Effects of X-Irradiation on Artificial Blood Vessel Wall Degradation by Invasive Tumor Cells

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ABSTRACT

Artificial vessel wall cultures, constructed by growing arterial endothelial cells on preformed layers of rat smooth muscle cells, were used to evaluate the effects of X-irradiation on tumor cell-induced tissue degradation. Bovine endothelial cells had radiation sensitivities similar to those of rat smooth muscle cells. Preirradiation of smooth muscle cells, before the addition of human fibrosarcoma (HT 1080) cells, did not increase the rate of degradation by the invasive cells. However, the degradation rate was decreased if the cultures were irradiated after the addition of HT 1080 cells. The presence of bovine endothelial cells markedly inhibited the destructive abilities of fibrosarcoma cells, but preirradiation of artificial vessel walls substantially decreased their capabilities to resist HT 1080-induced lysis. These findings suggest that the abilities of blood vessels to limit extravasation may be compromised by ionizing radiation.

INTRODUCTION

Malignant tumors are characterized by their abilities to invade normal surrounding tissues and to gain access to blood vessels and lymphatics. These properties ultimately allow them to travel to distant organs and to grow as metastases. The development of overt metastases diminishes the chance of cure in the majority of tumor patients, and, therefore, different therapeutic approaches have been developed to prevent this potentially lethal complication of cancer.

Vascular endothelial cells are intimately involved in the heterogeneous spread of tumors, both at sites where tumor cells enter the circulation from the primary tumor and at sites where tumor cells extravasate to form metastases. Radiation-induced vascular changes were observed as early as 1899 (5), and this harmful side effect is thought to be responsible for the serious radiation damage to normal tissue, which often hampers successful radiotherapy (12). Therefore, many studies using a variety of tissue systems have been performed to assess the radiosensitivities of endothelial cells, and they have been used to develop potential lethal irradiation methods.

Irradiation of tumor-bearing patients might be expected to influence the number and distribution of metastases by interaction with the vasculature. Prophylactic irradiation of the lungs has been administered to patients suffering from Ewing's sarcoma (14) or osteogenic sarcoma (2), since these tumors metastasize primarily to the lungs. The incidence of appearance of pulmonary metastasis was reduced by this treatment, although the results were inferior to those obtained with intensive chemotherapy. In studies with mice, it was found that i.v. administration of fibrosarcoma cells after irradiation of the lungs resulted in an increased incidence of pulmonary metastasis (3, 13, 18). In contrast, removal of the primary sarcoma tumor growing in the tail of mice prior to irradiation of the lungs resulted in a marked decrease of pulmonary metastasis (17). Therefore, the timing of prophylactic irradiation of the lungs during a given treatment regimen may be crucial for beneficial effect.

In the present study, we used an artificial vessel wall prepared in vitro to study the effects of irradiation on its subsequent degradation by human sarcoma cells. Irradiation of these structures prior to the seeding of the tumor cells resulted in an enhanced degradation of the vessel wall, whereas radiation of this artificial tissue after seeding of the tumor cells resulted in a markedly decreased degradation. These preliminary data resemble those found in animal studies and suggest that this in vitro model system may be used to further study the impact of irradiation on the sensitivities of vessel walls to degradation by invasive tumor cells.

MATERIALS AND METHODS

Cell Cultures. Bovine arterial endothelial cells (A1,C1-1) were used between passages 5 and 10 (8), and rat vascular smooth muscle cells (R22.CD.), at passages ranging from 13 to 18 (10). The human fibrosarcoma cell line HT 1080 (15) was used in all degradation studies between passages 40 and 46. All cell cultures were grown in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY), containing 10% fetal bovine serum (Irvine Scientific, Irvine, CA), 2% tryptose phosphate broth (Difco Laboratories, Inc., Detroit, MI), penicillin (100 units/ml), and streptomycin (100 µg/ml). They were maintained in 75-cm² plastic flasks and passaged regularly at subconfluency using trypsin-EDTA (Grand Island Biological Co.).

Production of Labeled Cultures. Smooth muscle cells were seeded into 35-mm dishes at 2 x 10⁶ cells/dish. The cultures were given ascorbic acid (25 µg/ml) on the following day and fresh ascorbic acid daily thereafter. Medium changes were performed twice weekly. L-(3,4-³H)-Proline (New England Nuclear, Boston, MA) was added at a final concentration of 1.0 µCi/ml 5 and 8 days after seeding. Radiolabeled proline was removed 12 days after seeding, and cultures were then used for degradative studies. The cell layer at this time contained visible amounts of extracellular matrix proteins.

Artificial blood vessel walls were constructed as described previously (8). In brief, smooth muscle cultures were prepared and labeled as described above. Endothelial cells (10⁶ cells/35-mm dish) were added to the preestablished smooth muscle cell layer 12 days after seeding of...
smooth muscle cells. Ascorbic acid was added daily, and the medium was changed twice weekly. The cultures were used for degradative studies 7 days following the addition of endothelial cells when the resulting structure had several of the morphological properties of a vessel wall.

Radiation. Radiation was performed on a General Electric Maxitron 250 at an energy of 140 kV and a current of 20 ma with a source-specimen distance of 50 cm. The measured half-value layer of the beam was 3 mm of aluminum, and the machine was calibrated with a Model 192 Capetoe exposure meter with a PR-06C (Farmer) chamber. All radiation is expressed as Gy, a unit of absorbed dose equal to 1 J/kg in any medium. One Gy corresponds to 100 rad. Radiation doses of 0 to 16 Gy were given at a dose rate of 0.76 Gy/min. All doses are reported as given doses and verified using thermoluminescent dose meters applied to dishes and a Victoreen Model 2800 thermoluminescent dose meter reader with a nitrogen flow.

Cell cultures were irradiated at room temperature and in room air immediately following removal from an incubator (37°, 5% CO2). Following radiation, they were replaced in the incubator for 30 min before further manipulations.

Survival Curves. Endothelial cells, smooth muscle cells, and artificial blood vessel walls were grown in 35-mm dishes. Endothelial and smooth muscle cells at both subconfluence (log phase of growth) and confluence and artificial blood vessel walls were radiated using the technique described above. Cultures were washed with calcium- and magnesium-free phosphate-buffered saline (140 mN NaCl, 4 mm KCl, 0.5 mm Na2HPO4, 0.15 mm KH2PO4) following irradiation and treated with 0.25% Viokase (Viobin Corp., Monticello, IL) containing 0.05% bacterial collagenase (Worthington type CLS) at 37° for 30 min. The suspension was then aspirated vigorously through a Pasteur pipet and returned to the incubator for another 30 min, followed by further aspiration. Samples of this single cell suspension (verified microscopically) were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL).

Cells (300/dish) were plated in 60-mm tissue culture dishes which had been coated with purified bovine fibrinogen (1.4 g/sq cm), and preincubated for 4 hr with medium containing 10% fetal bovine serum (11). Cells were grown for 14 days with medium changes performed on Days 6 and 10. After 14 days, the medium was removed, the dishes were washed with 0.9% NaCl solution, and the colonies were fixed with methanol (10 min) and stained with Giemsa stain (60 min). The number of macroscopic colonies/plate (approximately 50 cells/colony) was counted, and colony morphology was examined microscopically. Plating efficiencies at higher radiation doses were determined by plating 1800 cells/dish (4 to 6 Gy) or 3600 cells/dish (8 to 12 Gy) to compensate for increased kill.

Degradation of Radiolabeled Tissue Cultures. Labeled smooth muscle and artificial blood vessel wall cultures were prepared as described above and used to quantitate degradation by HT 1080 cells. HT 1080 cells (2 × 105/35-mm dish) were plated on the cultures, and ascorbic acid was added on a daily basis. Cell cultures were radiated either just prior to, or 24 hr after, the seeding of HT 1080 tumor cells, and the medium was changed every 48 to 72 hr thereafter. Samples (100 µl) of the supernatant medium were taken before medium changes, and the released radioactivity was determined following the addition of 5 ml of Biofluor (New England Nuclear). At the same time, samples were also taken from tissue cultures which did not contain tumor cells added. The results given in the text refer to the cumulative totals of solubilized radioactivity. Each experiment was repeated at least 3 times.

RESULTS

Radiation Effects on Smooth Muscle and Endothelial Cells. The artificial vessel wall is a complex structure of endothelial and smooth muscle cells and interstitial matrix, and the effects of radiation on individual endothelial and smooth muscle cell cultures were therefore investigated first. Confluent endothelial cells were radiated using 0 to 12 Gy (Chart 1). The colony-forming efficiency of control cultures of endothelial cells which received no radiation was approximately 30%, and the first effect of radiation was noted at 1.0 Gy, when the survival decreased to 68% of the control (Chart 1). Cell survival continued to decrease with increasing radiation doses and was 45% of control at 2 Gy and approximately 1% at 8 Gy. The D0 was calculated to be 1.4 Gy. Radiation of nonconfluent endothelial cell cultures resulted in similar decreases in survival (results not shown).

The colony-forming efficiency of smooth muscle cells which did not receive radiation was 34%, and radiation with 1 Gy decreased survival to 80% of control (Chart 1). The survival was 25% of control at 8 Gy, and the D0 was 1.6 Gy. Radiation of confluent smooth muscle cells resulted in a 4 to 15% higher survival when compared to nonconfluent cultures (results not shown). When the survival curves for confluent cell cultures were compared (Chart 1), endothelial and smooth muscle cells had similar sensitivities to radiation.

Radiation Effects on Artificial Vessel Wall Destruction. Earlier studies have shown that smooth muscle cultures were easily invaded and destroyed by HT 1080 cells (6). However, the presence of endothelial cells on artificial vessel walls inhibited the destruction of the underlying smooth muscle by HT1080 cells (6, 9). We therefore determined whether radiation of artificial blood vessels and their constituent cell types would decrease...
Effects of X-irradiation on Tissue Degradation

The effects of different radiation doses on the kinetics of hydrolysis of smooth muscle cell multilayers by HT 1080 cells are shown in Chart 2. Radiolabeled smooth muscle cultures irradiated with 0 to 16 Gy did not show any noticeable morphological changes under the phase-contrast microscope. Thus, although the cells were reproductively dead (Chart 1), they remained attached to the culture dishes after irradiation. When 2 x 10^5 HT 1080 cells were added to the culture after radiation, the fibrosarcoma cells completely degraded smooth muscle multilayers after 18 to 21 days of incubation. The rate and extent of hydrolysis of the smooth muscle cell multilayers by HT 1080 cells were not affected by preirradiation of the smooth muscle substrate. Results are shown for cultures which received 0, 4, and 8 Gy. Similar degradation kinetics of hydrolysis was observed with cultures receiving 1, 2, 12, and 16 Gy (data not shown).

The kinetics of hydrolysis of smooth muscle multilayers was affected if the cultures were radiated 24 hr after the addition of tumor cells (Chart 3). The fibrosarcoma cells were capable of the complete degradation of unirradiated smooth muscle cultures after 20 days of incubation. Cultures which received 4 and 8 Gy had a 22 and 65% decrease in hydrolysis rates, respectively. Experiments were also performed using 1 and 2 Gy with intermediate results (data not shown).

Subsequent experiments were carried out using an artificial vessel wall. Its degradation by HT 1080 cells has been studied extensively, and it has been found that endothelial cells markedly decreased the degradative activity of human fibrosarcoma cells (6).

The effect of radiation on the rate of hydrolysis of artificial vessel walls by HT 1080 cells is shown in Chart 4. Artificial vessel wall cultures were radiated with 0 to 16 Gy, and HT 1080 cells were added immediately after. The fibrosarcoma cells did not significantly degrade unirradiated artificial vessel walls, thus confirming previous studies (6). However, the resistance of the wall to destruction was decreased by preirradiation. This effect was first noted at 8 Gy and increased progressively with 12 and 16 Gy of radiation.

The role of endothelial and smooth muscle cells in the resistance of the artificial vessel wall to tumor cell degradation was investigated in the experiment shown in Chart 5. Confluent endothelial and smooth muscle cell cultures received either 0 or 8 Gy of radiation as individual cultures. The endothelial cells were then trypsinized and plated onto the smooth muscle cultures to form artificial vessel walls. The endothelial cells formed a confluent monolayer on the smooth muscle cell multilayer, but the process took 3 to 7 days longer than usual, and giant cells were noted microscopically. HT 1080 cells were seeded onto these cultures when the endothelial cells had reached confluency, and the hydrolysis of the artificial vessel was quantitated. No significant degradation of the artificial vessel wall by HT 1080 cells occurred in cultures in which neither endothelial nor smooth muscle cells had been irradiated. However, complete hydrolysis of the cultures was induced by HT 1080 cells when both cell types had been radiated. Radiation of either the endothelial or smooth muscle cells independently resulted in an intermediate level of hydrolysis of the artificial vessel wall by HT 1080 cells. Thus, radiation of both cell types was necessary to allow complete degradation of the artificial vessel wall by HT 1080 cells.
DISCUSSION

Several in vivo and in vitro studies have assessed the radiosensitivities of blood vessels and their constituent cells (for review, see Ref. 12). Different end points including morphological changes, functional changes, or reproductive cell survivals have been used so that wide variations in radiation sensitivities of vascular cells have been obtained. In the present in vitro study, we have investigated the effect of irradiation on the survival of vascular endothelial and smooth muscle cells and the influence of radiation on the degradation of an artificial vessel wall by human sarcoma cells.

The endothelial and smooth cells were found to have intermediate sensitivities to irradiation with D0s of 1.4 and 1.6 Gy, respectively. Similar results were reported by De Gowin et al. (4), who obtained a D0 of 1.6 Gy for human umbilical endothelial cells. Our results also compare favorably with the data of Reinhold and Buisman, who found a D0 of 1.7 Gy (16) for the rat capillary endothelium. These findings are in contrast to a recent study investigating the postirradiation proliferation of endothelial cells of the mouse mesentery (7). A D0 of more than 5.0 Gy was obtained, and a review of additional in vivo endothelial cell survival data reveals radiosensitivities with D0 values ranging from 2.4 to 10.3 Gy (12). These widely differing in vivo results do not allow more detailed comparison with our data.

The HT 1080 sarcoma cells degraded smooth muscle cell multilayers and their extracellular matrix within 3 weeks as described previously (6), and radiation of up to 16 Gy delivered to the smooth muscle cell cultures prior to seeding the tumor cells did not significantly influence their subsequent degradation. In contrast, radiation of these cultures after seeding of the tumor cells resulted in a significant decrease in the rate of matrix degradation. This effect was most likely due to the effect of radiation-induced tumor cell death and clearly indicated that the outcome of radiation treatment with respect to tumor cell on induced tissue destruction was markedly dependent on dose scheduling.

Artificial vessel walls consisting of endothelial cells grown on top of a multilayer of smooth muscle cells were very resistant to degradation by tumor cells as described earlier (6, 9). We have shown recently that bovine endothelial cells secrete a factor which inhibits the destruction of the smooth muscle cell matrix and also decreases the production and secretion of plasminogen activator by HT 1080 cells (6). Preirradiation of artificial vessel walls resulted in increased susceptibility of these structures to degradation by human sarcoma cells. Thus, the most likely interpretation of the present results is that irradiation of the endothelial cells in the artificial structure lessened their ability to secrete this inhibitory factor. It has been shown recently that irradiation of endothelial cells results in a diminished production of prostacyclin by these cells (1), so that radiation is already known to have significant effects on endothelial cell biochemistry.

Preirradiation of either of the component cell types of the artificial vessel resulted only in a moderate increase in the subsequent degradation rate by the sarcoma cells, whereas exposure of both cell types to irradiation prior to cocultivation resulted in a considerably increased susceptibility to hydrolysis by tumor cells. These results suggest that endothelial cell-smooth muscle cell interactions may be especially important in resisting tumor cell destruction. Further studies are underway to

Chart 5. Influence of radiation of endothelial and smooth muscle cells on the degradation of artificial vessel walls by HT 1080 cells. Artificial vessel walls consisting of endothelial cells grown on top of a multilayer of smooth muscle cells were very resistant to degradation by tumor cells as described earlier (6, 9). We have shown recently that bovine endothelial cells secrete a factor which inhibits the destruction of the smooth muscle cell matrix and also decreases the production and secretion of plasminogen activator by HT 1080 cells (6). Preirradiation of artificial vessel walls resulted in increased susceptibility of these structures to degradation by human sarcoma cells. Thus, the most likely interpretation of the present results is that irradiation of the endothelial cells in the artificial structure lessened their ability to secrete this inhibitory factor. It has been shown recently that irradiation of endothelial cells results in a diminished production of prostacyclin by these cells (1), so that radiation is already known to have significant effects on endothelial cell biochemistry.

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Chart 4. Degradation of radiated artificial vessel walls by HT 1080 cells. Artificial vessel walls labeled with [3H]proline were radiated with 0 (O), 8 (A), 12 (A), and 16 (G) Gy. HT 1080 cells were plated (2 x 10⁶ cells/35-mm dish) onto the artificial vessel wall immediately following radiation. Medium was changed every 2 to 3 days, and the release of radioactivity into the supernatant was determined at the indicated times after seeding.
evaluate the influence of timing of radiation treatment to an artificial vessel wall in regard to its susceptibility to degradation by tumor cells.

Our results obtained with artificial vessel walls are similar to those obtained with in vivo studies in mice, where an increased susceptibility to metastasis to lung or liver irradiated prior to i.v. administration of tumor cells has been observed (3, 13, 18). This radiation-induced effect of increased metastasis was temporary and disappeared after 2 days of recovery (18). The importance of the timing of irradiation of a certain organ to prevent metastatic disease was further illustrated in studies which showed that radiation treatment of an organ predilected for metastatic disease after removal of the primary tumor resulted in a decreased incidence of metastasis in such an organ (17). These in vivo studies also clearly demonstrate the importance of timing of prophylactic irradiation to an organ to prevent the development of secondary tumors. The lack of such studies may be responsible for the partial failures of prophylactic irradiation in recent clinical studies (2, 14).

Our in vitro model system presented in this study may therefore be useful to evaluate further the influence of irradiation on matrix degradation by tumor cells. Although the influence of host defense, coagulation factors, platelets, and blood flow on metastasis cannot be evaluated in this system, its relative simplicity may be advantageous to further understanding cell-cell interactions which seem to affect human cell invasion.

REFERENCES

Degradation by Invasive Tumor Cells

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