CONCISE REPORT

Irradiation Induces Release of von Willebrand Protein From Endothelial Cells in Culture

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Human umbilical vein endothelial cells in tissue culture were irradiated with doses between 0 and 40 Gy, and the released von Willebrand (vW) protein and that which remained associated with the cells was quantitated. Doses of 20 Gy and higher produced a statistically significant increase in amount of vW protein secreted. This release was present whether the cells were labeled continuously throughout the experiment or just prelabeled before irradiation. An increase in fibronectin secretion was not observed. The release response to radiation was slow, reaching significance close to 24 hours after irradiation. The release of vW protein was not due to cell lysis, because the secreted vW protein contained very little of the large 260-kilodalton vW precursor subunit present in cell lysates and the cells appeared intact by immunofluorescence staining.

WHEN A MALIGNANCY is treated with radiation, the normal tissues surrounding it are also irradiated and may be injured. In the lung, capillary endothelial cells and alveolar epithelial cells appear to be the most susceptible to radiation injury.[1] Within a few days of radiation injury, a variety of small vessel lesions occur, including increased capillary permeability,[2] vacuolization of the endothelium, and segmental separation of the endothelium from the basement membrane.[3] Following such endothelial cell injury, platelet thrombi may obstruct the capillary lumen.[3]

There is an increase in circulating von Willebrand (vW) protein in patients with acute lung injury,[4] and the appearance and action of vW protein in irradiated organs could be of pathogenic importance in thrombotic complications. von Willebrand protein is a large adhesive glycoprotein that mediates platelet attachment to subendothelium following vascular injury,[5] and this property is central to the processes of both physiologic hemostatic plug formation and pathologic thrombus formation. Von Willebrand protein is synthesized by megakaryocytes[6] and endothelial cells[7] and can bind to extracellular matrices of different cell origins.[8] In the endothelial cells, it is concentrated in specific organelles called Weibel-Palade bodies, which serve as storage and/or processing vesicles for vW protein.[9] It is possible that microthrombi observed after radiation therapy could be caused by release of vW protein from the Weibel-Palade bodies. We have therefore studied the release of vW protein from irradiated human umbilical vein endothelial cells in culture and have found that there is indeed a significant increase in secretion of this protein in response to irradiation.

MATERIALS AND METHODS

Cells and Cell Culture Conditions

Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously.[9,10] Cells were cultured in McCoy's 5A medium (Flow Laboratories, McLean, Va), containing 20% fetal bovine serum. For metabolic labeling, cells were grown in the presence of L-[^{35}S]methionine (25 μCi/mL, 12.3 Ci/mmol, Amersham Corp, Arlington Heights, Ill).

Antisera

The preparation and characterization of antisera against human vW protein used for immunofluorescence were described previously.[9,11] For immunoprecipitation, antiserum was purchased from Calbiochem-Behring Corp (San Diego).

Electrophoresis Gels

SDS-polyacrylamide gels were prepared as described by Laemmli.[12]

Irradiation of Endothelial Cells

Endothelial cell cultures were irradiated using a Shepherd model 81-14 Beam Irradiator, 663 KeV, 6,000 Ci, cesium^{137} source (J.L. Shepherd and Associates, Glendale, Calif) at a dose rate of 5.77 Gy/min. All cell cultures, including control cultures, were transported to the source and remained out of the CO_2 incubator for no longer than 30 minutes.

Purification of vW Protein and Fibronectin

Cells were lysed as described previously[13] so that the final concentration of ingredients was that of the radioimmunoprecipitation assay buffer[14] used for washing the immunoprecipitate. The cell lysate or culture medium samples were then incubated for 1.5 hours at room temperature with gelatin-Sepharose to purify fibronectin, which was eluted from the gelatin Sepharose with 6 mol/L urea in phosphate-buffered saline. Protein A-Sepharose CL4B (30 mg/25

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cm^2 flask), Pharmacia Fine Chemicals (Piscataway, NJ), was preincubated at room temperature for 30 minutes with 100 μL anti-vW protein antiserum before it was added to samples from which gelatin-Sepharose had been removed by centrifugation. The incubation was for 1.5 hours at room temperature. After extensive washing, protein A-Sepharose was boiled in electrophoresis sample buffer,[12] counts in the supernatant vW protein were determined, and the samples were analyzed by gel electrophoresis.

Immunofluorescence

Fluorescence microscopy of endothelial cells grown on glass cover slips was carried out as previously described.[9]

RESULTS

To determine the effect of radiation on the release of intracellularly stored vW protein the following experiment was performed. Human umbilical vein endothelial cells were labeled with [^{35}S]methionine for three days, during which time they achieved confluency. Culture medium was then exchanged for unlabeled medium and cells were irradiated. Two days later, vW protein and fibronectin were purified from the culture medium and from cell lysates. To account for any variation in cell numbers, the ratio of protein secreted to protein still associated with the cells was determined for each radiation dose. These values were then normalized by dividing them by the ratios obtained from control, nonirradiated cultures, which averaged 1.0 for vW protein and 2.6 for fibronectin. The normalized results from eight independent experiments are presented in Figure 1. We have observed an approximately 70% increase in the ratio ("medium/cell") of secreted vW protein to cell-associated vW protein with radiation doses of 20 Gy or above. There was a large increase in release of vW protein between 10 and 20 Gy, and the effect appeared to plateau above 20 Gy. No such increase was observed for fibronectin. There was a large variation in response between individual experiments probably because of the use of primary cultures from different donors rather than cell lines. Also, the intensity of the reaction to irradiation varies with the physiologic condition of the cells, which could have accounted for some of the variability.[15] We have examined the data by a two-factor between groups, fixed effect analysis of variance with several planned comparisons. The radiation-induced release of vW protein was significantly different from the null effect line (Fig 1), $P < .0001$, while the fibronectin release was no different ($P = .83$). For more detailed statistical analysis, see the figure legend. Thus, while irradiation caused the release of preformed vW protein from the endothelial cells, it did not cause the release of fibronectin, a glycoprotein that is synthesized but not stored by these cells.

A similar increase in the ratio of secreted vW protein to cell-associated vW protein was observed when metabolic labeling was continuous throughout the experiment (Fig 2). Again, there was no increase in fibronectin secretion (not shown). The irradiation effect on the endothelial cells that caused release of vW protein was very slow; no significant release was observed immediately after irradiation or six hours

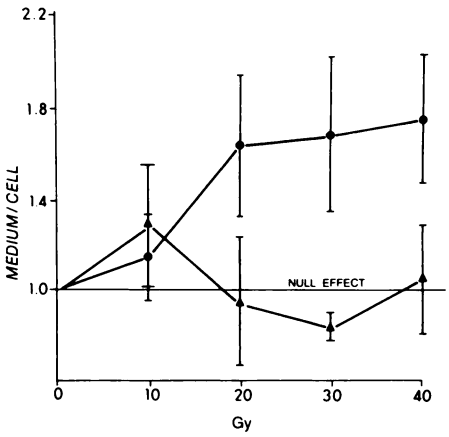

Fig 1. Release of preformed vW protein and fibronectin in response to irradiation. Metabolically labeled endothelial cells were placed in unlabeled medium and irradiated with 0, 10, 20, 30, or 40 Gy. Forty-eight hours later the normalized ratio, (culture medium/cell-associated), of purified vW protein (●) and fibronectin (▲) was determined. Points show the mean and standard deviation of the normalized ratio at each dose (n = 8 for vW protein, n = 4 for fibronectin). The effect of irradiation on release of vW protein between 20 and 40 Gy was not statistically different ($P = .71$), the curve appearing to plateau between these doses. The release of vW protein in response to 10 Gy was significantly different from all higher radiation doses ($P = .04$) and was not significantly different from nonirradiated controls ($P = .5$). On the other hand, the release of vW protein in response to 20 Gy was highly significantly different from controls ($P = .006$). Therefore, it appears that the radiation release effect started to occur somewhere between 10 and 20 Gy. There was no significant release of fibronectin upon irradiation.

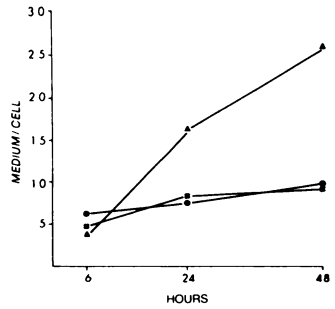

Fig 2. Time course of release of vW protein in response to irradiation. The data are from a typical experiment in which endothelial cells were metabolically labeled for 48 hours and then irradiated in fresh medium containing [^{35}S]methionine using the following radiation doses: 0 Gy (●), 10 Gy (X), and 40 Gy (▲). Von Willebrand protein was purified and the ratio of medium/cell-associated counts per minute was determined for every time point and plotted without normalization. The secretion of vW protein from 0 and 10 Gy-irradiated cultures was very similar; the release observed with 40 Gy was unchanged at six hours but was significantly higher at 24 and 48 hours after irradiation.

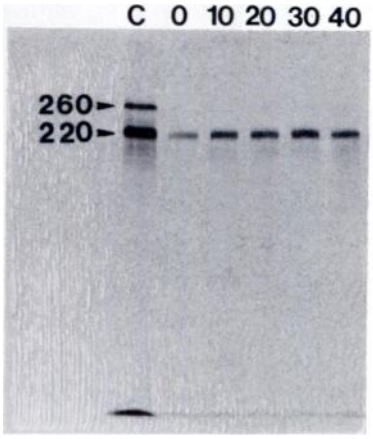

Fig 3. Analysis of vW protein subunits released from endothelial cells by radiation doses between 0 and 40 Gy. Von Willebrand protein was purified from control cells lysed just before irradiation. (C) and from media samples collected 48 hours after irradiation with 0, 10, 20, 30, and 40 Gy. Note the dominant 260 kDa precursor band (260) present in the cell lysate (C) in addition to the processed 220 kDa vW protein subunit (220), while in all the media samples, the only dominant band is the 220 kDa subunit.

later. There was noticeable release 24 and 48 hours after irradiation in comparison to nonirradiated controls.

The irradiation-dependent secretion of vW protein was not due to cell death or lysis. First, the released vW protein purified from culture medium contained very little of the large vW precursor[13,16] as analyzed by polyacrylamide gel electrophoresis (Fig 3), while cells that were lysed at the time of irradiation showed a prominent precursor band (Fig 3). The intracellular cleavage of precursor is very slow;[13] there was still some precursor present in the cells even after the 48 hours of chase (not shown). The polymeric composition of released vW protein also did not change with increasing radiation dose (not shown). These results indicate that irradiation did not cause an excessive amount of unprocessed, intracellular vW protein to appear as a result of cell lysis. Second, apparently normal cells containing Weibel-Palade bodies were observed at 15 minutes (not shown) and at 48 hours after irradiation by fluorescence microscopy (Fig 4).

Release of vW protein as a result of irradiation did not deplete the cells of Weibel-Palade bodies, and the effect on their number, if any, was limited (Fig 4). This indicates either that only few Weibel-Palade bodies released their contents upon irradiation or that Weibel-Palade bodies continued to be replenished by new synthesis.

DISCUSSION

The injury of human endothelial cells in culture by irradiation produced a slow release of vW protein (Figs 1 and 2). The large increase in effect between 10 and 20 Gy suggests that a threshold radiation dose exists, below which release was not detected, but above which the magnitude was independent of the radiation dose (Fig 1). A similar dose-release effect was observed for pulmonary surfactant from lungs of animals irradiated in vivo[17] and from isolated pulmonary type II cells following irradiation.[18]

The slow secretion of vW protein in response to irradiation (Fig 2) was probably due to release from a preformed cellular pool of the protein rather than to an increase in biosynthesis. This was demonstrated by the fact that an irradiation-stimulated increase in secretion of radiolabeled vW protein was observed even if the cells were transferred to unlabeled medium after irradiation (Fig 1), and therefore only the effect on preformed vW protein was observed. Furthermore, fibronectin, which is not stored in endothelial cells, did not show an increase in secretion even when the cells were incubated in labeled medium after irradiation (not shown), arguing against a general increase in biosynthesis as a result of irradiation.

Recently, two pathways for secretion of vW protein have been identified in human umbilical vein endothelial cells.[19] One pathway is directly linked to biosynthesis and can be blocked by cycloheximide. The other is from a storage compartment, most probably the Weibel-Palade bodies, and is inducible by the Ca^{++} ionophore A23187. One of the irradiation effects on cellular membranes is an increased membrane permeability to Ca^{++}.[15,20] It therefore appears likely that irradiation

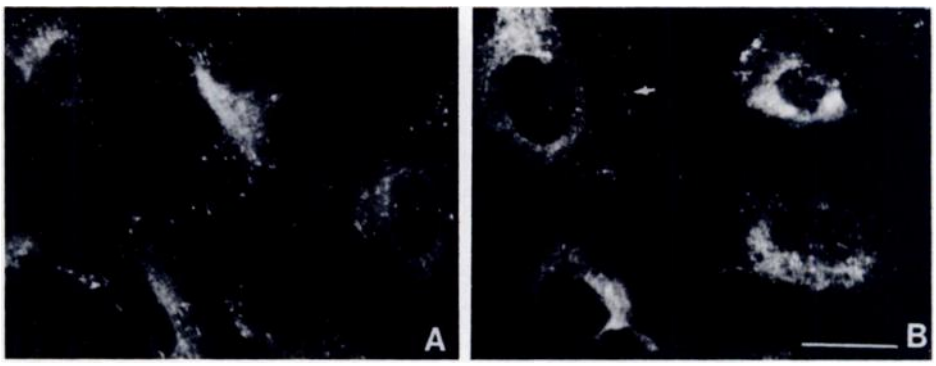

Fig 4. Immunofluorescent staining of permeabilized endothelial cells showing the distribution of Weibel-Palade bodies. (a) Control cells from four-day-old culture receiving no irradiation. (b) Cells irradiated with 30 Gy on day 2 and fixed 48 hours later. The arrow points to a Weibel-Palade body. All cells are well spread, and there is no apparent difference in Weibel-Palade body number and distribution between the control and irradiated cells. Bar = 20 μm.

can change the intracellular Ca^{++} concentration and thereby cause Weibel-Palade bodies to release their contents. We did not observe a massive depletion of Weibel-Palade bodies (Fig 4) as described with the use of the ionophore,[19] but rather a slow release (Fig 2), perhaps allowing for the cell to replenish its vW protein storage compartment by de novo synthesis.

The release of vW protein after irradiation could be of clinical significance, since there is considerable evidence of vascular alteration and even thrombosis in the microcirculation following radiation therapy. These effects are possibly caused in part by an increased concentration of vW protein released by the damaged endothelial cells, thereby contributing to acute and even late effects of radiation therapy.

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