Stimulation of Radiation-Impaired Plasminogen Activator Release by Phorbol Ester in Aortic Endothelial Cells (43137)

JANE RAYMOND,[1] SEI-CHUL YOON,[2] AND CHUNG-HSIN TS'AO

Departments of Medicine, Radiology, and Pathology, Northwestern University School of Medicine and Northwestern Memorial Hospital, Chicago, Illinois 60611

Abstract. **Ionizing radiation has been reported to affect the fibrinolytic activity of exposed tissue. With cultured bovine aortic endothelial cells, radiation suppresses the release of plasminogen activator to the conditioned media, with a concomitant increase in intracellular plasminogen activator. Thus study was undertaken to determine whether radiation-impaired plasminogen activator release can be modified by phorbol ester. We exposed cultured bovine aortic endothelial cells to a sterilizing dose of 10 Gy of γ-rays and found the treatment led to cell injury, as evidenced by an increased release of prelabeled chromium, and to a reduction of plasminogen activator in the conditioned media with elevated intracellular plasminogen activator in irradiated cells. Phorbol ester enhanced plasminogen activator activity in both sham-irradiated and irradiated endothelial cells. It was interesting to note that the increased plasminogen activator in phorbol ester-stimulated sham-irradiated cells was largely retained inside the cell, while it was released to the conditioned media in irradiated cells. Apparently, altered plasminogen activator activity of radiation-sterilized endothelial cells can be modified by exogenous stimuli.** [P.S.E.B.M. 1990, Vol 195]

The fibrinolytic system of normal and malignant tissue is involved in such biological processes as angiogenesis, wound healing, and tumor metastasis, in addition to the well-characterized clot lysis. Ionizing radiation exerts varying effects on the fibrinolytic activity of exposed tissue. Decreased activity has been described in lungs (1–4), liver (5, 6), skin (7), and blood vessels (8, 9) following radiation exposure. Increased activity, on the other hand, was noted in irradiated gastrointestinal mucosal cells (10, 11) and neurons (12). A decreased release of plasminogen activator (PA), with a concomitant increase in intracellular PA, was observed in cultured bovine aortic endothelial cells (BAEC) (13) receiving a sterilizing dose of radiation (14).

[1] Present address: Department of Medical Oncology, University of Pittsburgh, Pittsburgh, Pennsylvania 15240.

[2] Present address: Department of Radiology, Kangman St. Mary's Hospital, Catholic University Medical College, Seoul 137-040, Korea.

Received March 23, 1990. [P.S.E.B.M. 1990, Vol 195]
Accepted June 19, 1990.

0037-9727/90/1952-0213$2.00/0
Copyright © 1990 by the Society for Experimental Biology and Medicine

In the United States, half of all cancer patients receive radiation as either the primary therapy or as adjunct therapy. Studies of fibrinolytic changes and modification of those changes in irradiated tissues are not merely of academic interest. They signify the recognition of the importance of functional abnormalities in radiation-sterilized cells in the overall homeostasis of the organism. In this study, we sought to determine whether the impaired PA release from radiation-sterilized BAEC could be modified by exogenous stimulus.

Materials and Methods

Bovine Aortic Endothelial Cells. Frozen and thawed BAEC harvested from three thoracic aortas of slaughtered cattle were used in these studies. Cells were suspended in RPMI 1640 tissue culture medium containing 20% newborn calf serum and 10% dimethyl sulfoxide at a density of 5×10^6 cells/ml and frozen in liquid nitrogen at passages 0 (primary culture), 3, and 5. Thawed cells were grown in the same culture medium minus dimethyl sulfoxide in T-75 flasks. The endothelial nature of these cells was established by the presence of Factor VIII-related antigens. After reaching confluency, cells were trypsinized and passaged to 35-mm culture dishes. Cells in the 35-mm dishes reached >95% confluency 3–4 days later. Before being irradiated, the

standard culture medium was replaced with one containing only 2% newborn calf serum.

Irradiation of BAEC. Irradiation was accomplished by the use of a Philips 6000 Rhm ^{60}Co teletherapy unit, at 2.63 Gy/min for a total of 10 Gy. Usually, eight dishes of cells, subcultured from the same parent flask, were taken to the radiation therapy room. Four dishes of cells were irradiated at the same time, while the other four dishes of cells received no radiation (sham irradiated). Irradiated and sham-irradiated cells were returned to the Co_2 incubator for 3 days without disturbance. Experiments of cells were carried out on the fourth postirradiation day.

Protein and DNA Determinations. Cells in one of the four irradiated or sham-irradiated dishes were scraped into H_2O and frozen at −20°C. Protein and DNA concentrations in the sample were determined (13). Protein and DNA in this dish were considered to represent those in the other three sister dishes. Protein was assayed using the Lowry technique (15), and DNA was assayed according to method of Setaro and Morley (16).

Exposure of Cells to Phorbol Ester. At 72 hr postirradiation, the culture medium was removed and cells were rinsed once with Hanks' balanced salt solution (HBSS). Each dish of cells was incubated with serum-free medium with or without 15 n*M* or phorbol 12-myristate-13-acetate (PMA; Sigma Chemical Co., St. Louis, MO). PMA was first dissolved in absolute ethanol at a concentration of 1 μM and then diluted to 15 n*M* with serum-free medium. The same concentrations of ethanol were present in serum-free medium not containing PMA. Cells were incubated in this medium for 20 hr. The conditioned medium was collected and stored at −80°C. Cells were rinsed twice with HBSS, solubilized in 0.5% Triton X-100, and stored at −80°C.

Assay for PA Activity. The fibrin well method originally described by Unkeless *et al.* (17) was adopted. A sample of 0.1 ml of plasminogen-free bovine fibrinogen solution (1 mg/2.5 ml of H_20; Miles Laboratories, Elkhart, IN) containing 0.12 μCi of ^{125}I-labeled human fibronogen (Ibrin; Amersham Corp., Arlington Heights, IL) was pipetted to each of the 24 wells of a Costar tissue culture plate (Costar, Cambridge, MA). Fibrinogen solution was evaporated to dryness by placing the plate in a 37°C incubator for 18 hr. Fibrinogen, now coated on the bottom of the well, was clotted with 0.2 ml of reptilase (34 μg/well; Pacific Hemostasis, Bakersfield, CA). After 90 min at 37°C, reptilase was removed and the wells were washed three times with H_2O to remove free radioactivity. To each well was added 380 μl of RPMI 1640, 100 μl of 0.1% gelatin, 250 μl of test sample, and 20 μl of affinity-purified human plasminogen (50 microunits; Helena, Houston, TX). Plasminogen or test sample was excluded in control wells to determine nonspecific or spontaneous release of radioactivity. The plate was incubated at 37°C for 2 hr. Radioactivity in 500 μl of incubation fluid was determined with a gamma counter (BioData 20/20; Abbott Laboratories, North Chicago, IL). The amount of radioactivity in the incubation fluid was indicative of the degree of clot lysis. Clot lysis caused by test samples in the presence of plasminogen was considered to indicate PA activity in those samples. In each assay, a standard curve was constructed with urokinase (UK; Calbiochem, La Jolla, CA; 0 to 0.15 units/well). PA activity in test samples was read against the standard curve and was expressed as UK equivalents per unit of protein or DNA.

Radiation-Induced Cell Injury. To determine the extent of cell injury caused by γ-ray exposure, confluent BAEC were incubated with 1.5 ml of serum-free medium containing 2 μCi of $Na_2{}^{51}CrO_4$ (ICN Radiochemicals, Irvine, CA) for 4 hr at 37°C. Chromium-containing medium was then removed, and cells were washed six times with HBSS. After the last wash, 2% serum medium was added and cells were irradiated or sham irradiated. After 24 hr, radioactivity in the culture medium and in the cell lysate was determined with a gamma counter (18). The presence of increased radioactivity in the culture medium of irradiated cells was considered to indicate radiation-induced cell injury. Cell injury was divided into two categories: cell lysis and cell detachment. Cell lysis was represented by radioactivity in the supernatant of centrifuged (1000*g* for 10 min) culture medium. Cell detachment was the difference in radioactivity between the uncentrifuged and centrifuged aliquots of the same culture medium.

Data Analysis. In order to standardize the effects of radiation and PMA on PA activity of BAEC obtained from different aortas, PA activity of sham-irradiated or non-PMA-treated cells was arbitrarily assigned as being 1, with which the activity of irradiated and PMA-treated cells was compared by one-way analysis of variance. A $P < 0.05$ between experimental and control specimens was considered significant.

Results

Morphologic and DNA and Protein Changes in Irradiated Cells. No morphologic changes were discerned in BAEC monolayers immediately after 10 Gy of γ-ray exposure. At 24-hr postirradiation, the monolayers were disrupted by spaces left behind by detached cells and some of the attaching cells became elongated, losing their normal cobblestone appearance. By 72 hr, many cells were hypertrophic and the majority of them had resumed the cobblestone morphology. An uninterrupted monolayer with many large cells was reestablished (Fig. 1).

There was significant reduction of protein and DNA assayed on the fourth postirradiation day in irradiated dishes (Table I). The extent of reduction of

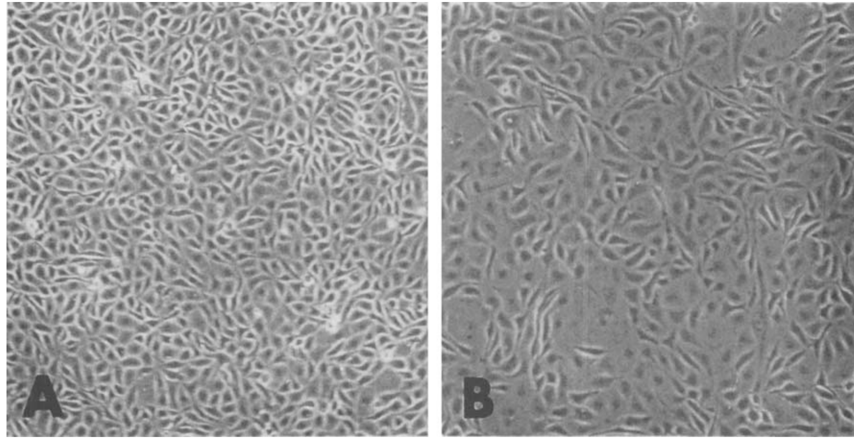

Figure 1. Morphology of BAEC 3 days after exposure to 0 (A) or 10 (B) Gy of γ-rays. Note the presence of many hypertrophied cells in the latter (original magnification, ×280).

Table I. Protein and DNA Contents in Sham-Irradiated and Irradiated BAEC Culture Dishes

	Contents (μg/dish) after:		*n*	*P*
	Sham irradiation	Irradiation		
Protein	305.6 ± 14.2	187.9 ± 9.9	8	0.0001
DNA	12.8 ± 1.0	7.4 ± 1.9	8	0.001

Table II. Radiation-Induced Cell Injury in the Form of Cell Detachment and Cell Lysis

	% Prelabeled chromium after:		*n*	*P*
	Sham-irradiation	Irradiation		
Cell lysis	36.7 ± 1.0	45.0 ± 3.0	6	0.03
Cell detachment	2.8 ± 0.5	5.9 ± 1.4	6	0.07
Combined	39.5 ± 1.3	51.1 ± 4.2	6	0.03

DNA was greater than that of protein. As a result, there was more protein per unit of DNA in irradiated specimens than in sham-irradiated counterparts.

Radiation-Induced Injury in BAEC. Examination under an inverted phase-contrast microscope revealed more detached cells in the culture media of irradiated dishes than in that of sham-irradiated dishes at 24 hr postirradiation and thereafter. On the basis of chromium release determined at 24 hr, the major form of injury caused by irradiation was cell lysis. Cell detachment was also increased in irradiated dishes, but the increase was not statistically significant. Overall cell injury, i.e., cell detachment plus cell lysis, was significantly higher in irradiated dishes (Table II).

Radiation Effect on PA Activity. PA activity was present in the conditioned medium as well as in the cell lysate of cultured BAEC. In irradiated dishes, there was a significant reduction in PA activity in the conditioned medium with an increase in PA activity in the cell lysate (Fig. 2). When PA activity was analyzed on the basis of cellular protein or DNA, the mean conditioned medium PA activity in irradiated samples was still lower than that in the medium of sham-irradiated counterparts but the difference was not statistically significant. Intracellular PA, i.e., PA in cell lysate, remained significantly higher in irradiated cells than in sham-irradiated cells (Fig. 3).

PMA Effect on PA Activity. Greater PA activity was consistently found in sham-irradiated and irradiated cells exposed to PMA for 20 hr, irrespective of whether the activity was analyzed on the basis of protein or DNA or per dish. PMA-stimulated PA in sham-irradiated cells was largely retained intercellularly with only a small increase in PA activity in the conditioned medium. In irradiated cells, the stimulated PA was released to the conditioned medium (Fig. 4).

Discussion

We demonstrated that irradiation of BAEC in tissue culture resulted in a reduction of PA in the condi-

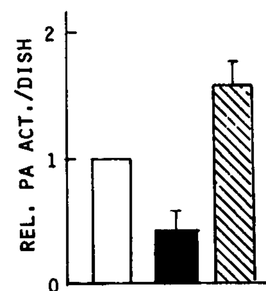

Figure 2. PA activity in the conditioned media (shaded bar) and cell lysates (hatched bar) or irradiated BAEC dishes. The activity was compared with that in sham-irradiated dishes (open bar) whose activity was designated as 1. There was a significant decrease in conditioned media-PA activity and an increase in cell lysate-PA activity in irradiated dishes. Mean ± SE, $n = 8$.

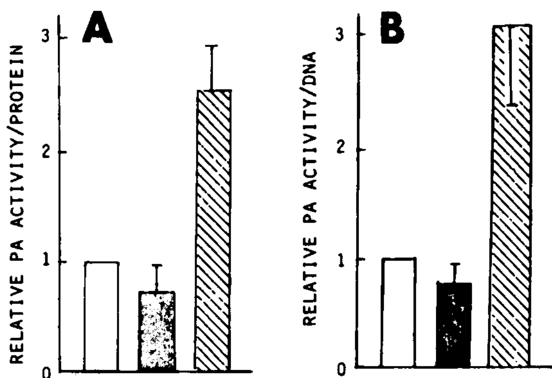

Figure 3. When PA activity in the irradiated dishes was analyzed on the basis of cellular protein (A) or DNA (B), the mean conditioned media-PA activity (shaded bars) in irradiated samples was reduced but it was not significantly different from that in sham-irradiated samples (open bars). Intracellular PA activity (hatched bars) remained significantly higher in irradiated cells than in sham-irradiated cells. Mean ± SE, $n = 8$.

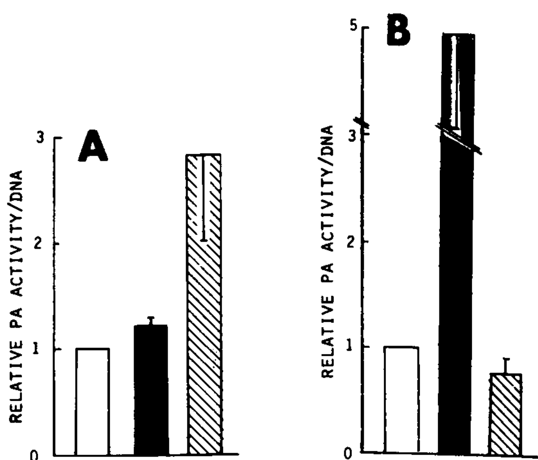

Figure 4. Effect of PMA on PA activity in sham-irradiated (A) and irradiated (B) BAEC. Conditioned media-PA (shaded bars) and cell lysate-PA (hatched bars) activity was compared with that in non-PMA-treated samples (open bars). PMA caused a significant increase in intracellular PA in sham-irradiated cells and stimulated the release of PA in irradiated cells. Mean ± SE, $n = 8$.

tioned medium. Reduced PA in conditioned medium coincided with an increase in intracellular PA activity. When total PA activity, i.e., PA activity in conditioned medium plus that in cell lysate, was analyzed on the basis of units of protein or DNA, the activity was actually increased in the irradiated samples. Since DNA generally agrees with cell count, these findings indicate higher PA activity in individual irradiated cells than in individual sham-irradiated cells. Recently, Rosen *et al.* (19) demonstrated enhanced protein synthesis in irradiated BAEC. The increased PA in irradiated cells observed in the present study could have, at least partly, resulted from increased synthesis of this serine protease. In spite of increased cellular PA, the release of this enzyme appears to have been impaired by γ-ray exposure. Another radiation effect on cultured BAEC was increased cell injury, as evidenced by elevated levels of prelabeled chromium in the conditioned medium. It should be noted that increased chromium release may result from greater nonspecific cell injury, whereas decreased PA release may reflect a radiation-induced impairment of a specific cellular function.

PA is involved in a number of biologic activities, mostly through its conversion of plasminogen to plasmin. Of the many biologic activities in which the fibrinolytic system participates, one is the resolution of fibrin clots. Intimal and subintimal fibrin deposition and fibrinoid necrosis of blood vessels is a common finding in irradiated organs (20–22). Fibrin formation and its persistent presence are important aspects in thrombogenesis and arterosclerosis. The predisposition of irradiated vessel to arterosclerosis (23) may partly be due to a suppressed release of PA from irradiated endothelial cells.

The tumor promoter phorbol ester has a wide range of biologic effects. Two of those effects are the induction of synthesis and the stimulation of release of PA in several tumor cell lines (24–26), as well as in endothelial cells (27). In the present study we found increased PA activity in both sham-irradiated and irradiated endothelial cells treated with PMA. The increased PA in PMA-treated sham-irradiated cells was largely retained inside the cells, while in PMA-treated irradiated cells, it was released to the conditioned media. The different PMA effects on the status of PA in sham-irradiated and irradiated cells may reflect a yet unidentified radiation effect on the cellular PA release mechanism that has become sensitive to PMA stimulation.

Endothelial cells normally have a very slow turnover rate. Cells receiving a sterilizing dose of radiation and not lysed or detached from the vessel wall could remain there for an extended period of time. The fact that the PA activity of these cells could be stimulated by PMA suggests that other metabolic functions of radiation-sterilized cells could also be modified by exogenous stimuli.

1. Fleming WH, Szakacs JE, King ER. The effect of gamma radiation on the fibrinolytic system of dog lung and its modification by certain drugs relative to radiation pneumonitis and hyaline membrane formation in lung. J Nucl Med **3**:341–352, 1961.
2. Gerber GB, Dancewicz AM, Bessemans B, Casale G. Biochemistry of late effect in rat lung after hemithoracic irradiation. Acta Radiol Ther Phys Biol **16**:447–445, 1977.
3. Dancewicz AM, Mazanowska A, Gerber GB. Late biochemical changes in the rat lung after hemithoracic irradiation. Radiat Res **67**:482–490, 1976.
4. Ts'ao C, Ward WF, Port CD. Radiation in rat lung. III. Plasminogen activator and fibrinolytic inhibitor activities. Radiat Res **96**: 301–308, 1983.
5. Reed GB, Cox AJ. The human liver after radiation: A form of reno-occlusive disease. Am J Pathol **48**:597–611, 1966.
6. Henderson BN, Bicker HI, Johnson RJ. Loss of vascular fibrin-

olytic activity following irradiation of the liver—An aspect of late radiation damage. Radiat Res **95**:646–652, 1983.
7. Essa E, Casarett G. Effect of epsilon-amino-n-caproic acid (EACA) on radiation-induced increase in capillary permeability. Radiology **106**:679–688, 1973.
8. Astedt B, Bergantz S, Svanberg L. Effect of irradiation on the plasminogen activator content in rat vessels. Experientia **320**: 1466–1467, 1974.
9. Svanberg L, Astedt B, Kullander S. On radiation-decreased fibrinolytic activity of vessel walls. Acta Obstet Gynecol Scand **55**: 49–51, 1976.
10. Stenberg B, Risberg B, Peterson H. Irradiation and gastrointestinal fibrinolysis: An experimental study in the rat. Eur J Clin Invest **10**:139–141, 1980.
11. Stenberg B, Risberg B, Zettergren L. Localization of tissue fibrinolysis in the gastrointestinal tract. Thromb Haemost **42**:1417–1424, 1979.
12. Soreq H, Miskin R. Plasminogen activator in the rodent brain. Brain Res **216**:361–374, 1984.
13. Ts'ao C, Ward WF: Acute radiation effects on the content and release of plasminogen activator activity in cultured aortic endothelial cells. Radiat Res **101**:394–401, 1985.
14. Rhee JG, Lee I, Song CW. The clonogenic response of bovine aortic endothelial cells in culture to radiation. Radiat Res **106**: 182–189, 1986.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1981.
16. Setaro F, Morley CGD. A modified fluorometric method for determination of microgram quantities of DNA from cells or tissue culture. Anal Biochem **71**:313–317, 1976.
17. Unkeless JC, Tobia A, Ossowski L, Quigley JP, Rifkin DB, Reich E. An enzymatic function associated with transformation of fibroblasts by oncogenic virus. J Exp Med **137**:85–111, 1973.
18. Zhou MH, Dong Q, Ts'ao C. Susceptibility of irradiated bovine aortic endothelial cells to injury. Am J Pathol **133**:277–284, 1988.
19. Rosen EM, Vinter DV, Goldberg ID. Hypertrophy of cultured bovine aortic endothelium following irradiation. Radiat Res **117**:395–408, 1989.
20. Fonkalsrud EW, Sanchez M, Zerubavel R, Mahoney A. Serial changes in arterial structure following radiation therapy. Surg Gynecol Obstet **145**:395–400, 1977.
21. Slauson DO, Hahn FF, Chiffelle TL. The pulmonary vascular pathology of experimental radiation pneumonitis. Am J Pathol **88**:635–654, 1977.
22. Haselton PS, Carr N, Schofield PF. Vascular changes in radiation bowel disease. Histopathology **9**:517–534, 1985.
23. Lindsay S, Kohn HI, Dakin RL, Jew J. Aortic arteriosclerosis in the dog after localized aortic X-irradiation. Cir Res **10**:51–60, 1962.
24. Crutchley DJ, Cananan LB, Maynard JR. Induction of plasminogen activator and prostaglandin biosynthesis in HeLa cells by 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res **40**:849–852, 1980.
25. Ferraiuolo R, Stoppelli MP, Verde P, Bullock S, Lazzaro P, Blasi F, Pietropaolo TC. Transcriptional induction of urokinase in cultured human kidney carcinoma cells by tetradecanoyl-phorbol-acetate. J Cell Physiol **121**:368–374, 1984.
26. Band V, Karlan BY, Zurawski VR Jr, Littlefield BA. Simultaneous stimulation of urokinase and tissue-type plasminogen activators by phorbol esters in human ovarian carcinoma cells. J Cell Physiol **138**:106–114, 1989.
27. Levin EG, Loskutoff DJ. Comparative studies of the fibrinolytic activity of cultured vascular cells. Thromb Res **15**:869–878, 1979.