Regulation of Protein Kinase C by Ionizing Radiation

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ABSTRACT

Members of the protein kinase C (PKC) gene family have been shown to play an important role in tumor promotion and regulation of cell growth. Experiments were designed to examine the effects of different qualities of ionizing radiation administered at a variety of doses and dose rates on the expression of PKC-specific mRNA in confluent Syrian hamster embryo cells. The results of these experiments showed that low-linear energy of transfer (LET) radiations (such as X-rays and γ-rays) can induce increased expression of PKC mRNA within 1 h after radiation exposure. Levels of expression of PKC mRNA were increased 4- to 6-fold over unirradiated controls. Dose effects were evident, with increased accumulation of PKC mRNA at higher doses (ranging from 6 to 200 cGy). Induction of PKC mRNA occurred at a time when total cellular transcription was reduced following irradiation. Similar exposure of the cells to fission spectrum JANUS neutrons, however, had little effect on PKC mRNA expression. Modest induction (2-fold compared to untreated cells) occurred when irradiations were at very low dose rates (0.5 cGy/min). These results suggest that induction of PKC mRNA may be a step in the transformation process caused by ionizing radiation. In addition, they demonstrate that different qualities of radiation may regulate PKC differently.

INTRODUCTION

The PKC gene family encodes a group of Ca++-, phospholipid-dependent serine/threonine-specific protein kinases (1, 2). PKC is activated by the binding of 1,2-diacylglycerol and its analogues (such as TPA) (3). The difference in activation between 1,2-diacylglycerol and its analogue TPA is evident in the prolonged effect of TPA caused by the long half-life of TPA within the cell (4, 5). Activation of PKC is evidenced in multiple ways: in translocation of PKC from the cytosol to the membrane; increased intracellular concentrations of the enzyme; increased intracellular PKC-specific kinase activity; and increased expression of PKC mRNA (4-8). Other reports have documented different effects of neutrons and γ-rays on DNA damage and repair following exposure of cells to radiation (9, 10). However, little work has examined differential transcriptional responses of cells to different qualities of radiation. In the experiments reported here, we examined expression of PKC-specific mRNA at various times following exposure of SHE cells to a variety of doses and dose rates of two different qualities (high versus low LET) of ionizing radiation.

Previous work from our laboratory has shown that some transcriptional responses (especially expression of actin and tubulin mRNAs) are similar in the first 3 h following exposure of cells to high- and low-LET radiations (11). However, this report documents induction of β-PKC within the first hour following exposure to either X-rays or γ-rays (low LET), but little change in expression of this gene following exposure of SHE cells to fission spectrum neutrons (high LET). The induction of PKC mRNA by low-LET radiations occurred at a time when general transcription within the cell was reduced. These results suggest that the cellular responses to high- and low-LET radiations may be quite different and that PKC activation may play a role in the transforming effects of low-LET radiations.

MATERIALS AND METHODS

Cells and Culture Conditions. In all experiments, we examined modulation of gene expression by ionizing radiation in SHE fibroblasts; these are normal, diploid cells that can be neoplastically transformed by low doses of ionizing radiation (12, 13). All cell cultures were established in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml). Cells were grown to confluence; 48 h prior to irradiation, they were placed in medium containing 1.0% fetal calf serum to maintain the cells in plateau phase. Studies of preirradiated SHE cells grown under this protocol showed them to be a mixed population of fibroblasts with >90–95% of the cells in the G0-G1 stage of the cell cycle. Cells for the present experiments were from passage 2.

Radiation Treatments. Cells plated in 100-mm Petri plates containing 10 ml medium were irradiated with 60Co γ-rays, 50 kVp X-rays, or fission spectrum neutrons (0.85 MeV) from the JANUS reactor. All irradiations were performed at room temperature on cells in plateau phase (>95% G0-G1 cells); equitoxic doses of neutrons, X-rays, and γ-rays were selected on the basis of survival data (13). Doses and dose rates were chosen on the basis of the significant frequency of morphological transformation as determined in a 10-day colony assay (13). Control cells were taken to the radiation chamber but not exposed to irradiation. Plates of cells were then incubated at 37°C for 1 or 3 h after irradiation but prior to harvest of the RNA.

Measurement of Transcription Levels. Total cumulative RNA synthesis in nuclei of SHE cells at various times postirradiation was determined in transcription run-on experiments. Nuclei were harvested at 4°C as previously described (14). In brief, SHE cells were lysed with 1% Triton X-100 in hypotonic solution, and nuclei were obtained by centrifugation through a pad containing 2 mM sucrose. Newly synthesized RNA was measured by pulse-labeling isolated nuclei in vitro for 15 min with [α-32P]UTP (16). RNA was purified by digestion with DNase I (5 μg/ml) and proteinase K (50 μg/ml) followed by precipitation at 4°C with trichloroacetic acid. Trichloroacetic acid-insoluble counts were measured from nuclei purified from cells at various times after irradiation. Equal numbers of nuclei were used for each point, with spectrophotometric techniques used to standardize for DNA content (15). Where needed, α-amanitin (10 μg/ml) was added to the transcription mix as a specific inhibitor of RNA polymerase II activity (15). All results represent experiments performed at least three times.

Purification of RNA and Northern Blots. RNA was prepared by isolation in 6 M guanidine isothiocyanate, extraction with phenol, and precipitation from 3 M sodium acetate, pH 6.0 (14, 16, 17). Poly(A)+ RNA was isolated by oligodeoxynucleotide cellulose column chromatography until no unbound material absorbing at 254 nm was detected. RNA was stored as an ethanol precipitate at −20°C. Routinely we found that poly(A)+ RNA represented 5–10% of the total RNA fraction.

RNA was separated by using formaldehyde agarose gel electrophoresis as described previously (18). Poly(A)+ RNA samples were dena-
tured in 50% formamide-1.9 M formaldehyde-0.2 M 3-(N-morpholino)propanesulfonic acid-50 mM sodium acetate-1 mM disodium EDTA (pH 7.5) for 15 min at 55°C and then separated on 1.2% agarose gels in 0.2 M 3-(N-morpholino)propanesulfonic acid-50 mM sodium acetate-1 mM disodium EDTA-2.2 M formaldehyde. Parallel lanes containing rRNA (Escherichia coli and mouse) and RNA ladder (BRL Laboratories, Bethesda, MD) markers were stained with ethidium bromide and photographed under UV light for use in sizing.

Northern transfers were performed as described (18). Blots were hybridized to 32Pnick-translated or oligo-labeled cDNA probes. Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 25–50 mM sodium phosphate (pH 6.5), 0.2% SDS, 0.2% bovine serum albumin, 0.2% ficoll, 0.2% polyvinylpyrrolidone, and 50 μg/ml sonicated denatured herring sperm DNA at 43°C. Prior to hybridization, all labeled probes were heat denatured at 90°C for 5 min. After hybridization, nonspecific binding was reduced by washing the hybridized blots three times for 1 h each at 43°C in 45 mM sodium citrate (pH 7.4), 0.45 M NaCl, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 μg/ml herring sperm DNA (sonicated, denatured), and 0.1% SDS and then three times for 1 h each in 1.5 mM sodium citrate (pH 7.4), 15 mM NaCl, 50 μg/ml herring sperm DNA (sonicated, denatured), and 0.1% SDS. The blots were then dried and exposed to X-ray film at -20°C.

In some experiments, the same blot was washed and hybridized successively to several different probes. Each probe was eluted by washing for 24 h in distilled, deionized water at 43°C, and blots were checked for total removal of the labeled probes by 24-h exposure to X-ray film. Blots were washed three times in hybridization buffer before rehybridization to a different probe. All blots are representative of results from three independent experiments. Relative quantitation of the hybridized probe was determined by using a Hirschmann microdensitometer.

Although equal amounts by weight, as determined by spectrophotometry, of RNA were loaded into each well of a given gel, we found sufficient variation from one preparation to another to make poly(A)+ analysis essential. mRNA analysis systems measuring the molar concentration of RNA with 3' poly(A) tails (Molecular Genetics Resources, Tampa, FL) were used for all poly(A)+ RNA preparations, and only RNA samples showing equimolar concentrations of poly(A)+ RNA were loaded onto the same gel.

cDNA Clones. We gratefully acknowledge the following people, who made clones available to us. Actin cDNA clones were obtained from Dr. A. Minty (Pasteur Institute, Paris, France), and α-tubulin cDNA was obtained from Dr. C. Veneziale (Mayo Clinic, Rochester, MN). PKC probes were provided by Dr. J. Knopf (Genetics Institute, Cambridge, MA). The β-PKC probe used in this work should hybridize to all β-PKC isoforms (19).

RESULTS

Induction of PKC mRNA by Irradiation. SHE fibroblasts were irradiated with equitoxic doses (13) of X-rays (75 cGy) and JANUS fission spectrum neutrons (21 cGy), both administered at a dose rate of 12 cGy/min, and poly(A)+ RNA was harvested at 1 or 3 h postirradiation. These RNA samples were analyzed for relative expression of PKC-specific mRNA by using Northern blots. Microdensitometric scanning of these blots, evident in Fig. 1, revealed rapid induction of mRNA for PKC within 1 h following exposure to X-rays, while exposure to neutrons caused an initial decrease in PKC mRNA with a return to control levels by 1 h postirradiation. Within the duration of the experiment, levels of PKC mRNA never returned to control levels after X-ray treatment. An equitoxic dose (90 cGy) of γ-rays caused induction of PKC mRNA similar to that of X-rays (data not shown; see below).

It should be noted that using Northern blot hybridizations probed for expression of α-, γ-, δ-, and ε-PKC in untreated and neutron- and γ-ray-exposed SHE cells, we could detect none of these transcripts in any of the RNA preparations derived from these cells (data not shown).

Dose/Dose Rate Effects on PKC mRNA Accumulation. Many dose responses and dose rate responses have been observed in cells exposed to ionizing radiations. We wished to determine whether the observed differential response of cellular PKC mRNA expression to ionizing radiations could be attributed to the different doses and dose rates being examined. We selected γ-ray and JANUS neutron doses and dose rates on the basis of the duration of the irradiation, because Fig. 1 clearly indicates the importance of kinetics in the PKC mRNA response.

Table 1 documents results from microdensitometric scans of Northern blots examining PKC mRNA accumulation after exposure to various doses and dose rates of neutrons and γ-rays. All results are based on PKC mRNA content in untreated cells (which is set at 1.0). All RNA samples were harvested from the cells 1 h after the completion of the irradiation. Results in Table 1, then, compare equitoxic doses of JANUS neutrons and γ-rays for the ability to induce PKC-specific mRNA. From the results, it is apparent that γ-rays are somewhat more effective in inducing PKC mRNA than are JANUS neutrons. In all cases, induction of PKC mRNA was modest.
Table 2 outlines the results of experiments designed to examine the effects of different γ-ray doses on PKC mRNA expression. We used a single dose rate, which had previously been shown to induce marked PKC mRNA accumulation. These results establish that as the γ-ray dose increases, the amount of PKC-specific mRNA similarly increases. This was apparent at both 1 and 3 h postirradiation. Similarly, results of experiments examining the effects of neutron dose and dose rate on PKC mRNA accumulation are presented in Table 3. Again, Northern blots were analyzed by microdensitometry as described above, and results were reported relative to PKC mRNA expression in untreated cells. We chose to use high and low dose rates for these experiments because of the observed low-dose rate effect of neutrons on cell transformation and viability (20, 21). Modest induction of PKC mRNA was observed in response to neutron exposure. A significant dose response or dose rate response was not readily apparent. It is also noteworthy that the levels of PKC mRNA in neutron-exposed cells did not reach the levels observed in γ-ray- or X-ray-exposed cells, especially in cells exposed to high dose rate γ-rays or X-rays.

Effects of Radiation on Total Transcription. The documented effects of ionizing radiation could be caused by generalized effects on cellular transcription. For that reason, we examined total transcription in isolated nuclei from untreated and irradiated (with γ-rays or neutrons) cells. Nuclei were pulse-labeled in vitro with [32P]UTP for 15 min prior to harvest of the RNA. Transcription was calculated relative to untreated controls (set at 100%). Fig. 2 documents the effects of various doses of neutrons (given at a dose rate of 0.1 cGy/min) and γ-rays (at a dose rate of 1 cGy/min) on total cellular transcription. All measurements were made on nuclei harvested 1 h after the completion of the irradiation. We did not examine effects of higher doses of neutrons at this dose rate because of the length of time required for the irradiation itself and the potential variation in results that this might cause.

The results in Fig. 2 demonstrate that low-dose neutrons administered at a low dose rate cause significant inhibition of transcription, far more than is observed at higher neutron doses or at any of the γ-ray doses. In Fig. 3, we report results of experiments examining the transcriptional effects of neutrons or γ-rays administered at higher dose rates (12 and 14 cGy/min, respectively). In these experiments, we did not observe the dramatic low-dose transcriptional inhibition detected in cells irradiated with neutrons at the low (0.1 cGy/min) dose rate. In addition, for neutrons, transcriptional effects were similar at 1 and 3 h postirradiation. For γ-rays, however, by 3 h postexposure, transcription began to recover to control levels.

DISCUSSION

This paper describes induction of PKC mRNA by low-LET radiations such as γ-rays and X-rays, within 1 h after treatment of the cells. This induction shows dose and dose rate dependence. JANUS fission spectrum neutrons, a high-LET radiation, had little effect on PKC mRNA. These results suggest potential differential regulation of mRNA expression/transcription by high- and low-LET radiations. Examination of the effects of these two radiation qualities on total transcription demonstrated a more dramatic transcriptional suppression and a longer time for transcriptional recovery for neutrons relative to low-LET radiations. These results suggest differential gene
regulation by high- and low-LET radiations.

The PKC gene family has been implicated in tumor promotion because it has been shown to be activated and induced by TPA and other tumor promoters. The high level of induction of PKC by low-LET radiations, such as γ-rays and X-rays, implicates tumor promotion as an event associated with low-LET radiations. This is particularly significant in light of findings by Housey et al. (22) and Persons et al. (23) demonstrating that increased expression of PKC in the absence of other cellular changes is sufficient to cause a loss of cell growth control. The fact that neutrons have a limited effect on PKC mRNA induction implies that neutrons may transform cells via an alternate pathway. This is further supported by work done by Han and Elkind (21) demonstrating that transformation frequencies with TPA/X-ray treatment are lower than those for TPA/neutron exposure in other fibroblastic cells, when equitoxic doses of X-rays and neutrons are compared. While the general response of cells to an initial radiation insult has remained uncharacterized, our results suggest that a specific pathway of phosphorylation via PKC may actually be initiated after exposure to radiation.

The level of PKC induction by X-rays and γ-rays (4- to 6-fold) is similar to that reported for other genes induced by DNA-damaging agents. Reports from Fornace's group have demonstrated that most genes induced within the first 4 h after DNA damage are induced approximately 2- to 4-fold (24-26). Few genes documented as being induced by DNA-damaging agents have been functionally characterized (except ubiquitin and c-fos), so that a pattern of the cellular response to radiation based on the identified induced gene products is difficult to generate. However, our findings and those of others suggest that a series of gene response groups are apparently induced, in some cases by multiple damaging agents (perhaps in a generalized stress response) and in other cases by specific damaging agents (perhaps as a specific response to damage by a particular agent). Induction of PKC may be somewhat specific for low-LET radiations, or the level of induction required for a cellular response to neutron exposure may be significantly less than that required for γ- or X-ray exposure.

We have observed dose and dose rate effects on total transcription in response to both high- and low-LET radiations. Interestingly, PKC mRNA is induced in a time frame during which total transcription within the cell is decreasing. The fact that particular doses of neutron radiation are more effective in inhibiting transcription when administered at low dose rates is reminiscent of previous findings by Hill et al. (20) documenting higher transformation rates in fibroblasts under similar conditions. This suggests, in fact, that neutrons administered at a low dose rate may be more damaging to cells than those administered at a high dose rate.

The sensitivity of total transcription as a measure of radiation-mediated damage is remarkable, particularly when neutron effects are examined. Clearly, doses as low as 1 cGy, when administered at low dose rates, can have a significant effect on cellular transcription. In most cell types, total transcriptional activity does not vary significantly from one experiment to another. This detection of transcriptional inhibition at low doses may prove to be a useful monitor for radiation exposure, especially for neutrons, where evidence of transcriptional inhibition is more long lived than for γ-rays.

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