Volume 330, number 1, 41–45 FEBS 12928 September 1993

Effects of ionizing irradiation on endothelial cell transglutaminase

Gil Korner[a], Varda R. Deutsch[b], Israel Vlodavsky[a], Amiram Eldor[b,*]

[a]*Department of Oncology, Hadassah University Hospital, Jerusalem, Israel*
[b]*Department of Hematology, Hadassah University Hospital, POB 12000, Jerusalem, Israel 91120*

Received 4 June 1993; revised version received 14 July 1993

The activity of transglutaminase (TG) was measured in cultures of bovine aortic and capillary endothelial cells (EC) following exposure to γ-irradiation. Resting confluent EC express significant TG activity which fluctuates in growing cells This activity was increased by 2-fold following non-lethal irradiation. The increase in TG activity was dose dependent up to 20 Gy and reached a plateau at 24–36 h after irradiation. Immunohistochemical studies showed a prominent increase in cytoplasmic TG following irradiation. Western blot analysis of whole cell extracts showed no increase in total cellular TG. Kinetic studies demonstrated that the affinity of the enzyme to its substrate was not altered, but the V_{max} was increased. TG has previously been shown to be stored in an inactive form in EC membranes. This activity could be recovered in normal EC, but not in irradiated EC, by the addition of potassium thiocyanate and dithiothreitol or 0.8 M NaCl. An inhibitor of TG was previously demonstrated in the 100,000 × g particulate fraction of EC. Following irradiation, a significant decrease in this inhibitory activity was demonstrated. These results imply that the post-irradiation enhancement of TG activity may be caused by activation of a latent cellular enzyme. This elevated TG activity may cross-link adjacent cytoplasmic and membrane proteins and may thus play an active role in the enhanced apoptosis observed following irradiation of EC.

Endothelial cell; Transglutaminase; Irradiation

1. INTRODUCTION

Early radiation injury is characterized by vascular damage, and the initial site of damage appears to be the endothelial lining of the vessel wall [1–3]. In capillaries, early post-radiation changes consist of endothelial cell (EC) swelling and sloughing and formation of mural thrombi, hyalinization and fibrosis [4]. In larger arteries, radiation has been shown to accelerate the development of atherosclerosis [4–7]. Significant morphological and functional anomalies also occur in the endothelial lining of vascular segments and in cultures of EC subjected to radiation, including the loss of their thromboresistant properties [8]. Factors which influence the interaction between blood-borne cells and the endothelium are expressed and/or released from the irradiated endothelium. These include the von Willebrand factor, prostacyclin, platelet activating factor (PAF), plasminogen activator, chemotactic factors and mitogens (PDGF, bFGF) [8–13].

Bovine aortic EC contain Ca^{2+}-dependent tissue-type transglutaminase and an inhibitor which blocks the enzyme via ionic interaction [14]. Transglutaminase (TG) catalyses the Ca^{2+}-dependent acyl transfer reaction between -γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, resulting in new γ-amides of glutamic acid and ammonia [14–17]. It has been suggested that TG is involved in the regulation of cell growth and differentiation [15–17]. Its activity varies considerably depending on the state of cell proliferation, and is usually increased with decreasing cell growth [14–17]. Ionizing irradiation exerts an antiproliferative effect associated with a significant reduction in the clonogenic capacity of cultured endothelial cells [18]. In the present study we investigated the effect of ionizing irradiation on EC TG activity.

Corresponding author. Fax: (972) (2) 434 434.

Abbreviations: TG, transglutaminase (*R*-glutaminyl-peptide, amine-γ-glutamyl transferase, EC 2.3.2.13); EC, endothelial cells; BAEC, bovine aortic endothelial cells; BAC, bovine adrenal capillary endothelial cells; TED, Tris–EDTA–DTT.

2. MATERIALS AND METHODS

2.1. *Materials*

[2,3-^{3}H]Putrescine dihydrochloride (17.4 Ci/mmol) and carrier-free $Na^{125}I$ were purchased from New England Nuclear (Boston, MA). *N,N*-Dimethylated casein was from Calbiochem-Behring Corp. (San Diego, CA). Rabbit immunoglobulin (IgG) directed against human Factor VIII was obtained from Accurate Chemicals (Westbury, NY). Tissue culture dishes were from Costar Co. (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), antibiotics, glutamine and trypsin were obtained from Grand Island Biological Co. (Grand Island, NY) Nitrocellulose sheets (BA-85) were from Schleicher and Schuell (Keene, NH). Reagents for gel filtration, gel electrophoresis and electroblotting were from Bio-Rad (Richmond, CA). Affinity-purified fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, goat anti-rabbit IgG (affinity purified), molecular weight standards and all other chemicals were of analytical grade, purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. *Cells*

Bovine aortic EC (BAEC) and bovine adrenal capillary (BAC) EC were established and maintained in culture as previously described [19,20]. Partially purified brain-derived basic fibroblast growth factor (bFGF, 100 ng/ml) was added to the cultures every other day during the phase of active cell growth until the EC reached confluence.

2.3. *Radiation*

Confluent EC were irradiated as described [8,12]. Briefly, cells from stock cultures were dissociated with 0.05% trypsin, 0.02% EDTA, and seeded into 35-mm dishes at 4×10^4 cells/dish. 3–4 days after reaching confluency the medium was replaced by fresh medium in which serum was replaced with 0.1% human serum albumin. The cells were irradiated by a cobalt-60 Gammacell 220 (Atomic Energy of Canada) at a dose rate of 50 cGy/min. The cells were fed with fresh medium and maintained in culture for various time periods prior to the experiments. Cell counting was performed with a Coulter counter, and cellular viability was evaluated by the Trypan blue exclusion test. Three dishes were used for each data point.

2.4. *Preparation of endothelial cell extracts*

All steps were carried out on ice with ice-cold buffers. Cell cultures were washed twice with ice-cold phosphate-buffered saline (PBS), once with 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT) (TED buffer) supplemented with 150 mM NaCl (TED NaCl buffer), and then scraped into 1 ml/dish of TED NaCl buffer. The cells were sedimented by centrifugation ($250 \times g$ for 5 min at 4°C), resuspended in TED buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF) to yield a protein concentration between 1.5 and 2.5 mg/ml. The cells were lysed by repeated (three times) freezing and thawing. The efficiency of cell disruption was verified by light microscopy and the Trypan blue exclusion test.

2.5. *Fractionation of cell lysates*

Cell lysates were centrifuged (4°C, 5 min) at $15,000 \times g$ and the supernatant was collected. The pellet was resuspended and washed twice by re-centrifugation in TED buffer. The final pellet was resuspended by sonication for 15 s in the same buffer to yield a protein concentration of 1.5–2.5 mg/ml. For fractionation at $100,000 \times g$, cell lysates were centrifuged (4°C, 1 h) at $100,000 \times g$. The supernatants were removed and saved. The pellet was resuspended and washed twice by recentrifugation in TED buffer and the final pellet was resuspended in TED buffer by repeated sonication (10 s, three times) to yield a protein concentration of 1.5–2.5 mg/ml. The $100,000 \times g$ pellet was then treated for 2 h at room temperature with 50 μg/mg DNase and RNase. The samples were centrifuged for 1 h at $100,000 \times g$, resuspended in a small volume of TED buffer to yield a protein concentration of 1 mg/ml, and dialysed exhaustively against TED NaCl buffer.

2.6. *Transglutaminase assay*

TG activity was assayed by [^{3}H]putrescine incorporation into dimethylated casein, as previously described [14]. The final reaction mixture contained 50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, 15 mM DTT (in some cases 5 mM as noted), 0.1 μCi/0.5 mmol putrescine, 50 mM NaCl, 800 μg dimethylated casein and 30–200 μg protein of the unknown sample. Under these conditions there was a linear relationship between the incorporation of putrescine into casein and the amount of cell extracts assayed for up to 15 min. TG activity is expressed as nmol [^{3}H]putrescine incorporated into acid-insoluble protein/min/mg cell extract protein. Protein was determined by the method of Bradford [21] and the results were analysed statistically by Student's *t*-test.

2.7. *Western blots of transglutaminase*

Cell lysates were mixed with an equal volume of sample buffer (50 mM DTT, 1 mM EDTA, 8 M urea, 3% (w/v) SDS, 50 mM Tris-HCl, pH 6.8), and applied onto 10% SDS-PAGE gels [22]. The separated proteins were electroblotted onto nitrocellulose membranes at 0.4 A for 1 h. The blot was saturated with 5% non-fat milk in PBS followed by 1% non-immunized goat serum. TG antiserum [14], diluted 1:250, was then added and incubated for 2 h, followed by a 1 h incubation with ^{125}I-labelled affinity-purified goat anti-rabbit IgG (5×10^5 cpm/ml) [14].

3. RESULTS

3.1. *Effect of radiation on endothelial TG activity*

Bovine aortic EC (BAEC) were irradiated with 12.5 Gy and maintained in culture for 24 h. TG activity was determined in cell lysates and conditioned medium. The activity of EC TG was critically dependent on the Ca^{2+} concentration, with a maximum activity obtained above 2 mM Ca^{2+}. The calcium concentration used in the following experiments was 10 mM. TG activity in sham-irradiated BAEC was 3.92 ± 0.4 nmol/min/mg protein. Irradiated cells showed a 2- to 3-fold enhanced TG activity which was dependent on the dose of irradiation (Fig. 1). No TG activity was detected in medium conditioned by BAEC. The maximal dose of radiation was 20 Gy, because at higher doses a marked detachment of EC from the culture dish was observed. A similar effect of radiation was observed in bovine adrenal capillary EC (data not shown).

TG activity in confluent BAEC increased gradually following feeding with fresh medium, reached a maximum at 6–7 h and decreased to basal activity by 24 h [14]. Increased TG activity was observed in irradiated (12.5 Gy) BAEC already at 1 h after feeding, and at 24 h post-radiation a 2- to 3-fold stimulation of TG activity was obtained: (7.8 ± 0.8 nmol/min/mg proteins in irradiated cells compared to 3.1 ± 0.4 in non-irradiated cells ($P < 0.01$).

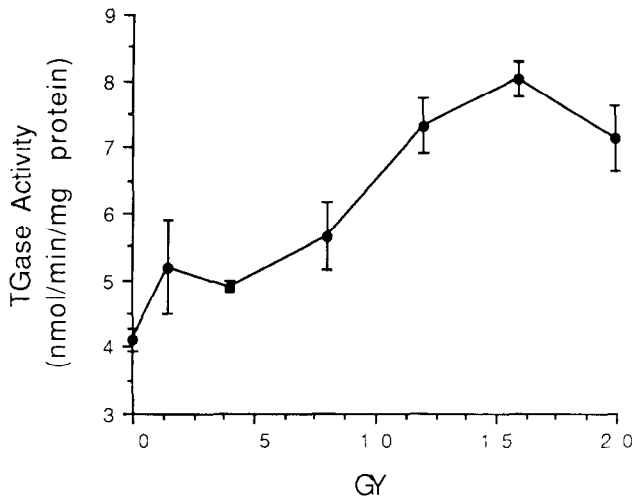

Fig. 1. Effect of γ-irradiation on TG activity in bovine aortic endothelial cells. Duplicate cultures of BAEC were exposed to increasing doses of γ-irradiation. After 24 h, cells were harvested and TG activity in cell lysates was determined as described in section 2. Each point in this and subsequent experiments represents the mean ± S.D. of four determinations. Each experiment was repeated at least three times.

3.2. *Effect of radiation on TG* K_m *and* V_{max}

The apparent K_m and V_{max} values of the transamidation reaction as a function of putrescine concentration were determined in 50 μg protein aliquots from irradiated (12.5 Gy) or sham-irradiated BAEC, 24 h after irradiation. Five different concentrations of putrescine were employed, ranging from 0.08 to 1.2 mM. The K_m and V_{max} were determined from Lineweaver–Burk double reciprocal plots (Fig. 2). The K_m averaged 0.2263 mM and was similar for irradiated and non-irradiated cells. The V_{max} was 10.5 nmol/min/mg protein in non-irradiated cells and 18.18 nmol/min/mg protein in irradiated (12.5 Gy) cells ($P < 0.01$). Experiments performed in the presence of a protein synthesis inhibitor (1–5 μg/ml cyclohexamide) were unsuccessful because the increased sensitivity of irradiated BAEC resulted in a marked detachment from the culture dish.

3.3. *Cellular distribution of TG activity following irradiation*

Irradiated and sham-irradiated BAEC cultures were exposed to mono-specific rabbit antiserum raised against a highly purified rat liver TG [23] which was previously shown to cross-react with BAEC TG [14]. Cells exposed to 12.5 Gy and examined 24 h later showed a strong fluorescent staining throughout their cytosol (Fig. 3). Sham-irradiated cells exhibited only a faint intensity of the fluorescent stain (not shown). Western blot analysis of sham-irradiated cultures was performed on cell lysates following separation of proteins on 10% SDS-PAGE. The TG protein band (80 kDa) had a similar intensity in irradiated and control cells (Fig. 4). These experiments suggest that, although more TG molecules reacted with the anti-TG antibodies in the cytosol of irradiated cells (as demonstrated by immunofluorescence), the total number of TG molecules was not affected by irradiation, as evident by Western blot analysis of cell lysates.

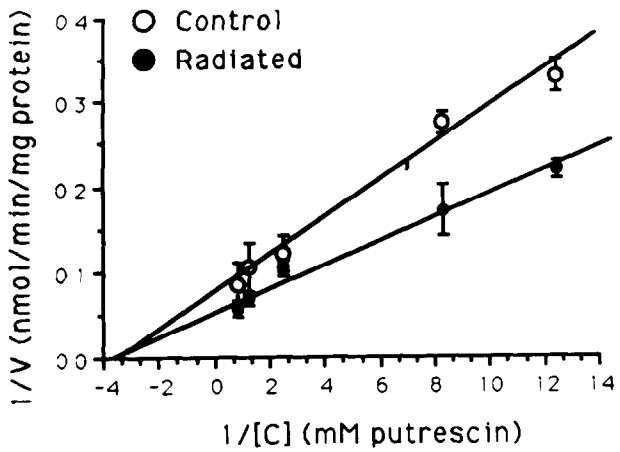

Fig. 2. Effect of γ-irradiation on the K_m and V_{max} of BAEC TG. Lineweaver–Burke double reciprocal plots of TG activity as a function of putrescine concentration. BAEC cultures were exposed to sham (○) or to 12.5 Gy γ-irradiation (●). 24 h later, cultures were harvested and cell lysates were prepared. Duplicate aliquots, each of 50 μg protein, were assayed for TG activity in the presence of increasing concentrations (0.08–1.2 mM) of putrescine.

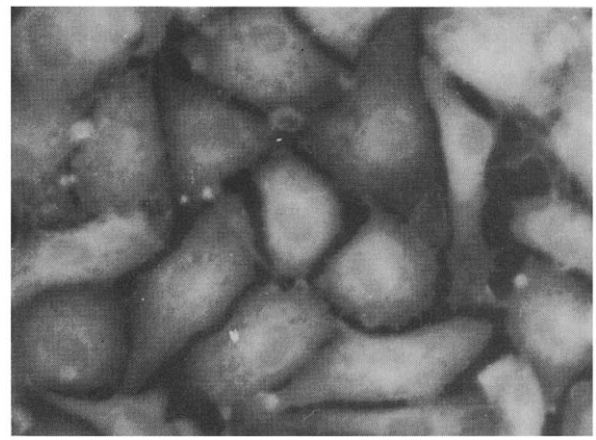

Fig. 3. Immunofluorescent staining of TG in irradiated BAEC. Confluent BAEC grown on glass cover-slips were exposed to 12.5 Gy γ-irradiation, fixed and stained by immunofluorescence using rabbit anti-rat liver TG antiserum (1:200) followed by FITC-conjugated goat anti-rabbit IgG (1:30). Non-specific staining with non-immune rabbit serum and FITC-conjugated goat anti-rabbit IgG yielded a faint background staining.

3.4. *Effect of irradiation on TG activity in BAEC membranes*

We have previously shown that a major part of the EC TG activity is found in the 15,000 × *g* supernatant fraction, with only a small percentage of the total cellular activity remaining in the particulate pellet [14]. The enzyme in the 15,000 × *g* particulate fraction is maintained, in part, in a latent form by an ionic interaction with its inhibitor and can be recovered following the addition of 0.8 M NaCl or 75 mM KSCN [14]. BAEC were irradiated with 15 Gy, and 24 h later a 15,000 × *g* particulate fraction was prepared. Sham-irradiated cells

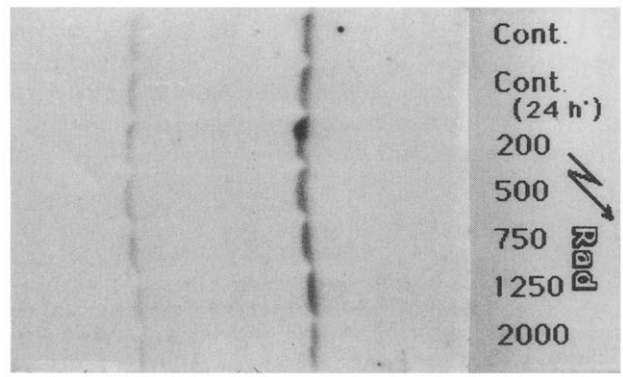

Fig. 4. Immunoblot analysis of TG in irradiated BAEC. BAEC cultures were exposed to various doses of γ-irradiation and harvested 24 h afterwards. Sham-irradiated control cultures were harvested at zero time and at 24 h. Aliquots (50 μg protein) from cell lysates were subjected to 10% SDS-PAGE electrophoresis followed by immunoblotting, as described in section 2.

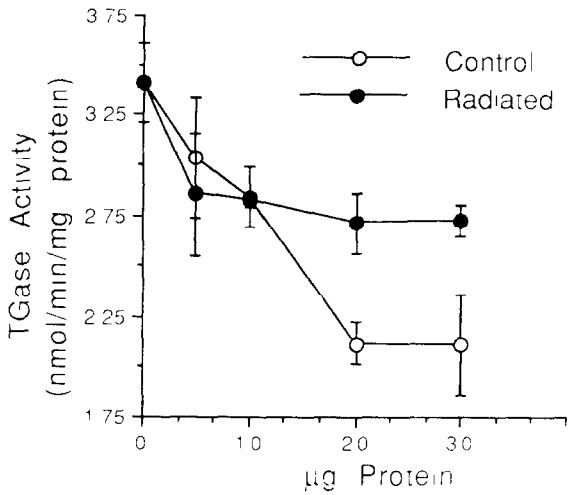

Fig. 5. Effect of γ-irradiation on the ability of BAEC 100,000 × *g* particulate fraction to inhibit TG activity. 100,000 × *g* particulate fractions from BAEC cultures exposed to sham (○) or 15 Gy irradiation (●) were prepared. Increasing amounts (5–30/μg protein) of these fractions were added to 20 μl of control BAEC 100,000 × *g* supernatant (3.5 TG units). TG activity was assayed in duplicate aliquots after incubation at 4°C for 1 h and adjustment of the reaction volume to 120 μl with TED NaCl buffer.

served as controls. Incubation (1 h, 4°C) of the particulate fraction with 0.8 M NaCl or with 75 mM KSCN plus 25 mM DTT liberated significant TG activity (128 and 65 nmol/min/mg protein, respectively) from the membranes of sham-irradiated cells, but there was little (28 nmol/min/mg protein) or no release of TG activity from the particulate fraction of irradiated cells by NaCl or KSCN + DTT, respectively.

We have previously demonstrated that the TG inhibitor found in the membranes of BAEC and glioma C_6 cells is located in the 100,000 × *g* particulate fraction. This inhibitor abrogates TG activity derived from the cytosol of EC or rat liver cells [14,17]. In the following experiment, a 100,000 × *g* particulate fraction was prepared from control and irradiated (15 Gy, 24 h) BAEC. This fraction was allowed to interact with the enzyme present in aliquots of the 100,000 × *g* supernatant of sham-irradiated cells and the residual TG activity was determined. Fig. 5 shows a markedly reduced inhibitory activity contained in the 100,000 × *g* particulate fraction following irradiation of endothelial cells.

4. DISCUSSION

The experiments described in this paper clearly show that radiation injury in cultured EC is associated with a significant enhancement of TG activity. This radiation effect, which was dose- and time-dependent, was observed in EC derived from capillary (adrenal) and a large artery (aorta). Kinetic studies revealed no change in the enzyme K_m in irradiated cells, while the V_{max} increased by almost 2-fold. These results indicate that, while the affinity of the enzyme towards its putrescine substrate was not affected by radiation, the reaction rate was significantly increased, suggesting that additional intracellular enzyme molecules became available for the transamidation reaction. This effect is not radiation specific, since exposure of cultured glioma cells to trypsin or neuraminidase, as well as cell fusion with Sendai virus, also triggered the activation of TG [23]. Activation of TG by trypsin and detergents was also described by Birckbichler et al., using cultured WI-38 human embryonic lung cells [24]. The rapid activation of glioma TG induced by injurious agents is, in all likelihood, due to release of a latent membrane-bound enzyme [23]. Likewise, radiation may trigger the activation of a latent or 'cryptic' form of TG. A similar example exists with ornithine decarboxylase, which is partially membrane bound and is activated upon cellular stimulation, a process that does not require protein synthesis [25]. In bovine aortic EC, most of the TG activity is found in the 15,000 × *g* soluble fraction, while only 4–22% of the activity is found in the particulate fraction [14]. TG activity in BAEC increased when the cells reached confluence, and was stimulated even further when their proliferation was arrested [14]. Our data on the effect of radiation on endothelial TG activity are consistent with this pattern of activation. Immunofluorescent studies revealed that irradiated EC displayed more TG antigen, as evident by the strong fluorescent staining of the cytosol. However, immunoblot analysis of the same cell lysates did not show any detectable increase of the TG band intensity following irradiation. In previous experiments, we have demonstrated that the intensity of the TG band is proportional to the quantity of the enzyme protein. A 2-fold increase in TG protein could be detected [14,17]. These results suggest that while the number of TG molecules accessible in EC to the anti-TG antibodies is increased following irradiation, the total number of TG molecules was not affected. Radiation injury may thus involve activation of cryptic TG molecules. Indeed, analysis of the bound non-active form of TG, found primarily in the 15,000 × *g* particulate fraction of EC, revealed that radiation significantly decreased the amount of the membrane-associated enzyme. In these experiments, release of the membrane-bound TG was carried out by treatment of the 15,000 × *g* particulate fraction with 0.8 M NaCl or KSCN + DTT. These results suggest that in irradiated cells the enzyme is liberated from membrane macromolecules.

Bovine aortic EC contain an inhibitor of TG which is found in the 100,000 × *g* particulate fraction and interacts with the enzyme via ionic interactions [14]. The content and/or activity of this inhibitor was decreased significantly in irradiated BAEC (Fig. 5). Our experiments suggest that, following irradiation, TG activity increases as a result of an enhanced mobilization and/or activation of a latent cryptic membrane-bound enzyme.

Radiation also decreases the TG-inhibitory activity present in the $100,000 \times g$ particulate fraction of EC, leading, in part, to the observed increase in TG activity.

Radiation injury is associated with arrest of cell proliferation due to breakages in nuclear DNA and apoptosis [26]. Association between an increased TG activity and programmed cell death (apoptosis) has been demonstrated by several investigators [27,28]. It has recently been reported that exposure of vascular EC to ionizing irradiation induces apoptosis in these cells [29]. The cross-linking of proteins by elevated TG activity in irradiated cells may be one of the first steps in programmed cell death, occurring as early as 24 h post-irradiation. Greenberg et al. showed that BAEC TG is capable of cross-linking α_2-plasmin inhibitor to fibrinogen, and that the resulting complex is 40-fold more resistant to degradation by plasmin [30]. Radiation, which impairs some of the thromboresistant properties of EC, is associated with enhanced tendency for thrombotic occlusions in capillaries and with premature atherosclerosis in large vessels [1–3]. The enhanced TG activity observed following radiation may also be operative in prothrombotic events which occur in small and large blood vessels.

Acknowledgements This work was supported by grants from the Israel Ministry of Health awarded to A.E., and from the Israel Cancer Research Fund to V.R.D.

REFERENCES

[1] Law, M.P. (1981) Adv. Radiat. Biol. 9, 37–73
[2] Fajardo, L.F. and Berthrong, M. (1988) Pathol. Ann. 23, 297–330.
[3] Eldor, A., Fuks, Z. and Vlodavsky, I. (1991) Sang Thrombose Vaisseaux 3, 411–416.
[4] Calvo, W., Hopewell, J.W., Reinhold, H.S. and Yeung, T.K. (1988) Br. J. Radiol. 61, 1043–1052.
[5] Stewart, J.R. and Fajardo, L.F. (1971) Radiol. Clin. North Am. 9, 511–531.
[6] Amronin, G.D., Gildenhorn, H.L., Soloman, R.D., Nadkarni, B.B. and Jacobs, M.L. (1964) J. Atheroscler. Res. 4, 325–334.
[7] McReynolds, R.A., Gold, G L. and Roberts, W.C. (1976) Am J. Med. 60, 39–45.
[8] Eldor, A., Vlodavsky, I , Hyam, E., Atzmon, R. and Fuks, Z. (1983) Prostaglandins 25, 263–279.
[9] Sporn, I.A., Rubin, P., Marder, V J. and Wagner, D.D. (1984) Blood 64, 567–570
[10] Polgar, P., Hahn, G. and Taylor, L. (1985) in· Biochemistry of Arachidonic Acid Metabolism (Wem Lands Ed.) pp. 161–173, Nijhoff, Boston.
[11] Ts'ao, C. and Ward, W.F. (1985) Radiat. Res. 101, 394–401.
[12] Matzner, Y., Cohn, M., Hyam, E. and Razin, E., Fuks, Z., Buchanan, M.R., Haas, T.A., Vlodavsky, I. and Eldor, A. (1988) J. Immunol. 140, 2681–2685.
[13] Witte, L.D., Fuks, Z., Heimovitz-Friedman, A., Vlodavsky, I., Goodman, D.S. and Eldor, A. (1989) Cancer Res. 49, 5066–5072.
[14] Korner, G., Schneider, D.E., Purdon, M.A. and Bjornsson, D (1989) Biochem J. 262, 633–641.
[15] Birckbichler, P.J., Orr, G.R , Patterson Jr., M.J., Conway, E. and Carter, H.A. (1981) Proc. Natl. Acad. Sci. USA 78, 5005–5008.
[16] Jetten, A.M., George, M.A., Pettit, G.R , Herald, C.L. and Rearick, J.I. (1989) J Invest. Dermatol. 93, 108–115
[17] Korner, G. and Bachrach, U. (1987) J. Cell. Physiol. 130, 44–50
[18] Haimovitz-Friedman, A., Vlodavsky, I., Chaundhuri, A., Witte, L. and Fuks, Z. (1991) Cancer Res. 51, 2552–2558.
[19] Gospodarowicz, D., Moran, J., Braun, D. and Birdwell, C.R. (1976) Proc. Natl. Acad. Sci. USA 73, 4120–4124.
[20] Schweigerer, L., Neufeld, G., Freidman, J., Abraham, J.A , Fiddes, J.C. and Gospodarowicz, D. (1987) Nature 325, 257–259
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] King, J. and Laemmli, U.K. (1971) J Mol. Biol. 62, 465–473.
[23] Korner, G and Bachrach, U. (1985) J. Cell. Physiol 124, 379–385.
[24] Birckbichler, P.J., Orr, G.R., Conway, E. and Patterson Jr., M.K. (1977) Cancer Res. 37, 1340–1344.
[25] Koenig, H , Goldstone, A.D. and Lu, C.Y. (1983) Proc. Natl. Acad. Sci. USA 80, 7210–7214.
[26] Bryant, P.E (1988) Int. J. Radiat. Biol 48, 55–60.
[27] Dell'Orco, R.T., Anderson, L.E., Conway, E. and Birckbichler, P.J. (1985) Cell Biol. Int. Rep. 9, 945–956.
[28] Fesus, L., Thomazy, V. and Falus, A. (1987) FEBS Lett. 224, 104–108.
[29] Lorand, L. (1988) Adv. Exp. Med. Biol. 231, 79–94.
[30] Greenberg, C S., Achyuthan, K.E., Borowitz, M.J and Shuman, M.A. (1987) Blood 70, 702–709.