Effect of Ionizing Radiation on AP-1 Binding Activity and Basic Fibroblast Growth Factor Gene Expression in Drug-sensitive Human Breast Carcinoma MCF-7 and Multidrug-resistant MCF-7/ADR Cells*

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We studied the effect of ionizing radiation on the activation of the AP-1 transcription factors and the regulation of basic fibroblast growth factor (bFGF) gene expression in drug-sensitive human breast carcinoma (MCF-7) cells and its drug-resistant variant (MCF-7/ADR). Northern blot and gel mobility shift assays showed that 135 Gy of ionizing radiation induced c-jun and c-fos gene expression, AP-1 binding activity, as well as bFGF gene expression in MCF-7/ADR cells. In MCF-7 cells, however, we observed little/no induction of bFGF gene expression and AP-1 binding activity after the stress. Nevertheless, MCF-7 cells transfected with plasmids containing c-jun gene contain high levels of bFGF protein. H-7 (60 μg/ml), a potent protein kinase C (PKC) inhibitor, inhibited the stress-induced AP-1 binding activity and bFGF gene expression in MCF-7/ADR cells.

Corroborating this observation, overexpression of PKCα induced bFGF gene expression in MCF-7 cells. Taken together, these results suggest that stress-induced bFGF gene expression is mediated through the activation of PKC and AP-1 transcription factors. Differences in the levels of PKC activity and AP-1 binding factors may be responsible for differential expression of bFGF among breast cancer cell lines. Although there are large differences in response to ionizing radiation between MCF-7 and MCF-7/ADR cell lines, we observed no significant differences in radiocytotoxicity between them.

It has been known for many years that alteration of the micro- or macroenvironment of a cell can trigger the highly complex cellular stress management system. Recent studies have shown that, in addition to the well known stress proteins, environmental stresses such as ischemia/hypoxia (Plate et al., 1993; Millauer et al., 1994), tumor promoters (Winkles et al., 1992), and radiation (Witte et al., 1989; Haimovitz-Friedman et al., 1991) are all stimuli for triggering the synthesis of angiogenic factor(s). The observation of the induction of these angiogenic proteins under these circumstances could have significant implicatons on the process of tumorgenosis. Upon these stresses, tumor cells may induce neovascularization by synthesizing and releasing diffusible tumor-derived angiogenic factors such as vascular endothelial growth factor and bFGF.

A fundamental question which remains unanswered is how the stresses stimulate the synthesis of these angiogenic factors. DNA sequencing studies suggest that angiogenic factor-related genes such as bFGF contain AP-1 cis-acting regulatory elements (TPA response element: TRE) (Kim et al., 1989a, 1989b; Shibata et al., 1991). In vitro DNA binding and in vivo footprinting experiments demonstrated that these regulatory elements are recognized by AP-1 transcription factors (jun and fos family proteins) (Angel et al., 1988; Deng and Karin, 1993). The activity of AP-1 transcription factor is regulated by the induction of jun and fos gene transcription and post-translational modification of their products (Binetruy et al., 1991; Boyle et al., 1991). Several researchers have reported that c-jun and c-fos genes are expressed in response to a wide range of stresses including heat shock exposure (Andrews et al., 1987; Buhk et al., 1990), UV irradiation (Angel et al., 1985; Hollander and Fornace, 1989; Stein et al., 1992), ionizing radiation exposure (Sherman et al., 1990; Hallahan et al., 1991a), and treatment with chemical agents (Andrews et al., 1987; Hollander and Fornace, 1989; Shibanuma et al., 1990) in mammalian cells. Moreover, these stresses also increase AP-1 binding activity (Piette et al., 1988; Hallahan et al., 1993). Thus, we hypothesized that the stress-induced activation of AP-1 transcription factors is responsible for the expression of the bFGF gene as a result of exposure to ionizing radiation. In this study, we also investigated differences between drug-resistant and -sensitive human breast carcinoma MCF-7 cells in response to ionizing radiation. We observed that the radiation-induced expression of jun and fos genes, AP-1 binding activity, as well as bFGF gene expression occurred more prominently in drug-resistant MCF-7/ADR cells compared to drug-sensitive MCF-7 cells. Nonetheless, there was no significant differences in the cytotoxic effects of ionizing radiation between these two cell lines.

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Cell Culture—Human breast carcinoma (MCF-7) and its multidrug-resistant subline (MCF-7/ADR) cells (S. H. Kim, Henry Ford Hospital, Detroit, MI) were cultured in McCoy's 5a medium with 10% iron-supplemented bovine calf serum (HyClone Laboratories, Logan, UT) and 26 mm sodium bicarbonate for monolayer cell culture. Two or 3 days prior to the experiment, cells were plated into 35-mm Petri dishes, T-150 flasks, or T-75 flasks. The Petri dishes/flasks containing cells were kept in a 37 °C humidified incubator with a mixture of 95% air and 5% CO2.

Radiation Exposure—Cells were irradiated at room temperature with a GE Maximar 250-III orthovoltage x-ray unit (General Electric, Chicago, IL) at a dose rate of 1.17 Gy/min. The tube voltage was 250 kVp, current was 15 mA, and added filtration was 0.25 mm Cu.

Northern Blot Analysis—Relative levels of c-jun, c-fos, and bFGF mRNA were determined using the Northern blot technique. Total cellular RNA was extracted by the LiCl-urea method of Tushinski et al. (1977). For RNA analysis, 3 µg of total RNA was electrophoresed in a 1% agarose-formaldehyde gel (Lehrach et al., 1977). The RNA was blotted from the gels onto nitrocellulose membrane, and baked at 80 °C for 2 h in a vacuum oven. Membranes were prehybridized at 42 °C in 50% formamide, 1× Denhardt's solution, 25 mM KPO4 (pH 7.4), 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM Na3C6H5O7), 50 µg/ml denatured and fragmented salmon sperm DNA. Hybridizations were conducted at 42 °C in prehybridization solution containing 10% dextran sulfate and radiolabeled appropriate cDNA probes (Oncogene Science, Uniondale, NY) at a concentration of 4 × 106 cpm/ml. After hybridization, blots were washed and placed into a stainless steel cassette with intensifying screens and autoradiographed. The films were scanned with a computerized laser scanning densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA). Quantitative measurement was performed with this instrument.

Preparation of Nuclear Proteins—Cells in T-150 culture flasks were rinsed twice with cold phosphate-buffered saline (PBS) and scraped into 10 ml of PBS. Cells were collected by centrifugation at 250 × g for 10 min. The pellet was resuspended in buffer A (10 mM HEPES, pH 8.0, 0.1 M sucrose, 50 mM NaCl, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol, and 0.5% Triton X-100) and incubated on ice for 10 min. The nuclei were collected by centrifugation for 10 min at 1000 × g. The nuclear pellet was rinsed twice with 5 ml buffer A and resuspended in 0.5 ml of buffer B containing 0.5 mM NaCl. After 30 min of incubation on ice with occasional gentle agitation, the nuclei were pelleted at 2000 × g for 10 min and supernatant was brought to 45% saturation with (NH4)2SO4. The precipitated proteins were collected by centrifugation in a microcentrifuge at 14,000 rpm for 30 min, and finally the pellet was resuspended in 100 µl buffer B containing 100 mM NaCl. The protein content of the resulting nuclear extract was determined by Bradford assay (Bradford, 1976).

Gel Mobility-shift Assay—A double-stranded oligonucleotide, the TRE-like sequence (GAG TTT AAA CTT TTA AAA GTT GAG TCA CGG CTG GTT G) of the human bFGF gene (Shibata et al., 1991), was used in the binding reaction. The binding assay contained 2–5 µg of nuclear extract, 1 µg of poly(dI-dC)·poly(dI-dC), and 0.5 ng of 32P-labeled double-stranded oligonucleotide in 10 mM HEPES, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, and 7 mM β-mercaptoethanol. The assay mixture was incubated at room temperature for 15 min in a final volume of 25 µl. After the incubation, 5 µl of 6× dye solution (0.1% bromphenol blue, 30% glycerol) was added to the reactions and the samples were immediately loaded and electrophoresed on a nondenaturing 4.5% polyacrylamide gel for 2.5 h at 140 V in 0.5× TBE (1× TBE: 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA). After electrophoresis, gels were dried in a slab gel dryer for 1.5 h at 80 °C and exposed on Fuji RX film.

Immunoprecipitation of AP-1 Binding Factor—Involvement of c-jun un AP-1 binding activity was determined by immunoprecipitation technique. Affinity purified polyclonal antibody (0.5 µg) against c-jun un protein (Oncogene Science) was added to 5 µg of nuclear extract and incubated at room temperature for 1 h. After the incubation, 2.5 µg of affinity purified anti-rabbit IgG was added to the samples and incubated overnight at 4 °C. Protein-antibody complexes were removed by centrifugation in a microcentrifuge at top speed for 10 min, and the supernatant was used in gel mobility shift assays.

RESULTS

Radiation-induced c-jun and c-fos Gene Expression—Northern blots in Figs. 1A and 2A show the level of c-jun and c-fos mRNA after x-ray exposure in adriamycin-sensitive (MCF-7) and -resistant (MCF-7/ADR) cells. Note that differences in mRNA after x-ray exposure in adriamycin-resistant MCF-7/ADR cells (S. H. Kim, Henry Ford Hospital, Detroit, MI) were in the range of 59–77%.

Polyacrylamide Gel Electrophoresis (PAGE)—Samples were mixed with 2× Laemmli lysis buffer (1× buffer: 2.4 µl glycerol, 0.14 µl Tris, pH 6.8, 0.21 µl sodium dodecyl sulfate (SDS), 0.3 µl bromphenol blue), and boiled for 5–15 min. Protein content was measured with BCA protein assay reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 µl β-mercaptoethanol and an equal amount of protein sample. X-ray was applied to a pre-denatured PAGE. Electrophoresis was carried out on 10–18% linear gradient SDS-polyacrylamide gels (Walker 1984).

Western Blot—Proteins that were separated by SDS-PAGE were transferred onto a nitrocellulose membrane by electroblotting. The transfer was performed at a current of 0.12 A and 30 V for overnight. The membrane was incubated in blocking solution (5% BSA) for 1 h at 25 °C, then washed, and then incubated with 1:1000 dilution of polyclonal antibodies (Oncogene Science, 1:130 dilution), the bFGF monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:50 dilution), or the protein kinase C (PKC) α polyclonal antibody (Life Technologies, Inc., 1:500 dilution). After incubation with the primary antibody, the membrane was washed, and incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG in the diluting solution (1:2000) for 1–2 h. The membrane was then stained using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Drug Treatment—1-(5-Isoquinolinesulfonyl)-2-methylpipеразине (H-7), N-[2-guanidinoethyl]-5-isoquinolinesulfonamide (HA1004), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma. Drug treatment was accomplished by aspirating the medium and replacing it with medium containing the drug. The drug treatment was terminated by aspiration and rinsing with Hanks' balanced salt solution.

Measurement of PKC Activity—Amerham's PKC enzyme assay system was used to determine PKC activity. Cells were mixed with extraction buffer (50 mM Tris·HCI, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% w/v β-mercaptoethanol, 0.5 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin), sonicated, and then centrifuged. Supernatants were saved and mixed with glycogen for storage. Protein content was determined by the Bradford method (1976). The reaction buffer (calcium buffer, lipid, peptide buffer, and dithiothreitol buffer) was added to the sample (50–100 µg of protein). The reaction was started by adding 122-µl TATP and continued for 15 min at 25 °C. The reaction was terminated by adding the stop reagent.
detected in MCF-7/ADR cells (Figs. 1 and 2).

Radiation-induced AP-1 Binding Activity—Gel mobility-shift assays in Fig. 3 show that AP-1 binding activity to a TPA responsive element (TRE; AP-1 binding site) sequence of the bFGF promoter was enhanced by radiation or TPA. We also observed that the level of radiation-induced AP-1 binding activity in MCF-7/ADR cells was much higher than that of MCF-7 cells. The AP-1 binding activity was shown to be highly specific to TRE in competition assays when the addition of 200 molar excess of unlabeled AP-1 abolished the radioactive signal from $^{32}$P-labeled AP-1 transcription factors and TRE complex (AP-1 detected in MCF-7/ADR cells (Figs. 1 and 2).

Fig. 1. Accumulation of c-jun mRNA after x-irradiation in MCF-7 or MCF-7/ADR cells. Cells were irradiated with 135 cGy and incubated at 37°C for the intervals (0.25, 0.5, 1.25, 3, 6, and 8 h) indicated at the bottom of each lane. Cells were harvested and RNA was isolated. An equal amount of RNA (30 µg) was loaded onto each lane of an agarose-formaldehyde gel for separation. After separation, RNA was blotted onto a nitrocellulose membrane, hybridized with $^{32}$P-labeled probes for c-jun and GAPDH mRNA, and autoradioographed (panel A). The autoradiography of c-jun mRNA was analyzed with a densitometer (panel B). C, RNA from untreated control cells. GAPDH, GAPDH probe was used to verify the equivalent amounts and integrity of RNAs loaded in each lane. Autoradiograms were all from the same blot, which was stripped and rehybridized with different probes.

Fig. 2. Northern blots of c-jun and c-fos mRNA after x-irradiation in MCF-7 or MCF-7/ADR cells (panel A) and quantitative analysis of c-jun mRNA (panel B). Cells were irradiated with 75 cGy and incubated at 37°C for the intervals (0.25, 0.5, 1.25, 3, 6, and 8 h) indicated at the bottom of each lane. Northern blot analysis was performed as described in Fig. 1. C, RNA from untreated control cells. GAPDH, GAPDH probe hybridized to the same blot to verify RNA uniformity.

Fig. 3. Detection of AP-1 binding activity in nuclear extracts from x-irradiated MCF-7 or MCF-7/ADR cells. Cells were irradiated with 135 cGy and incubated at 37°C for the intervals indicated at the top of each lane (0.25–3 h). The gel mobility-shift assay was performed with a $^{32}$P-labeled AP-1 oligonucleotide and nuclear extracts (5 µg) prepared from irradiated cells. Competition assays were performed by adding a 200-fold molar excess of an unlabeled AP-1 or an unlabeled SP1 oligonucleotide. C, nuclear extracts from untreated control cells. TPA, nuclear extracts from cells treated with 1 µM TPA for 1 h. Closed arrow indicates AP-1 binding activity. Open arrow indicates free $^{32}$P-labeled oligonucleotide fragment (FREE).

Data presented in Fig. 5 show that x-radiation induces bFGF mRNA. Although the bFGF gene is known to be a single copy and is located on human chromosome 4 (Mergia et al., 1986), primer extension analysis indicates that one start site is used to transcribe three sizes of bFGF mRNA (1.4, 3.7, and 7 kb) (Shibata et al., 1991). Our data from Northern blot assays confirmed the presence of three sizes of bFGF mRNA. Radiation elevated the level of bFGF mRNA in MCF-7/ADR cells. However, bFGF gene expression was not detected in MCF-7 cells. This variance may be due to the differences in the level of AP-1 transcription factor, and PKCα. This hypothesis was tested as described below.

Involvement of c-jun in the Regulation of bFGF Gene Expression—Data from transfection experiments demonstrated that MCF-7 cells that were transfected with the plasmids pRSV-c-jun or pCMV-c-jun containing the human c-jun gene contained an elevated level of bFGF protein (Fig. 6). These results were consistent with observations in MCF-7/ADR cells, which contain relatively high levels of c-jun and bFGF proteins (ADR in Fig. 6). Note that multiple sizes of bFGF protein originate from the use of the AUG codon as well as novel CUG translation initiation codons (Florkiewicz and Sommer, 1989). Consistent results were also obtained from MCF-7/ADR cells transfected with the pcMV-c-jun DNA vector, which contains a DNA binding domain defective c-jun mutant gene (data not shown). The elevated level of mutant c-jun protein resulted in the reduction of the AP-1 binding activity and bFGF protein level (data not shown).

Involvement of PKCα in the Regulation of bFGF Gene Expression—The Western blot in Fig. 7A demonstrated that the amount of PKCα in MCF-7/ADR cells was 6.5-fold higher than that in MCF-7 cells as determined by densitometer. Fig. 7B also showed a 5–7-fold elevation in the level of PKC activity in MCF-7/ADR cells compared to that of MCF-7 cells. The possible
involvement of PKC in the regulation of bFGF gene expression was studied by employing H-7, a potent PKC inhibitor (Figs. 8–10) or treatment with PKC analogues (60 μg/ml) significantly suppressed the levels of c-Jun, whereas the drug did not affect the GAPDH mRNA level (H-7 in Fig. 8). Data from gel mobility-shift assay demonstrated that treatment with H-7 (60 μg/ml) significantly suppressed the radiation-induced AP-1 binding activity (lane 1 in Fig. 9). In contrast, HA1004 (60 μg/ml), an H-7 analogue that is a potent inhibitor of cAMP and cGMP-dependent protein kinases and a weak inhibitor of PKC, did not suppress the radiation-induced increase in the AP-1 binding activity (lane 2 in Fig. 9). Northern blot and quantitative analysis in Fig. 10 demonstrated a 2-fold or more reduction of the 7 kb bFGF mRNA level in the presence of 60 μg/ml H-7. To confirm the role of PKCa in the regulation of bFGF gene expression, MCF-7 cells were transfected with plasmid pSV2M(2)6 containing bovine PKCa cDNA (Fig. 11). As compared to either parental MCF-7 or MCF-7-vector cells, PKCa was overexpressed approximately 5-fold in MCF-7-PKCa cells as determined by densitometric analysis (Fig. 11A). Interestingly, MCF-7-PKCa cells displayed bFGF gene expression as detected by Northern
Effect of bFGF is shown to be mediated by PKC activation and it can be abrogated by H-7 (Haimovitz-Friedman et al., 1994). Thus, our results raised a potentially important question. Given the large differences in the level of PKCα and the response of AP-1 factors and bFGF to ionizing radiation between MCF-7 and MCF-7/ADR cell lines, are MCF-7/ADR cells resistant to the cytotoxic effects of ionizing radiation? Second, can the effect be blocked by a PKC inhibitor? Data from Fig. 12A showed that there was no inherent differences in radiosensitivity between MCF-7 and MCF-7/ADR cells as displayed by cell survival curves. In addition, survival curves were still essentially superimposable for all radiation doses in the absence/presence of H-7 in both cell lines (Fig. 12, B and C). At the present time, only speculation can be made concerning these discrepancies. The lack of radioprotection in MCF-7/ADR cells suggests that extracellular bFGF rather than intracellular bFGF may have an important role in the radioprotection.

**DISCUSSION**

Our data from Figs. 1-3 demonstrated that ionizing radiation activated the expression of jun and fos genes and increased AP-1 binding activity in human breast cancer cells, particularly multidrug-resistant MCF-7/ADR cells. Our observations are consistent with results obtained in human epithelial cells (Hallahan et al., 1991a) and human sarcoma cell line RIT-3 (Hallahan et al., 1993). Several researchers have also reported that UV radiation or H₂O₂ treatment stimulates the expression of both c-jun and c-fos, as well as AP-1 binding activity in HeLa S3 cells (Devery et al., 1991). Transcriptional activation of the c-jun gene is exerted through the distal and proximal AP-1 binding sites (TRE) in its promoter (Angel et al., 1988). In vitro DNA binding and in vivo footprinting experiments demonstrated that TRE is recognized by either c-jun homodimers (Angel et al., 1988; Deng and Karin, 1993) or c-jun/activating transcription factor-2 heterodimer (van Dam et al., 1993, Herr et al., 1994). The transcriptional activity of these binding factors is stimulated by the phosphorylation of c-jun (Binetruy et

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**Fig. 9.** Effect of H-7 or HA1004 on the radiation-induced AP-1 binding activity in MCF-7/ADR cells. A, untreated control cells. B, cells were irradiated with 135 cGy and nucleoproteins were extracted immediately after irradiation. C, cells were irradiated and nucleoproteins were extracted 0.5 h after irradiation. D, cells were treated with 60 µg/ml H-7 for 1 h before, during, and after irradiation. Nucleoproteins were extracted 0.5 h after irradiation. E, cells were treated with 60 µg/ml HA1004 for 1 h before, during, and after irradiation. Nucleoproteins were extracted 0.5 h after irradiation. The gel mobility-shift assay was performed as described in Fig. 3. Closed arrow indicates AP-1 binding activity.

**Fig. 10.** Effect of H-7 on bFGF gene expression. NO DRUG, cells were irradiated with 135 cGy and incubated at 37°C for the intervals (0.25, 0.5, 1, 2.5, 3, 6, and 8 h) indicated at the bottom of each lane. H-7, cells were treated with 60 µg/ml H-7 for 1 h before, during, and after irradiation. Incubation intervals after irradiation were shown at the bottom of each lane. Northern blot analysis (panel A) and quantitative analysis of 7-kb mRNA (panel B) were performed as described in Fig. 1. Arrows indicate the location and size of bFGF mRNAs (7, 3.7, 1.4 kb) on the left side of the panel. C, RNA from untreated control cells. GAPDH, GAPDH probe was used to confirm the amount and integrity of RNAs loaded in each lane. See legend of Fig. 1 for further details.

**Fig. 11.** Expression of bFGF gene in MCF-7 cells which were transfected with the plasmid containing the bovine PKCα cDNA. Panel A, Western blot analysis was performed as described in Fig. 7. MCF-7, parental MCF-7 cells; MCF-7-vector, MCF-7 cells were transfected with pSV-M(2)6 vector without the insert of PKCα gene. MCF-7-PKCα, MCF-7 cells were transfected with pSV-M(2)6 vector containing PKCα encoding cDNA; MCF-7/ADR, multidrug-resistant MCF-7/ADR cells. Molecular weight (10 3) is shown on the right. Panel B, Northern blot analysis of mRNA from x-irradiated MCF-7-vector and MCF-7-PKCα cells. Cells were irradiated with 135 cGy and then incubated for various times (0.25–8 h) indicated at the bottom of each lane. Northern blot analysis was performed as described in Fig. 1. The apparent reduction of the bFGF mRNA level in MCF-7-PKCα 3 h after irradiation was the result of underloading of the sample. Arrows indicate the location and size of bFGF mRNAs (kb) or rRNA on the right or left side of the panel, respectively. C, RNA from unirradiated control cells. GAPDH, internal standard GAPDH mRNA.

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can be activated by UV and x-irradiation (Radler-Pohl et al. 1992) also known as the extracellular signal-regulated protein kinase activation. Mitogen-activated protein kinase, is rapidly increased in response to cell stimulation with various cell types. The TCF, which belongs to the family of E26 transformation-specific (Ets) proteins (Dalton and Treisman, 1992), is 1992). The TCF, which belongs to the family of E26 transformation element. The SRE is recognized and stably bound by a homodimer of serum response factor (Treisman, 1986). The binary SRE-serum response factor complex interacts with another factor, the ternary complex factor (TCF) (Treisman, 1992). The TCF, which belongs to the family of E26 transformation-specific (Ets) proteins (Dalton and Treisman, 1992), is homologous to Elk-1 (Hipskind et al., 1991). The activity of TCF is rapidly increased in response to cell stimulation with various agents, such as growth factors, which lead to mitogen-activated protein kinase activation. Mitogen-activated protein kinase, also known as the extracellular signal-regulated protein kinase (ERK) can be activated by UV and x-irradiation (Radler-Pohl et al., 1993; Stevenson et al., 1994). It appears to be responsible for the phosphorylation of TCF (Gille et al., 1992; Marais et al., 1993) and subsequently its transcriptional activity (Zinck et al., 1993). The activity of v-sis-inducible factor, which binds to serum induction element is also enhanced by phosphorylation. However, it is regulated by a different signaling pathway: a cytoplasmic protein tyrosine kinase (Nordheim et al., 1994).

Stress-induced AP-1 binding activity is due to the posttranslation modification of both newly synthesized and preexisting J un and Fos proteins (Boyle et al., 1991; Smeal et al., 1991). cJ un contains three domains: a DNA binding domain, a transcription activation domain, and a regulatory domain. In the unstimulated state, cJ un is constitutively phosphorylated by glycogen synthase kinase-3 or casein kinase II at serines (Ser-243 and Ser-249) and threonines (Thr-231 and Thr-239) close to its C-terminal DNA binding domain. Phosphorylation in this region markedly reduces the DNA binding and transcription ability of cJ un (Boyle et al., 1991). Dephosphorylation in this region occurs upon stresses such as phorbol ester tumor promoter TPA treatment (Boyle et al., 1991) and UV irradiation (Devery et al., 1992) and results in enhanced AP-1 binding activity. Boyle et al. (1991) reported that TPA-activated PKC is responsible for site-specific dephosphorylation of cJ un. It has been known that PKC can be activated by stress such as heat shock (Wooten, 1991) and ionizing radiation (Hallahan et al., 1993). Our data (Fig. 7) and Lee et al. (1992) showed that MCF-7/ADR cells contain markedly elevated amounts of PKCα. These observations suggest that differences in the level of PKCα and the level of AP-1 transcription factors (cJ un and c-Fos) may be responsible for the differential effects of stress on various cell types.

Several researchers have suggested that PKC mediates stress-induced bFGF gene expression. Tumor promoters such as phorbol 12-myristate 13-acetate, mezereon, and phorbol 12,13-didecanoate activate PKC. These promoters induce the accumulation of bFGF mRNA and its protein in human dermal fibroblasts (Winkles et al., 1992). The enhancement of bFGF gene expression by these tumor promoters is reduced by treatment with H-7. These results are consistent with our observations, which demonstrate the reduction of x-ray-induced bFGF gene expression by H-7 (Fig. 10). Although the dose of H-7 (60 μg/ml) we use is relatively specific for PKC, we cannot rule out H-7 as a general kinase inhibitor. Nonetheless, these results and data from Fig. 11 strongly indicate that the x-ray-induced bFGF gene expression is likely mediated through activation of PKC. Data from Figs. 3, 5, 9, and 10 also show a good correlation between the AP-1 binding activity and an increase in bFGF gene expression. Moreover, Fig. 6 shows that bFGF level was elevated in cells which were transfected with plasmids containing human cJ un cDNA. Taken together, our data have indicated that the enhancement of bFGF gene expression is related to an increase in PKC and AP-1 binding activity after ionizing radiation exposure.

We believe that many critical questions still remain to be answered to understand the mechanisms of regulation of bFGF gene expression after x-irradiation. However, our proposed model will provide important information to understand how environmental stresses induce bFGF gene expression and subsequently lead to tumor angiogenesis. This model will also provide a framework to study the critical steps in tumor development and metastasis.

Fig. 12. Effect of H-7 on the radiation dose-survival curves of MCF-7 and MCF-7/ADR cells. Panel A, x-ray survival curves for untreated MCF-7 and MCF-7/ADR cells. Panel B, MCF-7 cells were treated with H-7 (60 μg/ml) for 1 h before and during irradiation. Panel C, MCF-7/ADR cells were treated with H-7 (60 μg/ml) for 1 h before and during irradiation. NO DRUG, untreated control cells. Point, mean of four separate experiments; Error bars, one standard deviation of the data for each point.

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