Effects of Irradiation on the Release of Growth Factors from Cultured Bovine, Porcine, and Human Endothelial Cells

Larry Witte, Zvi Fuks, Adriana Haimovitz-Friedman, Israel Vlodavsky, DeWitt S. Goodman, and Amiram Eldor

ABSTRACT

The effects of radiation on the release of mitogenic factors into the media of cultured endothelial cells of bovine, porcine, and human origins were studied. Although unirradiated controls revealed a significant background activity, single doses of irradiation (20–60 Gy) resulted in a dose-related increased release of growth factor activity, measured by the mitogenic effects of the conditioned media on both 3T3 mouse fibroblasts and unirradiated endothelial cells serving as target cells. Receptor binding competition assays for the platelet-derivated growth factor receptor revealed that 12–28% of the total mitogenic activity was due to platelet-derivated growth factor-like mitogens. Mitogenic assays using endothelial cells and specific antibody mediated inhibition assays suggested that another component of the mitogenic activity was due to a fibroblast growth factor-like factor. Although radiation resulted in a significant increase in cell death, the enhanced growth factor activities did not appear to result from cell lysis-related leakage of intracellular stores of growth factors. Instead, our data suggest that the growth factors were synthesized de novo and secreted at elevated levels by the cells which maintained postradiation a high level of metabolic activity. Time course studies demonstrated that the growth factors accumulation in the conditioned media started within the first 24 h after radiation and reached a plateau within 72 h after treatment. Radiation-induced release of endothelial cell-derived growth factors may be involved in the pathogenesis of both early vascular damage and the late fibrosis which represents a prominent feature of late radiation damage in normal tissues.

INTRODUCTION

Recent histopathological studies have shown that radiation injury to slowly proliferating normal tissues is characterized in early phases by microvascular damage, while atrophy and fibrosis are prominent features of late radiation damage (1–3). Such changes were described as typical for the skin (4), lung (5), liver (6), and the central nervous system (8). Ultrastructural studies have suggested that capillary endothelial cells are the most sensitive targets for irradiation in the vessel wall. Several cellular changes have been described in the capillary endothelium including swelling, vacuolization, vesicle formation, and segmental cellular sloughing, accompanied by increased capillary permeability (3). The acute changes are followed by formation of platelet thrombi which frequently obstruct the capillary vascular lumen (1–3, 5, 6). Some studies have also described postradiation proliferation of endothelial and smooth muscle cells in small caliber arterioles, followed by general thickening of the vessel wall (1–3). All of these processes eventually lead to hyalinization and obliteration of small blood vessels, decrease of blood flow to the irradiated tissues, anoxia, necrosis, and replacement of normal tissue elements by fibrosis (1–3).

Further details of the cellular and subcellular effects of ionizing radiation on the vascular endothelium have recently been investigated using in vitro cultures of endothelial cells. Several studies demonstrated damage to the endothelial cell clonogenic capacity with D0 values of 101–165 cGy calculated from the radiation dose survival curves (9–15). Radiation was also found to affect some of the endothelial cell functions associated with blood coagulation and thrombolysis. These include an enhanced production and release of von Willebrand protein (16), initial increased release and subsequent decreased capacity to produce prostacyclin (17–20), and a decreased capacity to secrete plasminogen activator (21, 22). Other radiation-induced alterations in endothelial cell function include inhibition of calcium-dependent amino acid transport, elevation of 5’ nucleotidase activity, inhibition of the prostaglandin E2-stimulated cyclic adenosine monophosphate synthesis (23), and production and secretion of a lipoygenase pathway product, which was characterized as a chemoattractant to neutrophils but different in structure from the LTB4 and 13-HODE leukotriens (24).

Whether other endothelial cell functions are also affected by irradiation is unknown. Recent studies have shown that cultured endothelial cells produce and secrete several types of peptide growth factors which are either distinct from or similar to the PDGF (25–28). Enhanced secretion of PDGF-like peptides over basal levels was found when endothelial cells were stimulated by thrombin (29), the coagulation factor Xa (30), phorbol esters and bacterial endotoxin (31), and by TNF and IL-1 (32). In addition, cultured endothelial cells were also shown to have the capacity to synthesize FGF, but it has been claimed that this factor remains mostly intracellular because of the lack of a demonstrated consensus signal peptide sequence in its nuclear genome (33–35). We have previously described some characteristics of the mitogenic activity released from normal cultures of porcine aortic endothelial cells (36). Time-course studies showed that the mitogenic activity was released steadily during the first 24 h and reached a plateau at about 48 h. The mitogenic activity was partially destroyed when heated to 56°C for 30 min, while the remainder was resistant even to heating to 75°C for 15 min or to 100°C for 2 min. Ultrafiltration experiments showed that mitogenic activity was retained by membranes with a M, cut-off of up to 30,000 and had a high affinity to the resin Blue Sepharose. In the present study, we report that ionizing radiation enhances the release of mitogenic factors from cultured endothelial cells. Our data suggest that the mitogens secreted in response to radiation are produced by cells which survive, at least in the short term, the lethal effects of irradiation and remain metabolically active. The initial characterization of the conditioned media growth factors reveals that both PDGF-
like and FGF-like peptides are produced and secreted after irradiation. We also discuss the possible involvement of these factors in the pathogenesis of early and late radiation changes in blood vessels.

**MATERIALS AND METHODS**

Cells. BAEC were established in culture as previously described (37). Stock cultures were grown in DMEM supplemented with 10% bovine calf serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). Partially purified brain derived fibroblast growth factor (FGF, 100 ng/ml) was added to the cultures every other day during the phase of active cell growth and was removed after the endothelial cells reached confluence. HUVEC were a gift from Dr. B. B. Weksler, Cornell University Medical College, NY, and were cultured as reported previously (38). PAEC were established as previously described (26).

Radiation. Confluent endothelial cells were irradiated as described previously (19). Briefly, stock cultures of endothelial cells were dissociated with 0.05% trypsin 0.02% EDTA and seeded on 35-mm dishes (Falcon) at 4 x 10⁵ cells/dish. Seven to eight days after reaching confluence the medium was changed to fresh medium in which serum was substituted by 0.1% human serum albumin. The cells were then irradiated in a cobalt-60 Gammarcell 220 (Atomic Energy of Canada) at a dose rate of 50 cGy/min. The conditioned medium from each dish was collected at various periods of time later after irradiation and frozen for later evaluation of its mitogenic activity. The endothelial cell monolayers were dissociated with trypsin/EDTA for cell counts (performed with a Coulter Counter) and evaluation of cellular viability by the trypan blue exclusion test. Four dishes were used for each data point.

Mitogenic Activity Assay. The mitogenic activity of the conditioned media was evaluated by mitogenic assays using several types of target cells. When the assays were performed with BALB/3T3 (clone A31) mouse fibroblasts (American Type Culture Collection), the cells were grown to near confluency in 96-well microtiter plates (Falcon) and then induced to quiescence by 48-h exposure to DMEM supplemented with 5% human plasma derived serum deficient in serum mitogens prepared as previously described (26, 39). Aliquots (10-40 µl) of endothelial cell-conditioned media samples were added to the 3T3 cell cultures and incubated for 15 h at 37°C in a 10% CO₂ humidified incubator. The resulting mitogenic stimulation was then measured by pulsed with [³H] thymidine (0.25 µCi/well of [methyl-³H]thymidine, 2 Ci/mmol, New England Nuclear) for 3 h of incubation. The activity was determined by either fixation and processing of the cells for autoradiography as previously described (40) and assessing the labeling index as the percentage of cells with labeled nuclei, or by evaluation of the [³H] thymidine incorporated into DNA by liquid scintillation spectrophotometry of TCA extracts of the cells dissolved in 0.5 N NaOH as previously described (41). For each experiment a standard curve using increasing concentrations of calf serum (up to 10%) was generated. One unit of mitogenic activity was defined as the activity conferred by aliquots of the conditioned medium equivalent in magnitude to 50% of the maximal activity observed with calf serum in the concomitant standard curve.

When mitogenic assays were carried out with BAEC or PAEC, 1-2 x 10⁵ cells were plated per well in 24-well plates (Falcon) in culture medium consisting of DMEM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), glutamine (2 mM), and 10% fetal bovine serum (FBS). On Day 1 the medium was changed to fresh medium containing aliquots of the conditioned media to be assayed. The resulting mitogenic stimulation was measured by pulsed with [³H]thymidine (0.25 µCi/well) added on Day 3 for 2 h of incubation, followed by scintillation spectroscopy of TCA extracts of the cells dissolved in 0.5 N NaOH, as described above. In addition, for each experiment a standard curve using increasing concentrations of partially purified FGF was also generated. One unit of mitogenic activity was defined as the cpm observed with aliquots of the conditioned medium equivalent to 50% of the maximal activity produced by FGF in the concomitant standard curve.

PDGF Receptor Binding Competition Assay. Purified human PDGF was labeled with ¹²⁵I by the iodine monochloride method and competition binding studies were carried out essentially as described by Bowen-Pope and Ross (42). In brief, 3T3 mouse fibroblasts were plated at a concentration of 2 x 10⁴ cells per well in 24-well plates (Falcon) in 1 ml of DMEM supplemented with 10% calf serum. After 24 h incubation the medium was changed to DMEM supplemented with 5% plasma derived serum. Two days later the cell cultures were cooled to 4°C on ice, washed with cold binding medium (DMEM supplemented with 1 mg/ml of human serum albumin and 15 mM HEPES, pH 7.4) and incubated in 1 ml of binding medium containing 1 ng ¹²⁵I-PDGF (51,168 dpm/ng) plus conditioned media samples or known amounts of cold PDGF (0.5-32 ng) to generate a standard curve. Nonspecific binding was determined by including 500 ng/ml of cold PDGF in the binding medium. The cells were then plated on a rotating plate at 4°C for 3 h. The cells were washed five times in binding medium and dissolved in 1 ml of solubilization buffer (1% Triton X-100, 10% glycerol, 15 mM HEPES, 0.1 mg/ml bovine serum albumin in H₂O). The samples were counted in an LKB 1274 RIAGAMMA counter.

Antibody-mediated Inhibition of FGF-like Mitogenic Activity. The assay was performed using a neutralizing anti-bFGF rabbit polyclonal antibody (kindly provided by Dr. M. Klagsbrun, Harvard University, Boston, MA). This antibody was shown to block the mitogenic effects of bFGF on endothelial cells and its ability to stimulate neurite outgrowth in PC-12 cells (43). The target cells in the assay were BAEC. The cells (2 x 10⁴/well) were plated in a 24-well plate (Falcon) in supplemented DMEM as described above and incubated for 3 h at 37°C in a 10% CO₂ humidified incubator. Partially purified bovine brain FGF (50 ng/ml) or 200-µl samples of conditioned medium were then added with or without an IgG fraction of the rabbit anti-bFGF antisera (final concentration, 200 µg/ml). The cultures were incubated for an additional 72 h, pulsed with [³H]thymidine and the radioactivity of the TCA-precipitable DNA was determined by scintillation spectroscopy as described above.

Statistical Analysis. Statistical analyses of the data were conducted by standard procedures, using linear regression analysis and Student's t tests.

**RESULTS**

Effects of Radiation on Release of Mitogen Factors from BAEC. Fig. 1A shows the levels of mitogenic activity present in culture media of control unirradiated BAEC, or media collected 48 h after irradiation with single doses of 20, 40, and 60 Gy. The mitogenic activity was quantitated as the percentage of labeled nuclei counted in 3T3 fibroblasts for each concentration of endothelial cell-conditioned medium tested. Cultures irradiated with 20 Gy were nearly threefold more active in producing a 50% labeling index in 3T3 cells than unirradiated control conditioned medium, further increasing with 40 or with 60 Gy. There was a dose-response relationship for the release of mitogenic activity (Fig. 1B) with a plateau reached between 40 and 60 Gy.

Time Course of Release of Mitogen Factors from BAEC following Irradiation. The time course of the effect of radiation on the release of growth factor activity from cultured BAEC is shown in Fig. 2. Both the unirradiated control BAEC and the irradiated cells exhibited a linear time-related accumulation of mitogens in the conditioned media, but the rate of accumulation in the irradiated cultures was approximately twice that observed in the unirradiated controls. Thus, by 72 h the concentration of mitogenic activity in the media of cells irradiated with 20 Gy was 51.4 units/ml compared to 26.9 units/ml for the unirradiated controls. After 72 h unirradiated cells no longer released mitogenic factors but there was evidence of additional slow
EFFECTS OF IRRADIATION ON GROWTH FACTORS

Fig. 1. A, [\textsuperscript{3}H]thymidine incorporation in 3T3 cells following exposure to aliquots of conditioned medium from BAEC, 48 h after irradiation with single doses of 0 (○), 20 (□), 40 (▲), and 60 (●) Gy. Mitogenic activity was determined by autoradiography, as described under "Materials and Methods." ECCM, endothelial cell conditioned medium. The values shown are averages of triplicate determination. B, dose effect of radiation on the release of mitogenic factors from BAEC into the culture medium. The values are calculated from the data shown in A. One unit of mitogenic activity is defined as the ECCM activity equivalent to 50% of the maximal mitogenic activity achieved with calf serum. Bars, one standard error of the mean.

Fig. 2. Time course of the release of mitogenic activity by unirradiated BAEC (○) and by irradiated BAEC (20 Gy) (□). Aliquots of the conditioned media were removed at the times indicated, and the mitogenic activity was determined by liquid scintillation spectroscopy of the [\textsuperscript{3}H]thymidine incorporated into the DNA of 3T3 cells, as described under "Materials and Methods." In this experiment one unit of mitogenic activity of the conditioned media was represented by 17,440 cpm, determined from a concomitant standard curve. The values shown are averages of triplicate determinations.

release of growth factor activity from the irradiated BAEC between 72 and 96 h. The loss of secretion of mitogenic activity after 72 h in the control cultures may, however, reflect the effects of depleted medium and/or the build-up of released cell waste products, since mitogenic activity continued to be released from control cultures after a change to fresh serum-free medium (data not shown).

Effect of Radiation on BAEC Viability and the Correlation with Conditioned Media Mitogenic Activity. In the same experiments shown in Fig. 1, we also tested the effect of irradiation on endothelial cell viability. Despite the high radiation doses used in our study, the cultures did not show immediate evidence of cell death and lysis. During the first several hours after irradiation on the cells remained firmly attached to the culture dish as a monolayer, retained their normal morphology and exhibited exclusion capacity of the trypan blue dye (data not shown). However, within 24–48 h of radiation, increasing numbers of endothelial cells became rounded, detached from the dish, and no longer excluded trypan blue dye. Table 1 shows that irradiation with 20–60 Gy caused a 57–62% decrease in cell viability by 48 h. To differentiate between the possibility that the postradiation enhancement of the mitogenic activity resulted from leakage of intracellular reservoirs of mitogens started in the nonviable detached and lysing cells, versus the possibility of its being released from the surviving and the metabolically active cells which remained attached to the culture dish as a monolayer, we first determined the constitutive levels of intracellular mitogens of intact control BAEC lysed by three cycles of freezing and thawing. The results of this experiment show that approximately 18.3 units of mitogenic activity were found in lysates of 1 × 10\textsuperscript{6} unirradiated BAEC. As shown in Table 1, the amount of mitogenic activity which could be attributed to the cells which detached from the monolayer after irradiation (both lysed and floating nonviable BAEC) was estimated to represent only a small fraction (8–14%) of the total radiation induced increase in the mitogenic activity. These data suggest that the majority of the radiation-induced increased activity resulted from \textit{de novo} synthesis and secretion of the growth factors by cells which survived (at least in the short term) the lethal effects of irradiation and remained metabolically active.

Effect of Radiation on Release of Mitogenic Factors from HUVEC. Fig. 3 shows that HUVEC also released increased amounts of mitogenic factors following irradiation. Similar to BAEC, this increased release was radiation dose related. Fig. 3 also shows that, similar to BAEC, the release of the factors was time dependent after irradiation. At 3 h there was little if any significant release of activity over the basal secretion observed in the unirradiated controls, but highly significant increases occurred with all doses after 24–48 h of incubation. The mor-

Table 1 Effect of radiation on endothelial cell viability and mitogenic activity 48 h after irradiation

<table>
<thead>
<tr>
<th>Radiation dose (Gy)</th>
<th>Viability (cell number/dish × 10\textsuperscript{4}/plate)</th>
<th>Mitogenic activity (units/plate)</th>
<th>Estimated mitogenic activity derived from nonviable cells (units/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.06 ± 0.02</td>
<td>63 ± 7.2</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.46 ± 0.05</td>
<td>139 ± 14.9</td>
<td>10.9</td>
</tr>
<tr>
<td>40</td>
<td>0.41 ± 0.04</td>
<td>192 ± 19.7</td>
<td>11.9</td>
</tr>
<tr>
<td>60</td>
<td>0.40 ± 0.02</td>
<td>208 ± 10.1</td>
<td>12.1</td>
</tr>
</tbody>
</table>

\* The level of [\textsuperscript{3}H]thymidine incorporation which is one unit of mitogenic activity is 19,400 cpm.

Fig. 3. Effect of irradiation on the release of mitogenic activity from HUVEC. Cells were irradiated with single doses of 2.5, 5, 10, and 20 Gy. The conditioned media were removed at different points in time and assayed for mitogenic activity with 3T3 cells. The mitogenic activity was determined by liquid scintillation spectroscopy of the [\textsuperscript{3}H]thymidine incorporated into the DNA of the 3T3 cells, as described under "Materials and Methods." In this experiment, one unit of conditioned media mitogenic activity was represented by 18,564 cpm as determined from a concomitant standard curve. The values shown are averages of triplicate determinations.
Effect of Heat on Mitogenic Activity of Conditioned Media. Similar to BAEC and HUVEC, PAEC also exhibited enhanced mitogenic activity in the conditioned media after irradiation. Fig. 4 shows that the mitogenic factors in the conditioned media from both unirradiated and irradiated PAEC cultures were mostly heat sensitive, although some of the activity in both media was found to be resistance to heat. Heating to 56°C for 30 min destroyed all but 15 and 13.3% of the mitogenic activity in the unirradiated and 50 Gy-irradiated culture media, respectively. The heat-resistant fraction was not destroyed by further heating to 75°C for 15 min or 100°C for 3 min. These data suggest that the conditioned media contained at least two distinct mitogens distinguished by their sensitivity to heat. In experiments not shown, all of the mitogenic activity from both unirradiated and irradiated cultures was lost upon treatment with trypsin, suggesting peptide structures for the mitogenic factors in the conditioned media.

Characterization of PDGF-like Activity in Conditioned Media. To determine whether the heat-resistant component of the mitogenic activity could be attributed to a PDGF-like factor, we tested the capacity of the conditioned media to compete with radiolabeled PDGF for binding to its receptors on 3T3 fibroblast. The conditioned media for this experiment were generated from unirradiated and irradiated PAEC cultures. The data in Table 2 clearly indicate that the conditioned media contained a PDGF-like factor with a high affinity to the PDGF receptor. As expected from the heat sensitivity experiments, the relative fraction of the mitogenic activity attributed to a PDGF-like factor accounted for only a minor percentage (12%) of the total activity for the unirradiated controls, but it increased in proportion after irradiation (Table 2). After a dose of 25 Gy, the PDGF-like activity increased by 2.5-fold while 50 Gy caused over a 6-fold increase in PDGF-like activity released, representing 28.1% of the total mitogenic activity. Similar to the total increase in activity, the radiation-induced increase of the relative fraction of the PDGF-like factor over the non-PDGF-like mitogens was also dose related.

Characterization of the FGF-like Activity in Conditioned Media. To characterize the non-PDGF-like factor, the mitogenic effect of the conditioned media was tested with PAEC serving as target cells. In studies not shown we found that PAEC do not bind 125I-PDGF. Similarly, it has also been shown by others that endothelial cells do not have cell surface receptors for PDGF (42, 44) but do have receptors for FGF (45, 46) and respond to it with mitogenic responses (37). Fig. 5 shows that aliquots of conditioned media from both control unirradiated and irradiated cultures did confer mitogenic responses in PAEC and that the mitogenic activity after irradiation increased over the unirradiated basal levels in a dose-response relationship. The data from Fig. 5 is also shown in Table 2 under non-PDGF-like activity. As indicated approximately 78% of the total mitogenic activity in unirradiated PAEC-conditioned media was due to the non-PDGF-like mitogen. This activity also increased following irradiation with an increase of 1.9-fold after a dose of 25 Gy and of 2.3-fold after a dose of 50 Gy.

In an attempt to identify FGF as a component of the non-PDGF activity in a more specific manner, antibody-mediated inhibition tests of the conditioned media mitogenic activity were performed using a rabbit polyclonal antibody against bFGF. Fig. 6 shows that 200 μg/ml of the anti-bFGF rabbit IgG inhibited 80% of the mitogenic effect of 50 ng FGF on BAEC. No inhibition was found when preimmune rabbit IgG was used instead of the anti-bFGF antibody (data not shown). When conditioned media of irradiated cultures were used instead of FGF, 58% and 70% antibody mediated inhibitions were observed with conditioned media from cultures receiving 150 cGy and 5,000 cGy, respectively. These data strongly indicate the existence of FGF-like mitogens in the conditioned media.

Table 2 Effect of radiation on release of PDGF-like and non-PDGF-like mitogens from endothelial cells

<table>
<thead>
<tr>
<th>Gy</th>
<th>PDGF-likea</th>
<th>Non-PDGF-likeb</th>
<th>Totalc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.96 ± 0.12</td>
<td>6.30 ± 0.96</td>
<td>8.00 ± 1.16</td>
</tr>
<tr>
<td>2.5</td>
<td>0.80 ± 0.08</td>
<td>7.70 ± 1.03</td>
<td>10.60 ± 0.79</td>
</tr>
<tr>
<td>25.0</td>
<td>2.43 ± 0.41</td>
<td>12.10 ± 1.55</td>
<td>17.90 ± 2.16</td>
</tr>
<tr>
<td>50.0</td>
<td>6.09 ± 0.62</td>
<td>14.30 ± 0.91</td>
<td>21.70 ± 1.81</td>
</tr>
</tbody>
</table>

Units of mitogenic activity determined from a PDGF radioreceptor assay, b endothelial cell clonal growth assay, and c 3T3 mouse fibroblast mitogenic assay.
DISCUSSION

Our data show that endothelial cells produce and secrete under normal culture conditions at least two peptide growth factors, which are either similar or identical to PDGF and FGF. The quantitative evaluations (Table 2) suggest that most if not all the mitogenic activity detected in the conditioned media can be accounted for by these two growth factors. Our data also demonstrate, for the first time, the effect of radiation as an inducer of synthesis and secretion of these two growth factors in endothelial cells. Previous studies have already demonstrated the capacity of endothelial cells to produce and secrete PDGF-like mitogens. Endothelial cells in culture transcribe mRNA species that hybridize with probes for the c-sis homologous B chain of PDGF (47—49) and less frequently with probes for the A chain (50—54). PDGF-like products of such transcriptional mRNA activities have been detected in the media of nearly every type of endothelial cell tested so far (25—32). It has also been shown that several agents that stimulate and activate endothelial cells such as thrombin, phorbol esters, transforming growth factor-β (51, 54, 55), TNF (32, 53), IL-1, and bacterial endotoxin lipopolysaccharide (53) enhance the transcription of PDGF chains A and B mRNA in endothelial cells and increase the amounts of PDGF-like mitogens secreted into the medium. On the other hand, human recombinant γ-interferon (53), β-adrenergic agonists, such as isoproterenol and norepinephrine and agents that increase cellular cAMP such as forskolin (54, 55) suppress the transcription of mRNA for PDGF and the release of PDGF-like proteins from endothelial cells. Our data demonstrate the capacity of irradiation to stimulate endothelial cell secretion of increased amounts of PDGF-like molecules, which after 50 Gy and 48 h in culture reach a 6-fold increase over the basal levels of unirradiated controls (Table 2). However, in view of the large numbers of cells that show evidence of cell death and lysis with the high radiation doses employed in our study, a legitimate question may be asked whether the enhanced PDGF-like activity did not result from leakage from preexisting intracellular reservoirs of the mitogen during the process of cell death and lysis. The results of our freeze and thaw experiments (Table 1) and the quantitative data derived from the PDGF receptor binding competition experiments (Table 2) suggest that the majority of the increase in the PDGF-like activity resulted from radiation induced de novo synthesis of the factor by cells that survived the lethal effects of radiation and remained metabolically active. The dose-dependent change in the PDGF:FGF ratio also supports this hypothesis. However, our interpretation of these data is not conclusive beyond questioning. Therefore, to address this issue in a more specific manner, experiments are currently underway to evaluate the levels of transcriptional PDGF-specific mRNA in the cells that remain adhered to the culture plate and continue to exclude the trypan blue dye at various periods of time after irradiation.

Whereas the concentrations of PDGF-like mitogens in the conditioned media do indeed increase significantly after irradiation, the majority of the mitogenic activity still remains non-PDGF-like in nature. The positive identification of FGF as the main non-PDGF-like component is supported by our experiments that demonstrate the mitogenic effects of the conditioned media on both normal PAEC (Fig. 5) and BAEC (Fig. 6). It has been shown that endothelial cells do not have receptors for PDGF (42, 44) but express high numbers of FGF receptors (45, 46) and respond to basic FGF added to it exogenously (37). Furthermore, the mitogenic effect of the conditioned media on BAEC was blocked in our experiments by a specific rabbit polyclonal antibody against bFGF (Fig. 6). As in the case of PDGF, the data suggest that the FGF secreted after irradiation is a product of enhanced de novo synthesis by metabolically active surviving cells, but assessment of the levels of FGF-specific transcriptional mRNA in unirradiated and irradiated cells is required to support this hypothesis.

Previous studies utilizing specific cDNA probes for the bFGF gene (34, 35) demonstrated that nearly every type of endothelial cell tested exhibited synthesis capacity of FGF (56), but suggested that the FGF molecules remain mostly intracellular (33) due to the lack of a recognized consensus signal peptide sequence in the FGF gene (34, 35). However, recent studies present new evidence for the presence of FGF in conditioned media of endothelial cells. Utilizing heparin-Sepharose chromatography as a method of analysis, Vlodavsky et al. (33) were able to detect small amounts of FGF in the media of BAEC, although it represented less than 3% of the factor stored within the cells. The authors suggest that these small amounts of FGF found in the conditioned media could perhaps leak from cells undergoing detachment and lysis during the experimental manipulations of the cultures. Sato and Rifkin (57) also have recently published evidence for continuous release of endogenous bFGF from endothelial cells in culture and demonstrated its bioactive autocrine function in inducing cell movement, DNA synthesis and the production of plasminogen activator. These autocrine-like activities of bFGF were blocked by a specific anti-FGF antibody (57). Whereas these data do not conclusively prove that the secretion of endogenous FGF represents a constitutive function of endothelial cells, the data support our current observations that when sensitive biological methods are employed, the levels of bioactive FGF detected in the conditioned media are metabolically significant and may be involved in the regulation endothelial cell function in vitro.

The mechanism by which FGF is, in fact, secreted into the medium remains unknown. One possible suggestion is that FGF is released from cells in response to sublethal damage and leakage associated with cell membrane injury. Such a mechanism would be consistent with irradiated cells, but is perhaps less likely in unirradiated cultures. Another nontraditional secretory mechanism has been suggested (58, 59) which involves the formation of intracellular complexes of FGF with heparan
sulfate proteoglycan residues, found in endothelial cell cytoplasm and nucleus, which are then liberated into the medium. Although no direct evidence of such intracellular complex formation is available, this possibility is consistent with the binding patterns of FGF to extracellular matrix components (58, 59) and its storage in vivo in basement membranes of the cornea (60) and of blood vessels.4 Whereas the secretory pathway of FGF after irradiation is still unknown, the experiments currently underway evaluating the kinetics of radiation-induced transcription of mRNA for FGF and the regulatory mechanisms involved in this function will hopefully contribute to the clarification of this issue.

The biological significance of the enhanced secretion of growth factors from irradiated cells is still unknown. PDGF-like molecules possibly secreted from the intima of blood vessels after irradiation may serve as paracrine factors for the proliferation of smooth muscle cells observed in small caliber arteries after irradiation in vivo (1–3) and perhaps participate in initiating the postradiation fibrosis observed in chronic radiation damage in normal tissues (1–6). FGF secreted after irradiation may participate in the abnormal proliferation of endothelial cells reported to obliterate the lumen of small caliber arterioles in various organs (1–3). In addition, the secreted FGF may have a critical role in the repair of radiation induced damage in the irradiated endothelial cells themselves. We have recently shown that FGF is capable of inducing repair processes of radiation damage in endothelial cells (15). BAEC plated after irradiation for colony formation assays on an extracellular matrix (ECM) produced by a clone of PF-HR9 cells that contains high concentrations of bFGF, exhibited a Do value for the dose survival curve of 95 cGy and a Dq of 174 cGy. However, BAEC plated on an identical ECM except for its being bFGF free (produced by another clone of PF-HR9 cells) exhibited a similar Do value of 110 cGy but the threshold shoulder was completely eliminated. When exogenous FGF was added to the FGF-free ECM immediately after irradiation, the threshold shoulder was reconstituted, but it was progressively eliminated with delay of FGF addition during the first 24 hours postradiation. These data suggest that FGF is involved in the induction of repair of radiation induced damage in BAEC and that its effects are conferred during the early postradiation period. It is thus possible that radiation induced synthesis and secretion of FGF from endothelial cells may be critical for promoting repair of intimal radiation induced damage in blood vessels in vivo. This regulatory mechanism of radiation damage repair is currently being investigated.

ACKNOWLEDGMENTS

We thank Esther HyAm for her excellent technical assistance.

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