6, 24, 48, 72, and 96 hours incubation from 3 experiments. Percentage difference in cell numbers of irradiated HUVECs over controls was calculated and showed a similar negative percentage change occurred at each plating density (* indicates cell numbers of irradiated HUVECs were significantly different from controls, $P$=0.05; paired Student’s $t$ test).
expression on HUVECs might have been inversely related to alterations in cell number. However, linear regression analysis indicated that alterations in HUVEC cell numbers were unlikely to have mediated the observed increased CD31 expression (not shown).

**Cell Size**

Data on cell size (forward-scatter fluorescence) were pooled from 3 experiments. Irradiated HUVECs exhibited significant increases in cell size compared with controls at certain time points but the percentage differences relative to the controls at each time point were insufficient to account for the observed increases in CD31 expression (Figure 5).

**The Proportion of Cells in Each Phase of the Cell Cycle**

The proportion of cells in various phases of the cell cycle was determined for control and irradiated HUVECs plated at high density from FACS profiles of propidium iodide-stained DNA. The data from 3 experiments were pooled (Figure 6A to 6C). At all time points, most HUVECs were in the G1/G0 phase of the cell cycle. In control cultures the percentage in G1/G0 was approximately 70% throughout the study, whereas the numbers of irradiated HUVECs in G1/G0 exhibited small but statistically significant decreases compared with controls at 48, 72, and 96 hours (Figure 6A). There were no significant differences between control and irradiated HUVECs in S phase: approximately 8% to 9% were in S phase at any time (Figure 6B). The proportion of irradiated HUVECs in G2/M increased significantly compared with controls after 6 hours by approximately 1.5- to 2-fold but never exceeded 20% of the total cell population (Figure 6C).

**The Effect of Irradiation on mRNA Levels for CD31 in HUVECs**

For these experiments, HUVECs were plated at the higher cell density and irradiated with 5 Gy. Total RNA was extracted from control and irradiated cultures at 6, 24, 48, 72, and 96 hours after irradiation and subjected to Northern blot analysis for CD31 mRNA. Under the conditions used, a single band was detected in samples using the CD31 probe and quantified with a phosphorimager (Figure 7A). The blots were stripped and sequentially hybridized with GAPDH to correct for loading. A time-dependent increase in the intensity of the CD31 band can be seen in irradiated samples compared with corresponding bands in controls (Figure 7A). The values quantified from the CD31 bands were normalized to the GAPDH signal and the percentage difference exhibited by irradiated cultures over controls calculated. The results from 3 experiments were pooled and show changes in CD31 mRNA in irradiated HUVECs over controls with time averaging an increase of 70% (Figure 7B). The percentage increases in surface protein levels of CD31 (determined by FACS as in Figure 3) are expressed in the same format for comparison. Both follow a similar trend of upregulation over time. Analysis of CD31 mRNA levels with a Wilcoxon rank test confirmed that the percentage increase in CD31 mRNA levels in irradiated cultures over controls with time was statistically significant (P=0.024).
The Effect of Irradiation on CD31 mRNA
Alternatively Spliced Variants of the Cytoplasmic Domain in HUVECs

The possible occurrence of alternatively spliced variants of CD31 mRNA for the cytoplasmic domain of the protein was determined by RT-PCR in control and irradiated HUVECs using previously published primers. Total RNA was obtained from HUVECs plated at the higher cell density at 6, 24, 48, 72, and 96 hours after irradiation (5 Gy). After cDNA synthesis, each sample was subjected to PCR analysis. The products from a representative experiment are shown in Figure 8. A single band of approximately 260 bp was clearly visible in lanes corresponding to both control (1 to 5) and irradiated samples (6 to 10) but absent in the lane for the negative control (N) where water was used instead of cDNA. This fragment was not observed when PCR was carried out on genomic DNA extracted from whole blood or the native RNA samples (data not shown). Southern blot analysis was also carried out on the products of the PCR reactions in which their reactivity to the probe used for Northern blotting was determined. A single band of hybridization was detected corresponding to the 260-bp DNA fragment previously described in all HUVEC samples (data not shown). A product of 200 bp corresponding to the alternatively spliced cytoplasmic domain variant previously described was not observed on either ethidium bromide or Southern blots.

The Effect of Anti-CD31 Mab on the Transendothelial Migration of Leukocytes Through HUVEC Monolayers

These experiments were undertaken to investigate the functional role of CD31 in leukocyte transmigration through irradiated endothelium. HUVEC monolayers on collagen gels were irradiated with 5 Gy, incubated for 72 hours and then leukocyte transmigration assays performed on them. This dose and time period were chosen as our previous experi-

Figure 7. Northern blot analysis for CD31 mRNA in HUVECs. A, Autoradiographs obtained when total RNA extracted from control and irradiated HUVECs at 6, 24, 48, 72, and 96 hours after irradiation was separated by gel electrophoresis, capillary-blotted onto membrane, hybridized with a CD31 probe and then re-probed for GAPDH. The intensity of the CD31 bands in extracts from irradiated HUVECs shows an increase with time compared with controls. B, CD31 bands were quantified, values normalized using GAPDH and expressed as a percentage of controls. Values from 3 experiments were pooled and over the time course increased significantly by an average of 70% (P=0.024; Wilcoxon rank test). The data for percentage increase in surface CD31 protein levels on irradiated HUVECs (presented originally in Figure 3) is shown in the same format for comparison. Both follow similar trends of upregulation.

Figure 8. Analysis of cytoplasmic tail mRNA for the presence of alternatively spliced variants in irradiated HUVECs using RT-PCR. Total RNA was extracted from control (lanes 1 to 5) and irradiated (lanes 6 to 10) HUVECs after 6, 24, 48, 72, and 96 hours incubation, subjected to RT-PCR analysis using primers designed to amplify between exons 10 and 16 of the cytoplasmic domain of CD31 and the resulting products separated on an ethidium-stained agarose gel by electrophoresis. A product approximating 260 bp was observed in all samples (lanes 1 to 10) except in the negative control (lane N) as expected. A previously reported 200 bp product was not observed. The other bands shown correspond to the primers. Southern blot analysis of PCR products using a CD31 probe was also undertaken. A single band corresponding to the product observed on ethidium-stained gels was found in all samples but no additional bands were observed below this band (data not shown).
ments showed 5 Gy induced a marked upregulation of CD31 expression on HUVECs 72 hours after irradiation. The number of peripheral blood leukocytes that transmigrated through irradiated HUVEC monolayers into the underlying collagen gel was increased by 3- to 4-fold over controls ($P<0.007$; Figure 9). Incorporation of anti-CD31 Mab into the assay system markedly attenuated transmigration ($P<0.008$).

Immunostaining of Tissues With Mab to CD31
Mab to CD31 stained endothelial cells of blood vessels in all 21 irradiated skin tissues. However, intensity of staining varied considerably between tissues and within various parts of a section. Although blood vessels in normal tissues stained well, the intensity of staining was generally not as strong as in irradiated ones (Figure 10). Furthermore, in irradiated tissues, unlike normal controls, there was a marked accumulation of leukocytes and platelets, both of which were positive for CD31. When the tissue sections were semi-quantitatively ranked on the basis of intensity of staining exhibited by the endothelial cells in blood vessels, the sections from irradiated tissues received a significantly greater mean score than controls ($P<0.001$; Figure 11).

Discussion
U-937 cell adherence to irradiated HUVECs was significantly elevated over corresponding nonirradiated cells. Previously radiation-induced leukocyte adherence to endothelial cells has been attributed to expression of E-selectin and ICAM-1. Our flow cytometric analysis showed that irradiated HUVECs exhibited significantly increased expression of CD31 compared with controls. Furthermore, Northern blotting confirmed that significantly raised amounts of CD31 mRNA transcripts were detectable in irradiated HUVECs. Thus, the observed upregulation in the cell membrane levels of CD31 on HUVECs could be part of a general cellular response induced by irradiation. A number of other parameters were studied to examine their possible effect on the expression of CD31 in HUVECs. In this respect cell number, cell size and cell cycle stage are some of the important variables that might influence the results.

The alterations observed in cell numbers were expected as irradiation diminishes the clonogenic capacity of endothelial cells in vitro and causes their depletion in vivo from the vasculature. Differences in the level of cell proliferation demonstrated by control cultures at the different seeding densities presumably reflected differences in contact inhibition. The reduced rate of cell depletion at the lower plating density could be due to increased proliferation at this level of confluence. From Figures 3 and 4 it seemed that CD31 expression may be inversely linked to alterations in cell number because at both plating densities the percentage increase in CD31 expression was similar and the percentage decrease in cell numbers was similar. However, analysis of the data using linear regression confirmed that the increased
CD31 expression on irradiated HUVECs was not related to changes in cell numbers.

There was a possibility that irradiation could have affected HUVECs in such a way as to cause a selective accumulation of cells in 1 phase of the cell cycle in which their expression of CD31 was much higher. The results of the cell cycle analysis discount this suggestion because there was no marked alteration in the proportion of cells in a particular phase that would account for the change in CD31 expression. One of the morphologic changes that endothelial cells are reported to undergo in vivo, after irradiation, is swelling. It is conceivable that the upregulated levels of CD31 on irradiated HUVECs may have been due to an increase in cell surface area. However, the data from the flow cytometric analysis of forward scatter, which is proportional to cell size, showed that the increase in CD31 far exceeded any alterations in size. Our findings that irradiation induces increased CD31 expression with time, independent of initial cell plating density, changes in cell density or cell size, or changes in the proportion of cells in various phases of the cell cycle, led us to conclude that the upregulation of CD31 on HUVECs is a direct response to irradiation.

The observed rise in cell surface CD31 in HUVECs was accompanied by an increase in CD31 mRNA, suggesting that irradiation induced transcription of the CD31 gene and/or stabilization of CD31 mRNA. The fact that both CD31 mRNA and surface protein appeared to follow a similar trend of upregulation suggests that the increase in CD31 mRNA in irradiated HUVECs is responsible for the elevation in CD31 expression at the cell surface. Activation of the transcription factor NF-κB has been shown to occur in endothelial cells after exposure to ionizing radiation and a putative role for NF-κB in the radiation-enhanced expression of ICAM-1 on endothelial cells has been implied. Recent characterization of the promoter region of the CD31 gene has revealed the presence of potential NF-κB binding sites among those for other transcription factors. Whether NF-κB has a role in the mechanism of radiation-induced CD31 upregulation in endothelial cells remains to be elucidated.

CD31 transcripts may undergo alternative splicing of the portion that encodes the cytoplasmic domain. Therefore, it is possible that the upregulation of CD31 in irradiated HUVECs might have involved the induction of 1 or more of these variants. Previous characterization of the gene for human CD31 has shown that the cytoplasmic domain is encoded by exons 10 to 16. With the use of RT-PCR, human and murine spliced variants that lack exon 14 have been identified. Truncated versions of CD31 lacking the portion encoded by this exon are unable to mediate the aggregation of CD31 transfectants with mock transfected cells via a ligand-binding mechanism now thought to involve the activation of secondary non–CD31 adhesion receptors through CD31-mediated signal transduction. Hence, the induction of CD31 transcripts lacking cytoplasmic domain-encoding exons (such as exon 14) after radiation may lead to the expression of CD31 that has an altered capacity to mediate signal transduction, having important functional consequences. This possibility was examined using the RT-PCR technique with primer sequences previously published by Kirschbaum et al. A product of approximately 260 bp that bound cDNA probe for CD31, corresponding to the cytoplasmic domain without alternative splicing, was amplified from all samples. However, under the conditions used, we did not observe the exon 14–deficient variant (or any others) on either ethidium bromide-containing agarose gels or Southern blots. Whether this reflects our PCR conditions or suggests that irradiation does not trigger the production of alternative spliced cytoplasmic domain variants in HUVECs remains to be determined.

CD31 is implicated in the mechanisms mediating leukocyte transmigration. Anti-CD31 Mabs inhibit leukocyte transmigration through the endothelium in vivo and in vitro. Evidence from transendothelial migration assays suggests that it is a homophilic interaction between endothelial CD31 and leukocyte CD31, which is important for transmigration. We therefore investigated the functional role of CD31 in the transmigration of leukocytes through irradiated endothelium using an in vitro model. The fact that transmigration of peripheral blood leukocytes was upregulated when HUVEC monolayers were exposed to radiation implies that in vivo irradiation of endothelial cells activates mechanisms that mediate transmigration in addition to leukocyte adherence. Its inhibition after incorporation of anti-CD31 Mab suggests that radiation-induced leukocyte transmigration is CD31-dependent.

The irradiation dose given to HUVECs was comparable with regimes therapeutically given to patients with cancer. To validate in vitro findings of radiation-induced CD31 expression on endothelial cells, we semi-quantitatively examined CD31 expression in skin specimens taken from patients who had received radiotherapy. Our results showed that CD31 expression was raised on the endothelial cells of blood vessels in irradiated tissues compared with controls, providing additional support for our in vitro findings.

The upregulation of CD31 on irradiated endothelial cells in vivo may have important pathological consequences. Our results showing endothelial cell irradiation upregulates leukocyte transmigration in a CD31-dependent manner imply a role for radiation-induced CD31 expression in the mechanisms controlling radiation-mediated leukocyte transmigration in vivo. Endothelial CD31 can also regulate transmigration, a property likely to be compromised when endothelial cells are irradiated and may therefore promote leukocyte emigration. Recently, affinity modulation of CD31 has been
reported and if irradiation augments CD31 binding through this mechanism, it is possible that transmigration could be protracted, exposing the vasculature as well as tissues to any mediators capable of cell damage released by leukocytes, further exacerbating radiation-induced vascular injury.

The effect of irradiation on CD31 could also have consequences for endothelial cell–cell interactions. Endothelial cells express the heterotypic ligand (α,β3) for CD31 and Buckley et al speculate that CD31 homophilic and heterophilic adhesion could be involved in a signaling mechanism that regulates endothelial cell proliferation and differentiation. Thus, if radiation-induced CD31 upregulation disrupts this balance, it may hinder the ability of the vasculature to recover from radiation damage and potentially cause abnormal cell proliferation. Occlusive protrusions into the vascular lumen have been observed in irradiated blood vessels, apparently after endothelial cell proliferation. Whether the increased expression of CD31 we have noted in irradiated tissues had any causal relationship with other molecules such as α,β3 and von Willebrand factor, which are upregulated in endothelial cells as a result of angiogenesis or vascular injury, remains to be established. It has been suggested that the progressive loss of endothelial cells from irradiated blood vessels may be responsible for the development of platelet aggregates through binding von Willebrand factor deposited on the extracellular matrix. With CD31 being implicated in platelet adherence to the vascular wall, it is plausible that raised levels after radiation may contribute to the induction of platelet aggregation to the irradiated vasculature.

In conclusion, the marked effects of irradiation on the upregulation of endothelial cell CD31 is a novel finding and has therapeutic implications in ameliorating the effects of irradiation-induced normal tissue damage.

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References


Irradiation Induces Upregulation of CD31 in Human Endothelial Cells
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