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EARLY RESPONSE GENE INDUCTION FOLLOWING DNA DAMAGE IN ASTROCYTOMA CELL LINES

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Abstract—Early response genes (ERGs) are a group of genes with low or absent expression in quiescent cells that can be induced rapidly by a variety of proliferation and differentiation stimuli. c-*jun* and c-*fos* are prototypes for this group of genes. Recent evidence suggests that DNA damaging agents such as cytotoxic drugs and ionizing radiation can elicit strong ERG induction, suggesting that these genes may be involved in DNA damage repair and/or cell death. Paralleling this interest in drug-induced ERG expression is a growing body of evidence implicating ERG expression as important to the intrinsic function of the central nervous system. We therefore set out to explore the pattern of chemotherapeutic drug- and radiation-induced c-*jun* and c-*fos* expression in neuroectodermally (astrocytic) derived cell lines. We have demonstrated that various chemotherapeutic agents and ionizing radiation can induce c-*jun* in a time- and concentration-dependent manner. Furthermore, this induction can be prevented by pretreatment of the cells with agents that protect against DNA damage. Finally, we have demonstrated that c-*fos* and c-*jun* expression and induction are discoordinately regulated, reflecting a difference in astrocytic cell lines compared to hemapoietic cell lines. The possible relevance of these observations to the clinical resistance of astrocytic tumors to standard chemotherapy and radiation is discussed.

c-*jun* is a well characterized proto-oncogene, which was first identified in its oncogenic form (v-*jun*) in the acute transforming avian sarcoma virus [1, 2]. Recently, the normal cellular homologue, c-*jun*, has been identified as a gene encoding a component of the mammalian transcriptional activator complex AP1 [3–5]. c-*jun* is a member of a group of genes including *jun*-B, *jun*-D, c-*fos*, and *fos*-B that encode for related transcriptional factors [6–8]. The protein products of these genes have several common structural motifs including a leucine zipper and a stretch of basic amino acids [9–12]. The leucine zipper allows for protein–protein interactions between various members of these families of transcriptional factors thereby forming homo- and heterodimers. The basic amino acid domain of these dimers allows for protein–DNA binding to a heptameric DNA consensus sequence known as the 12-*O*-tetradecanoylphorbol 13-acetate (TPA) responsive element [13–17].

c-*jun* can be induced as an early event following cellular exposure to a variety of cytokines including platelet-derived growth factor, epidermal growth factor, transforming growth factor, tumor necrosis factor and nerve growth factor [18–22]. This property places c-*jun* into a larger group of genes known as the early response genes (ERGs)† [23, 24]. The ERGs are defined as a group of genes whose expression is low (or absent) in quiescent cells but can be rapidly activated by a variety of external stimuli. This regulation of expression is rapid, occurring from minutes to hours following exposure to the stimuli, with an equally rapid return of expression to baseline. This induction of ERG expression is thought to occur generally by transcriptional activation and is usually independent of *de novo* protein synthesis [25, 26]. The rapidity of induction followed by rapid transcriptional termination, in conjunction with the relatively short half-life of most ERG mRNA transcripts, suggests that ERGs play an important regulatory role in the response of a cell to external stimuli.

In addition to the typical proliferation and/or differentiating growth factors that induce c-*jun* expression, recent evidence suggests that certain cytotoxic stimuli can similarly induce c-*jun* expression [27–30]. In particular, several chemotherapeutic agents such as etoposide, arabinofuranosylcytosine and cisplatin, as well as ionizing radiation, have been shown to be potent inducers of c-*jun* expression in hematopoietic cells. This highly reproducible induction of c-*jun* in response to cytotoxic agents suggests that c-*jun* may be involved in a specific intracellular response to cellular damage. Whether this response is part of the repair process, programmed cell death, or some other process remains to be elucidated.

Paralleling the growing interest in ERG induction by proliferation, differentiation and cytotoxic stimuli has been a growing body of work by neurobiologists that suggest that ERG expression is important to the intrinsic function of the central nervous system (CNS) (reviewed in Ref. 31). Specifically, ERG

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† Abbreviations: ERGs, early response genes; hiFBS, heat-inactivated fetal bovine serum; ara-C, cytosine arabinoside; MMC, mitomycin C; MMS, methylmethane sulfonate; PBS, phosphate-buffered saline; DTT, dithiothreitol; CDDP, cisplatin; NAC, *N*-acetyl-cysteine; 3-Ado, 3-deazaadenosine; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

expression has been implicated in neuronal membrane depolarization and cell-to-cell signaling [32–37]. Because of our interest in cytotoxic drug- and radiation-induced activation of ERG expression, and the apparent importance of these genes in the CNS, we were interested in examining the effects of drugs and radiation in neuroectodermally-derived cells. Based on the hypothesis that drugs/radiation-induced ERG expression may be relevant to clinical correlates of cytotoxicity, we chose to study expression of these genes in several astrocytoma-derived cell lines since astrocytomas are the most common primary brain tumors in adults and are particularly refractory to standard chemotherapy and radiation [38, 39].

The results presented here are the first description of ERG expression in astrocytoma cell lines to our knowledge. They demonstrate that c-*jun* is constitutively expressed at high levels in quiescent cells but is highly overexpressed following DNA damage by a variety of agents. In contrast to hematopoietic cell lines, however, c-*fos* is not expressed, even following exposure to drugs and radiation. This suggests that DNA damage-induced ERG expression differs in astrocytic cells compared to many other cell types.

MATERIALS AND METHODS

Cell culture. The T98G human glioblastoma cell line (American Type Culture Collection) was grown at 37° in Eagle's Minimal Essential Medium (MEM) containing 10% heat-inactivated fetal bovine serum (hiFBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids. The A-172 human anaplastic astrocytoma cell line was grown in DMEM with 4.5 g/L glucose and 10% HiFBS. The CCF-STTG1 human astrocytoma cell line was grown in RPMI containing 10% hiFBS with glutamine (2.9 mg/mL). The KNS-42 astrocytoma cell line was grown in DMEM with 10% hiFBS supplemented with 1 mM sodium pyruvate. Cells in logarithmic growth phase were treated with various concentrations of drugs (see Results for exact concentrations) including cytosine arabinoside (ara-C), mitomycin C (MMC), methylmethane sulfonate (MMS), BCNU (carmustine), cisplatin, and hydrogen peroxide (H_2O_2). Cells were irradiated using either a Phillips RT 250 accelerator at 250 kV, 14 mA equipped with a 0.35 mm copper filter or a gamma cell 1000 (atomic energy of Canada, Ottawa) with a Cs137 source emitting at a fixed dose rate of 14.3 Gy/min as determined by dosimetry.

Northern blot analysis. Total cellular RNA was isolated as described previously [40]. RNA (20 μg) was loaded into each lane and separated on an agarose/formaldehyde gel. Separated RNA was transferred to nitrocellulose and probed with a ^{32}P-labeled DNA. The DNA probes included a 1.8 kb BamHI/EcoRI c-*jun* cDNA, and a 0.91 kb ScaI/NcoI fragment containing exons 3 and 4 of the c-*fos* cDNA. Autoradiographs were exposed for 24–48 hr unless otherwise noted.

Nuclear run-on assays. Treated T98G cells were trypsinized, pelleted at 600 *g*, and washed in cold phosphate-buffered saline (PBS). Cells (5×10^6) were resuspended in cold lysis buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM MgAc, 0.1 mM Na_2EDTA 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 10 mM Tris–HCl, pH 8.0]. Cells were homogenized with a glass/teflon pestle homogenizer five times up and down. Disrupted cell morphology was confirmed microscopically. Nuclei were pelleted and resuspended in 100 μL glycerol buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA). An equal volume of reaction buffer (10 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM DTT) was added to the nuclei. This suspension was then incubated at 26° for 30 min with 200 μCi [α-^{32}P]UTP (>800 Ci/mmol; Amersham Corp., Arlington Heights, IL). The transcription reaction was terminated by the addition of 40 U DNase I, 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM Na_2EDTA, 60 μg/mL yeast tRNA and 150 U/mL RNasin for 15 min at 26°. Proteinase K (750 μg/mL) and 1% (v/v) sodium dodecyl sulfate (SDS) were added for 30 min at 37°. Nuclear RNA was purified by phenol/chloroform extraction and then ethanol was precipitated three times in 2.5 M ammonium acetate. RNA was purified through a Sephadex G50 spin column equilibrated in and eluted with column buffer (0.3 M NaCl, 0.1% SDS, 1 mM Na_2EDTA, 10 mM Tris–HCl, pH 7.5).

Probes were generated by digesting plasmid DNAs containing various cloned inserts with restriction endonucleases as follows: (a) the 2.0-kb Pst-1 fragment of the chicken β-actin pA1 plasmid; and (b) the 1.8-kb BamHI/EcoRI fragment of the human c-*jun* cDNA from the pBluescript SK(−) plasmid. The insert DNA was denatured by heating to 65° for 15 min, separated in a 1% agarose gel, and transferred to nitrocellulose filters by the method of Southern [41]. The filters were prehybridized in 5× Denhardt's solution (40% formamide, 4× SSC, 5 mM Na_2EDTA, 0.4% SDS, and 100 μg/mL yeast tRNA) for 2 hr. Hybridizations were accomplished with 10 cpm of ^{32}P-labeled RNA per mL hybridization buffer for 72 hr at 42°. The filters were then washed out in 2× SSC and 0.1% SDS at 37° for 30 min, 10 μg/mL RNase A in 2× SSC at 37° for 20 min, and 0.1× SSC and 0.1% SDS at 42° for 30 min.

RESULTS

Whole cell RNA from quiescent T98G, A-172, KNS-42, and CCF-STTG1 astrocytoma cell lines was hybridized to a probe for the human c-*jun* gene. All four cell lines expressed c-*jun* message, although at different levels (Fig. 1A). The level of c-*jun* expression did not appear to correlate with cellular growth rate or morphologic differentiation.

We were next interested in whether a number of DNA damaging agents, each with a distinct mechanism of action, could induce c-*jun* expression. Except where otherwise indicated, all induction studies were performed with T98G cells. c-*jun* expression was induced by several DNA damaging agents including MMC (Fig. 1B), cisplatin (CDDP) (Fig. 1C), MMS (see Fig. 5), ionizing radiation (see Fig. 3A), ara-C and BCNU (data not shown). The

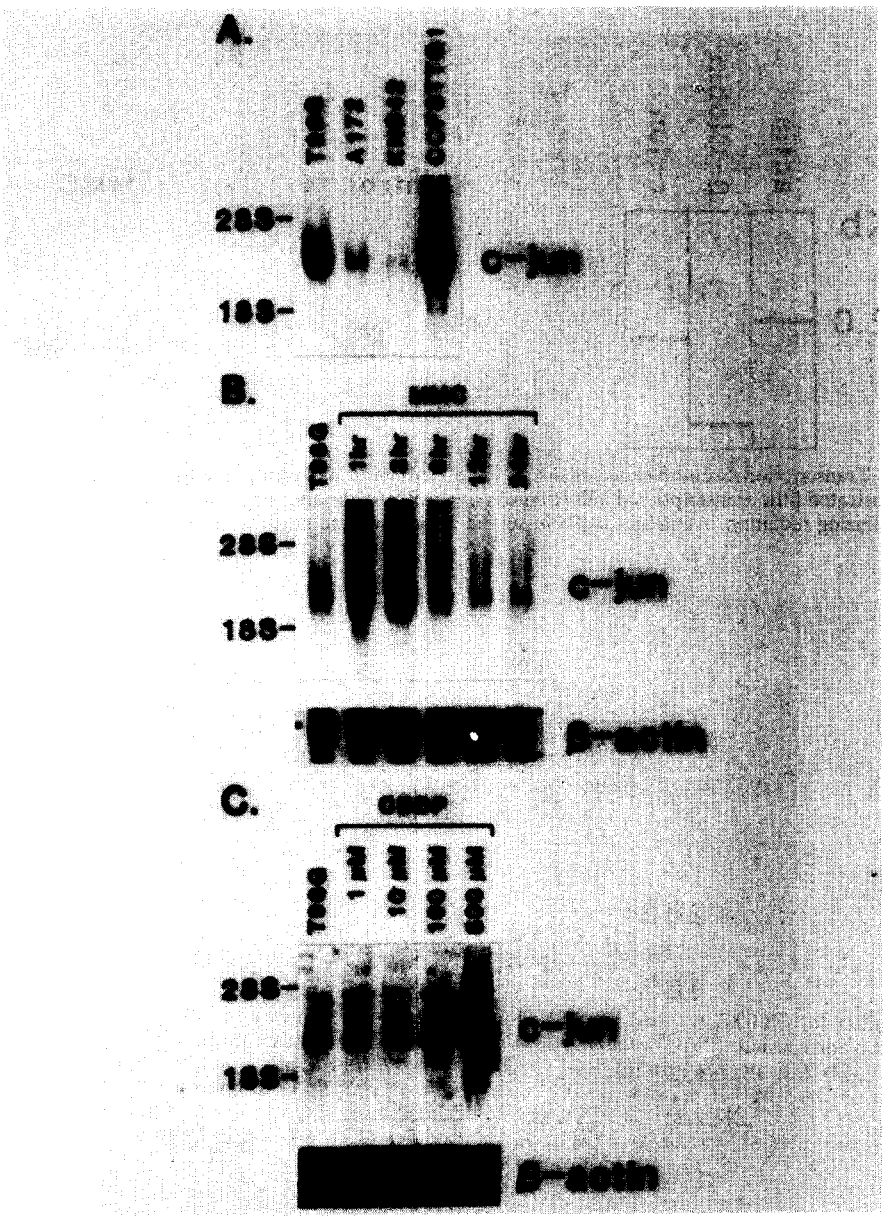

Fig. 1. c-*jun* mRNA expression in astrocytoma cell lines as demonstrated by Northern blot analysis (see Materials and Methods). All cells were harvested in the logarithmic growth phase. (A) Basal levels of c-*jun* mRNA expression in four astrocytoma cell lines; (B) time course of c-*jun* mRNA induction in T98G cells following exposure to 1×10^{-5} M mitomycin C (MMC); and (C) c-*jun* mRNA induction in T98G cells 3 hr following exposure to 1, 10, 100 and 500 μM cisplatin. β-Actin hybridization to washed Northern blot filters controlled for equal RNA loading in all induction experiments.

time course of c-*jun* induction by the cytotoxic drugs was consistent from one drug to another with a maximal response occurring 1–3 hr following drug exposure with return of c-*jun* to basal levels by 12–24 hr.

Nuclear run-on experiments demonstrated that most of the increase in c-*jun* transcripts following cellular exposure to cytotoxic drugs and radiation was largely secondary to post-transcriptional modulation of RNA rather than a large increase in gene transcription (Fig. 2).

We hypothesized that c-*jun* induction following exposure to ionizing radiation was the result of the generation of intracellular free-radicals. This idea was supported by the experimental observation that H_2O_2 could similarly induce c-*jun* (Fig. 3B). We tested this hypothesis further by examining the effect of the free-radical scavenger *N*-acetyl-cysteine (NAC) with and without radiation on c-*jun* induction. Radiation-induced c-*jun* expression was inhibited significantly by pretreatment with NAC (Fig. 4A). These experiments suggest that radiation-mediated

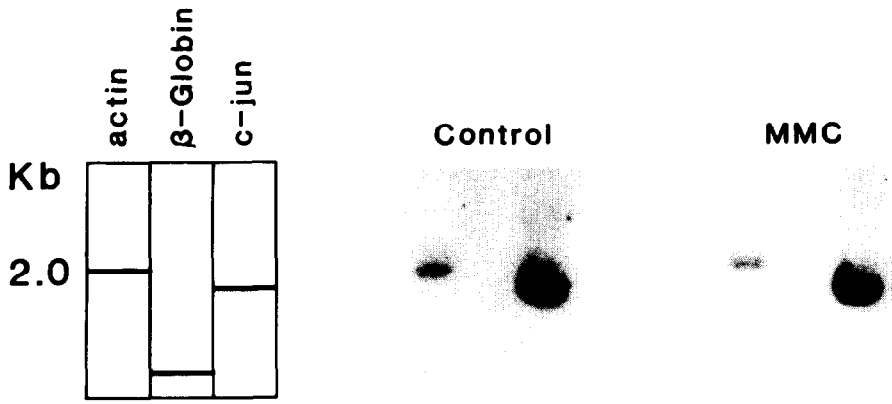

Fig. 2. Transcriptional run-on experiments. These experiments, as described in Materials and Methods, demonstrated little transcriptional induction of c-*jun* mRNA 3 hr after exposure of cells to 2000 cGy of ionizing radiation. Actin and β-globin served as positive and negative controls, respectively.

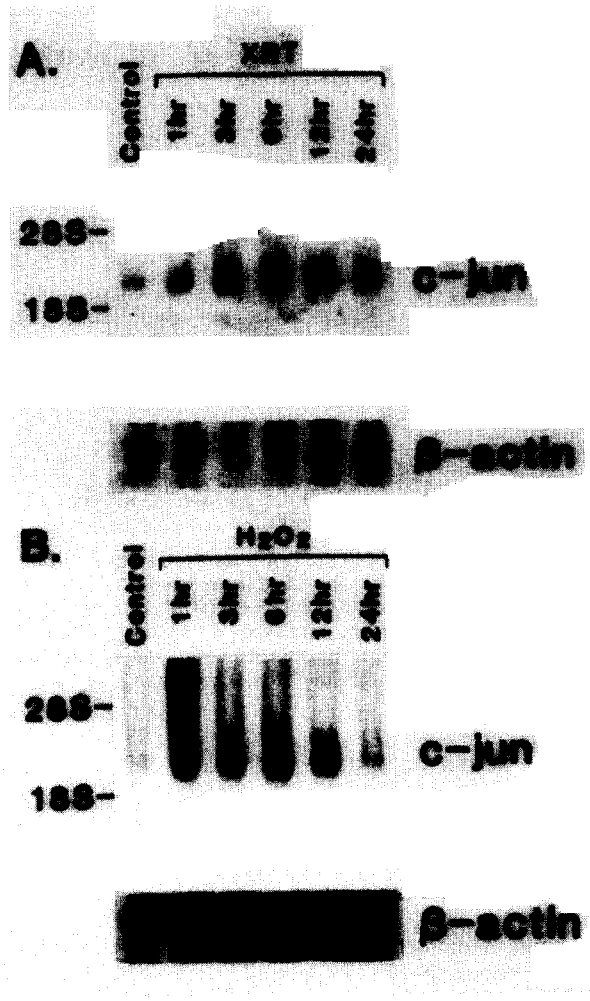

Fig. 3. (A) c-*jun* mRNA induction in T98G cells at 1, 3, 6, 12, and 24 hr following exposure to 2000 cGy of ionizing radiation. (B) c-*jun* mRNA induction in T98G cells at 1, 3, 6, 12, and 24 hr following exposure to 1.5×10^{-4} M H_2O_2.

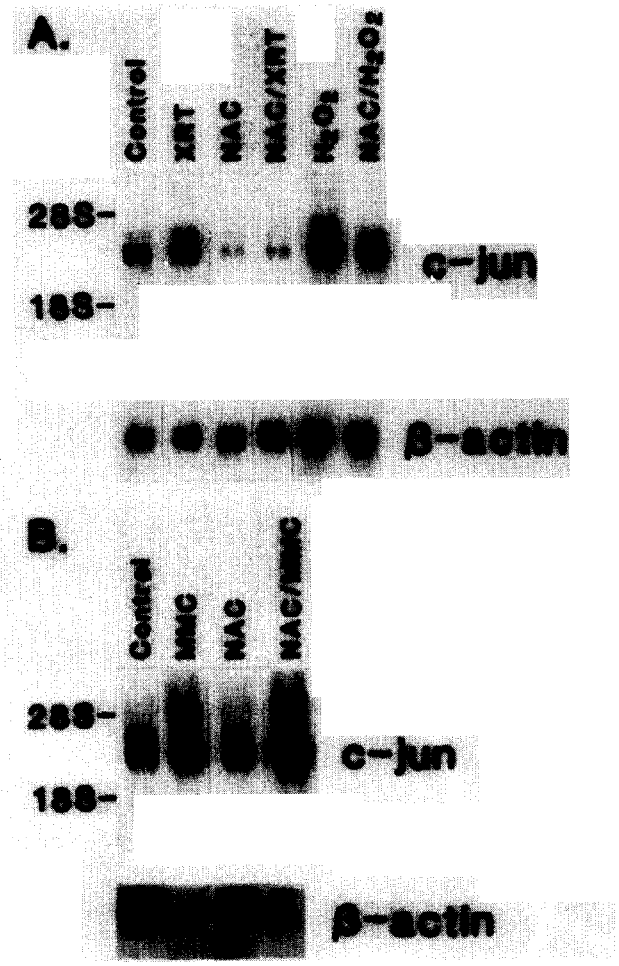

Fig. 4. (A) c-*jun* mRNA induction in T98G cells 3 hr following exposure to 2000 cGy of radiation (XRT), 1.5×10^{-4} M H_2O_2 with and without 2×10^{-5} M N-acetyl-cysteine (NAC). Lanes 4 and 6 show the effects of pretreatment of cells with 2×10^{-5} M NAC prior to exposure to 2000 cGy XRT 1.5×10^{-4} M H_2O_2, respectively. (B) Effect of pretreatment of cells with 2×10^{-5} M NAC on c-*jun* mRNA induction 3 hr after exposure of cells to 1×10^{-5} M MMC.

c-*jun* induction is a direct effect of free-radical generation.

We were similarly interested in the proximal steps leading to drug-induced c-*jun* expression. Although most of the agents examined in this report are thought to have different mechanisms of action, most are considered to have DNA alkylating properties. We therefore examined the ability of the demethylating agent 3-deazaadenosine (3-Ado) to block the induction of c-*jun* following exposure to MMS and MMC. 3-Ado caused a slight increase in the level of c-*jun* at 3 hr (Fig. 5) and 6 hr (data not shown) post-drug exposure. Nevertheless, 3-Ado significantly inhibited c-*jun* induction by both MMS and MMC at 3 and 6 hr. NAC, however, had no effect on MMC-induced c-*jun* expression (Fig. 4B).

In addition to c-*jun*, another ERG that we were particularly interested in studying was c-*fos*. Basal levels of c-*fos* expression were undetectable in all four astrocytoma cell lines (Fig. 6). To our surprise, c-*fos* could not be induced by any drug or radiation with the exception of a small increase at 1 hr after MMC exposure (data not shown). These data, therefore, suggest that c-*jun* and c-*fos* expressions have discoordinate regulation in response to DNA damaging agents in astrocytic cells.

DISCUSSION

In the present study, we have demonstrated high basal levels of c-*jun* expression in a variety of astrocytoma derived cell lines. We have also described in detail the ability of several cytotoxic drugs and ionizing radiation to induce expression of c-*jun* in a time- and concentration-dependent manner. In addition, we performed similar experiments examining the basal level of c-*fos* and its ability to be induced by radiation and cytotoxic agents. These experiments demonstrated a significant difference in regulation between these two proto-oncogenes in astrocytic cell lines.

Although similar experiments using hematopoietic

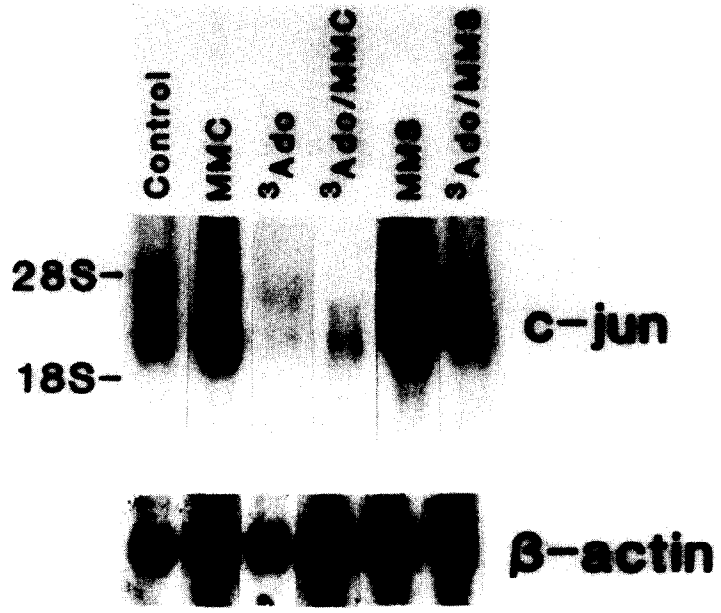

Fig. 5. c-*jun* mRNA induction 3 hr following exposure to 1×10^{-5} M mitomycin C (MMC), 1×10^{-4} M deazaadenosine (3-Ado), and 3×10^{-4} M methylmethane sulfonate (MMS). Lanes 4 and 6 show the effect of pretreatment of cells with 1×10^{-4} M 3-Ado prior to exposure to 1×10^{-5} M MMC and 3×10^{-4} M MMS, respectively.

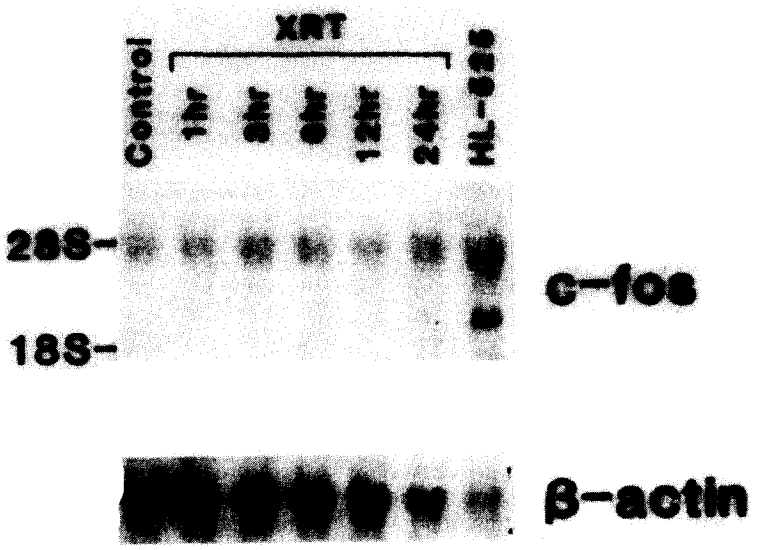

Fig. 6. c-*fos* mRNA induction in T98G cells at 1, 3, 6, 12, and 24 hr following exposure to 2000 cGy of ionizing radiation. HL-525 cells harvested at 6 hr following exposure to 2000 cGy of ionizing radiation served as a positive control.

cell lines have been described in the literature, this is the first such description of c-*jun* and c-*fos* inducibility in neuroectodermally derived cell lines (especially human tumor cell lines) to our knowledge. This is of particular interest to us given the apparent growing importance of ERGs in the function of the CNS.

The mechanisms by which c-*jun* is induced following cytotoxic drug exposure or ionizing radiation remain unclear. This is particularly perplexing given the fact that most of the drugs tested in this study have different mechanisms of action. Nevertheless, there is one common end result of all these agents, that being DNA damage. This type of eukaryotic cellular response may be similar to the induction of specific repair genes (i.e. RAD54) seen in prokaryotic cells after UV light-induced DNA damage [42, 43]. Our study provides evidence,

albeit indirect evidence, that DNA damage is a major stimuli for c-*jun* induction in human astrocytoma cell lines. First, based on the hypothesis that radiation-induced DNA damage is mediated by oxygen free-radicals, we demonstrated that inhibition of cellular free-radical formation with NAC could significantly inhibit radiation-induced expression of c-*jun* [44]. Secondly, we demonstrated that an agent capable of inhibiting DNA methylation, 3-Ado, could inhibit alkylating agent-induced expression of c-*jun*. MMS (and probably MMC) is known to be a direct methylating agent, yielding N^7-methylguanine as its major adduct [45]. This methylation accounts for approximately 80% of the total DNA alkylation. The alkylated purines and pyrimidines lead to the spontaneous or enzymatic breakage of N-glycosidic bonds leaving an apurinic/apyrimidinic site which can be cleaved by endonucleases. 3-Ado is a potent demethylating agent that can inhibit drug-induced DNA methylation [46]. The present study demonstrates that inhibition of drug-induced alkylation with 3-Ado significantly inhibited MMC-induced c-*jun* expression. Taken together, the results of the 3-Ado and NAC experiments suggest that inhibition of direct mediators of DNA damage can suppress the induction of c-*jun* seen following exposure of astrocytic cells to cytotoxic drugs and radiation.

Our inability to demonstrate c-*fos* induction following DNA damage is particularly interesting. In hematopoietic derived cell lines, we and other investigators have found c-*fos* to be coordinately regulated with c-*jun*. In astrocytic cell lines, however, c-*fos* was not induced by drugs or radiation despite high levels of c-*jun* induction. Although there is no definitive explanation for this, it is intriguing to postulate that c-*fos* may be regulated more tightly in neuroectodermally derived cells than in other cell types. This hypothesis is consistent with a growing amount of experimental data that have linked c-*fos* induction to membrane depolarization and neurotransmitter signaling within the central nervous system. Indeed, recent animal studies suggest that c-*fos*, and possibly other ERGs, play key roles in the response of post-mitotic neurons to trans-synaptic stimulation. Thus, neuroectodermally derived cells may possess transcriptional and/or post-transcriptional controls that restrict c-*fos* induction to a very limited number of external stimuli. This control of c-*fos* induction may not, however, be unique to neuroectodermally-derived cells since it has been reported that c-*fos* is similarly not induced by ionizing radiation in normal fibroblasts [47].

To date, the post DNA damaging signals that mediate ERG induction have not been elucidated. The different responses of c-*jun* and c-*fos* to drugs and radiation in astyrocytic cells may help to decipher the signals. For example, it has been demonstrated recently that nicotine and other membrane depolarizing agents such as potassium induce c-*fos* expression in neuroectodermally-derived cell lines via a calcium-dependent mechanism [32]. This is mediated by a *cis*-acting element on the c-*fos* promoter located approximately 60 base pairs from the mRNA cap site [48]. This calcium responsive element (CaRE) is identical to the cAMP response element (CRE) [49]. Indeed, c-*fos* can be induced to very high levels in PC-12 cells following a cAMP agonist exposure. Therefore, since c-*fos* is highly inducible in neuroectodermally-derived cells following activation of the cAMP/calcium signaling pathways, it is reasonable to hypothesize that these are not the signals mediating c-*jun* induction following DNA damage. Whether other known signaling systems, such as a protein kinase C, are involved remains to be determined. Furthermore, whether these differences in ERG induction following DNA damage relate in any way to the intrinsic drug and radiation resistance of glioma cells remains to be elucidated.

In summary, the present study demonstrates a consistently reproducible induction of c-*jun* expression in several astrocytoma cell lines following exposure to a variety of cytotoxic drugs and ionizing radiation. This induction can be substantially blocked by inhibiting the direct mediators of DNA damage. Interestingly, c-*fos* was not similarly induced, as it is in other hematopoietic cell lines. The use of neuroectodermally-derived cell lines in conjunction with hematopoietic cell lines may eventually help elucidate signalling events following DNA damage. Elucidation of these events may, in turn, provide clues as to why astrocytomas remain one of the most clinically refractory tumors to both chemotherapy and radiation.

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