Tumor Necrosis Factor Gene Expression Is Mediated by Protein Kinase C following Activation by Ionizing Radiation

Dennis E. Hallahan, Subbulakshmi Virudachalam, Matthew L. Sherman, Eliezer Huberman, Donald W. Kufe, and Ralph R. Weichselbaum

Department of Radiation and Cellular Oncology, University of Chicago and Pritzker School of Medicine, Chicago, Illinois 60637 [D. E. H., S. V. E., H. R., R. W.]; Laboratory of Clinical Pharmacology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115 [M. L. S., D. W. K.]; and Biological and Medical Research Division, Argonne National Laboratory, Argonne, Illinois 60439 and Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637 [E. H.]

ABSTRACT

Tumor necrosis factor (TNF) production following X-irradiation has been implicated in the biological response to ionizing radiation. Protein kinase C (PKC) is suggested to participate in TNF transcriptional induction and X-ray-mediated gene expression. We therefore studied radiation-mediated TNF expression in HL-60 cells with diminished PKC activity produced by either pretreatment with protein kinase inhibitors or prolonged 12-O-tetradecanoylphorbol-13-acetate treatment. Both treatments resulted in attenuation of radiation-mediated TNF induction. Consistent with these results, we found no detectable induction of TNF expression following X-irradiation in the HL-60 variant deficient in PKC-mediated signal transduction. The rapid activation of PKC following γ-irradiation was established using an in vitro assay measuring phosphorylation of a PKC specific substrate. A 4.5-fold increase in PKC activity occurred 15 to 30 s following irradiation, which declined to baseline at 60 s.

INTRODUCTION

TNF production is proposed to be an important mediator of the biological response to ionizing radiation. This cytokine is released into the medium of X-irradiated human sarcoma cell lines (1). The resulting cytotoxicity was reversed with monoclonal antibodies to TNF. The biological significance of X-ray-mediated TNF gene induction is demonstrated by the cytotoxicity of selected neoplastic cells and tumors, endotoxic shock and end-organ tissue damage (2), the associated pathogenesis of pulmonary fibrosis that occur in response to TNF in vivo (3). The direct cytostatic and cytotoxic effects of TNF on transformed cells in culture are associated with free radical production and subsequent DNA fragmentation (4, 5), which may be the mechanism of synergistic cell killing with ionizing radiation in some human tumor cell lines (6).

PKC-mediated signal transduction from cell surface receptors to the nucleus participates in the cellular response to external stimuli such as serum and growth factors (7, 8). One of the initial steps in this process is receptor-mediated activation of phospholipase C, which results in the hydrolysis of membrane phospholipids to diacylglycerol (8, 9). Diacylglycerol subsequently activates members of the phospholipid-dependent, serine/threonine-specific PKC family (7), which play a pivotal role in the regulation of the molecular response to growth factors and mitogens (10, 11). The participation of PKC in radiation-mediated gene induction has been suggested in recent studies. Down-regulation of PKC following prolonged TPA stimulation or PKC inhibition each results in attenuation of X-ray-mediated expression of transcription factor genes jun/AP-1 and Egr-1/zif-268 (12). In addition, X-ray-mediated transcriptional activation of the long terminal repeat of the Maloney murine sarcoma virus is also abrogated by each of these techniques (13).

TNF expression following X-irradiation is regulated at the transcriptional level in HL-60 promyelocytic leukemia cells (14). However, the mechanisms of radiation-mediated TNF transcriptional activation have not been determined. Mechanisms of TNF gene transcription in response to many external stimuli include activation of PKC by TPA indicating the importance of this signal transduction pathway in TNF production (15, 16). Furthermore, the promoter region of the TNF gene contains a TPA responsive element (17). We were therefore interested in studying the role of PKC-mediated signal transduction in radiation-induced TNF expression.

MATERIALS AND METHODS

Cell Cultures. HL-60 cells (American Type Culture Collection, Rockville, MD) were grown in 20% heat inactivated FBS (Rehautin, Purchase, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, and RPMI (GIBCO). HL-525 TPA-resistant variants of HL-60 cells were isolated by exposing wild-type cells to low concentrations of TPA (0.5 to 3 nm) through 102 passages 7 days apart (18). Cell line HL-205 is a variant of HL-60, which differentiates in response to TPA like wild type HL-60 cells. These cells were grown in heat inactivated 20% FBS in RPMI with 1 mM L-glutamine and 100 units/ml penicillin, 100 μg/ml streptomycin.

Isolation of Total Cellular RNA. HL-60 cells (2 × 10⁷) were X-irradiated with 20 Gy (GE Maxitron X-ray Generator, 2.1 Gy/min) as described previously (1). Control cells were treated with identical conditions but not irradiated. Cells were pretreated with protein kinase inhibitors H7 (100 μM) and HA 1004 (100 μM) (Seikagaku America, St. Petersburg, FL) (19, 20) for 1 h prior to irradiation. TPA 1 μM was added for 24 h to down-regulate PKC as described previously (21). During TPA stimulation, 50 nm were added 3 h prior to RNA extraction. Total cellular RNA was extracted as described by Chomczynski and Sacchi (22).

RNA Blot Hybridizations. Total cellular RNA (20 μg) was separated on a formaldehyde-1% agarose gel electrophoresis and transferred to nylomembrane (Gene Screen Plus) as described previously (1). Northern hybridizations were performed in 50% (v/v) formamide, 0.75 M NaCl, 0.075 M sodium citrate at 42°C. The TNF cDNA probe pE4 (23) and 7S probe were labeled with [32P]dCTP as described (1). Final washes
were with 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS at 68°C for 1 h.

Assay of the Phosphorylation Capacity of Protein Kinase C. HL-60 cells (2 \times 10^7) were incubated in RPMI 0.1% FBS for 24 h, pelleted in microfuge tubes, and irradiated with 20 Gy using a 60Co source (Gammacell 220) at a dose rate of 3.2 Gy/s, while a control tube underwent sham irradiation and simultaneous protein extraction. Protein was extracted on ice at 15-s intervals following irradiation by the addition of 0.4 ml of lysis buffer [Tris/HCl (pH 7.5) 20 μM, EDTA 0.5 mM, EGTA 0.5 mM, and 2-mercaptoethanol 10 mM (TEM with 0.5% Triton X-100, and 25 μg/ml each leupeptin and aprotinin]. Cells were homogenized in a Dounce homogenizer and incubated for 30 min on ice followed by centrifugation on separate cellular debris. Protein from 2 \times 10^7 cells was added to DEAE cellulose (0.5 g/column; Whatman DE52) columns prewashed with TEM. Protein was eluted with 2.5 ml of 0.2 M NaCl in TEM and collected on ice. Partially purified protein extract (25 μl) was added to 25 μl of TEM, 5 μl of phosphohistidyl serine and 10 mm phorbol ester in Triton X-100 mixed micelles; GIBCO) (24, 25), and 10 μl of 12P]ATP/substrate (5 \times 10^5 cPM/ml of 12P]ATP, 100 μM ATP, 250 μM synthetic peptide Gln-Lys-Arg-Pro-Ser(8)-Gln-Arg-Ser-Lys-Tyr-Leu, 5 mM CaCl2, 100 mM MgCl2, and 20 mM Tris pH 7.5) (GIBCO) (24). Following incubation for 5 min at 30°C, samples were dried on phosphocellulose and washed in 1% H3PO4 twice for 5 min followed by washing in H2O twice for 5 min. Scintillation counts of each sample and 10 μl of unwashed 12P]ATP/substrate were performed. To calculate the rate of 32P incorporation into the peptide substrate, 100 μM synaptic PKC specific inhibitor peptide (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn) (GIBCO) in 20 nm Tris 7.5 (26) was added to PKC assays prior to [12P]JATP/substrate and samples were incubated, washed, and counted as described above. This peptide shares the amino acid sequence of the regulatory domain of PKC and has been shown to be a specific inhibitor of this enzyme (26). PKC activity as determined by peptide phosphorylation was corrected for that which may have occurred from other protein kinases by the addition of a PKC specific inhibitory peptide (24). Background 32P incorporation was subtracted from that of assays without inhibitor and the rate of 32P incorporated into the peptide substrate was calculated (pmol/min) as described previously (24). Phosphorylation rates were normalized to 10^6 cells per assay.

Two-Dimensional Gel Analysis of Phosphoproteins. Cells were incubated in 0.1% serum for 48 h and [12P]orthophosphate (0.5 mCi/ml phosphate free minimum essential medium; GIBCO) was added for 45 min. Cells were washed with serum free Dulbecco’s modified Eagle’s medium and γ-irradiated as described above. Lysis buffer (0.5% SDS, 8 M urea, 50 mM 2-mercaptoethanol) was added at 15-s intervals on ice. Isoelectric focusing of 100 μg total cellular protein was performed using ampholines (LKB) pH 4.0 to 7.0, 20% 2.5–4.0, 60% 3.5–9.0, 20% 3.5–10.0 as described by O’Farrell (27). The second dimension consisted of 10% SDS-polyacrylamide gel electrophoresis as described by Laemmli (28). Gels were dried (Bio-Rad model 583) and autoradiographed at −80°C with intensifying screens. Control cells were treated with identical conditions but not irradiated.

RESULTS

Diminished Protein Kinase C Activity Abrogates Radiation-mediated TNF Expression. To determine whether ionizing radiation induces TNF transcription through a PKC dependent pathway, we analyzed radiation-mediated TNF gene expression following PKC depletion or inhibition, or in variant HL-60 cell lines that are deficient in signal transduction via the PKC pathway. TNF expression was undetectable in unirradiated control HL-60 cells, whereas radiation-mediated TNF expression occurred 3 h following X-irradiation (20 Gy) (Fig. 1). To demonstrate PKC dependence for X-ray-mediated induction of TNF expression, protein kinase inhibitors were added 1 h prior to irradiation. H7 is a potent inhibitor of both PKC and the cyclic nucleotide-dependent protein kinases, whereas HA1004 is a selective inhibitor of cyclic nucleotide-dependent protein kinases but has much less affinity for PKC (19, 20). H7 and HA1004 had no effect on cell viability as measured by trypan blue dye exclusion. Pretreatment of HL-60 cells with H7 (100 μM for 1 h) abrogated the radiation-mediated TNF expression (Fig. 1). In contrast, HA1004 (100 μM for 1 h) had no effect on radiation-induced TNF expression. To further demonstrate the requirement of PKC activation for X-ray-mediated TNF induction, PKC was down-regulated by exposure to TPA for 24 h as described previously (21). This approach abrogated X-ray-mediated TNF expression (Fig. 1). These data support the notion that PKC-dependent signal transduction is required for radiation-mediated TNF expression.

TNF transcriptional induction by TPA is a marker for monocytic differentiation of HL-60 cells. Variant HL-60 clones that exhibit different relative susceptibilities to TPA-induced monocytic differentiation have been established. The variant cell line HL-525 demonstrates no translocation of PKC during TPA treatment and has a deficiency in the PKC signal transduction pathway that precedes TPA-mediated c-fos induction and monocytic differentiation (29). Moreover, TPA does not induce TNF expression in this variant. Conversely, the cell line HL-205 demonstrates normal PKC translocation and transcriptional induction of c-fos and TNF in response to TPA stimulation. These cell lines were used to further study the role of

Fig. 1. TNF expression in X-irradiated HL-60 cells is attenuated following PKC inhibition. TPA lane is RNA from cells pretreated with TPA to down-regulate PKC prior to irradiation. The protein kinase inhibitor H7 and the selective inhibitor of cyclic nucleotide protein kinase HA1004 were added 1 h prior to irradiation.

PKC-dependent signal transduction in radiation-mediated TNF induction. Fig. 2 demonstrates that TNF is expressed following X-irradiation in HL-205 cells but not the TPA-resistant variant HL-525. Taken together, these results suggest that signal transduction via the PKC pathway is required for X-ray-mediated TNF induction.

Ionizing Radiation Increases the Phosphorylation Capacity of Protein Kinase C. To determine directly whether PKC is activated by ionizing radiation, we measured the phosphorylation capacity of PKC in γ-irradiated human tumor cell lines. The synthetic peptide, Gin-Lys-Arg-Pro-Ser(8)-Gin-Arg-Ser-Lys-Tyr-Leu, which is the major site of PKC phosphorylation of myelin basic protein, has been shown to be a specific substrate for PKC (24, 30). We utilized this substrate for in vitro assays of phosphorylation capacity of PKC. HL-60 cells were pelleted and irradiated with 20 Gy (320 cGy/s) and protein was extracted at 15-s intervals as indicated in Fig. 3. Control cells (sham-irradiated) were treated with identical conditions but not irradiated. The increase in PKC activity in γ-irradiated cells was compared to sham-irradiated controls (Fig. 3). The first protein extraction, performed 15 s following γ-irradiation, was found to have PKC activity that was 4.5-fold greater than that of control, whereas peak PKC activity at 30 s was 4.7-fold greater and returned to basal levels within 60 s. The phosphorylation capacity of PKC following γ-irradiation was compared to that produced by TPA stimulation and sham irradiation (Fig. 3).

To further demonstrate protein kinase participation in the cellular response to ionizing radiation, we analyzed protein phosphorylation using two-dimensional gel electrophoresis. Cells were incubated in 0.5% serum for 24 h, followed by labeling with \( ^{32} \)P orthophosphate for 45 min. Cells were then γ-irradiated as described above. Lysis buffer was added at the indicated times (Fig. 4). At 15 s following irradiation, protein phosphorylation was unchanged from that of unirradiated control cells, whereas phosphorylation occurred within 45 s and is compared to that produced by TPA stimulation (Fig. 4). A phosphoprotein that is similar to the M, 80,000 protein, which has been proposed to be a PKC specific substrate (MARKS protein), is indicated (Fig. 4, arrows) (31). Phosphorylation returned to basal levels at 60 s following γ-irradiation. Thus, in vivo phosphorylation of a PKC specific substrate occurs during peak enzymatic activity as shown in Fig. 3. These results further support PKC activation by ionizing radiation.

**DISCUSSION**

Transcriptional induction occurs in prokaryotes and yeast during the response to ionizing radiation (reviewed in Ref. 32). The mechanisms of transcriptional induction are best characterized in prokaryotes. DNA repair processes are initiated by DNA damage, which activates an enzyme (RecA) that in turn participates in transcriptional induction of genes encoding repair enzymes (33). In eukaryotes, DNA damage initiates the UV-response, which can be modified by the addition of protein kinase inhibitors implicating a reverse signal transduction path-
Activation of PKC by ionizing radiation may lead to phosphorylation of cytoplasmic proteins which subsequently enter the nucleus to initiate TNF transcription. Recent studies have demonstrated a M, 40,000 DNA binding protein that is located within the cytoplasm of untreated cells and is translocated into the nucleus of lymphoblastoid cells following X-irradiation to bind to the enhancer region of simian virus 40 (39). These data implicate signal transduction within the cytoplasm, which is supported by the present findings that protein kinase C activation is required for radiation-induced TNF expression. Further support of the requirement of PKC-mediated signal transduction preceding radiation-mediated transcriptional induction is the recent observation that PKC inhibition and down-regulation result in attenuation of X-ray-mediated expression of transcription factor genes jun/AP-1 and Egr-1/zif-268 (12). In addition, X-ray-mediated transcriptional activation of the long terminal repeat of the Maloney murine sarcoma virus is also abrogated by these techniques (13). This proposed mechanism of signal transduction is analogous to that observed following TPA-induced activation of NFkB in human lymphoblasts (40). NFkB is inactivated in the cytoplasm by binding to IkB, which is phosphorylated following PKC activation allowing NFkB translocation into the nucleus to initiate transcription. The activation of NFkB following DNA damage by UV-irradiation is abrogated by protein kinase inhibitors but not inhibitors of protein synthesis (34). The signal transduction pathway initiated following X-ray-mediated activation of PKC may thus involve post-translational modification of trans-acting factors leading to transcription.

We have previously demonstrated that radiation-mediated TNF gene expression results in TNF protein production (1). Thus, the biological significance of radiation-mediated PKC activation is the resulting TNF production. The potential importance of TNF induction in tumors and normal tissues is demonstrated by the wide range of effects of this cytokine on neoplastic cells and organ systems. The previously described consequences of radiotherapy such as pneumonitis, fatigue, anorexia, and vascular injury may be associated with radiation-mediated TNF induction. TNF induction following PKC activation is thus a novel mechanism of the cellular response to ionizing radiation. Herein, we demonstrate direct evidence that PKC is activated following X-irradiation of HL-60 cells by measuring the phosphorylating capacity of this enzyme. The relevance of this finding is demonstrated by the attenuation of radiation-mediated TNF induction following PKC depletion or inhibition. PKC inhibitors therefore represent a potential means of pharmacologically manipulating the cellular response to ionizing radiation. Furthermore, PKC is a non-DNA repair enzyme demonstrated to be activated immediately following X-irradiation. PKC activation and subsequent TNF induction serve as a model for subsequent investigations of the biochemical events required for radiation-mediated gene activation and the biological effects of X-rays.

REFERENCES

X-RAY ACTIVATION OF PKC MEDIATES TNF EXPRESSION


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