Induced gene expression and subsequent cytokine production have been implicated in the normal tissue injury response to radiotherapy. However, studies of radiation-induced gene expression have used single radiation doses rather than the fractionated exposures typical of the clinical situation. To study the effects of multiple radiation doses on gene expression, we investigated nuclear factor κB (NFκB) DNA binding activity in primary astrocyte cultures after one and two exposures to x-rays. After a single dose of x-rays (3.8–15 gray (Gy)), NFκB binding activity in astrocytes increased in a dose-dependent manner, reaching a maximum by 2–4 h and returning to control levels by 8 h after irradiation. In split-dose experiments, when an interval of 24 h was used between two doses of 7.5 Gy, the second 7.5-Gy exposure failed to induce NFκB activation. The period of desensitization induced by the first radiation exposure was dose-dependent, persisting approximately 72 h after 7.5 Gy compared with 24 h after 1.5 Gy. No changes in IκBa protein levels were detected. However, the presence of a transcription inhibitor prevented the desensitizing effect of the initial irradiation. Irradiation also prevented NFκB activation in astrocytes by a subsequent exposure to H2O2, but it had no effect on the activation induced by tumor necrosis factor-α. These data indicate that an initial x-ray exposure can desensitize astrocytes to the NFκB-activating effects of a subsequent radiation exposure. Furthermore, they suggest that this desensitization depends on gene transcription and may have some specificity for NFκB activation mediated by reactive oxygen species.

It is now well established that ionizing radiation not only kills cells but also alters the expression of specific genes in surviving cells. Among the functional consequences of radiation-induced modulation in gene expression is the increased production of certain cytokines, which has been suggested to be of particular relevance to the normal tissue injury that can result from radiotherapy for cancer (1–4). After irradiation of normal cells, genes reported to be induced include those coding for TNF-α (5, 6), interleukin-1β (6), interleukin-6 (7), transforming growth factor-β (2, 4), and basic fibroblast growth factor (8). These cytokines have a wide variety of cell type-dependent biological effects, and increases in their production can be expected to influence cells both inside and outside the radiation field. Thus, radiation-induced gene expression and subsequent cytokine production may play a critical role in determining the response of normal tissue to radiotherapy. At present, it is unclear whether the induction of a given cytokine in irradiated cells contributes to a tissue-specific recovery process or is actually involved in the pathogenesis of radiation-induced normal tissue injury. Regardless, modulation of cytokine activity, whether through replacement therapy or the use of inhibitors, represents a potential strategy for the amelioration of the normal tissue injury that can result from radiotherapy.

Investigations of radiation-induced gene expression in normal cells and tissues to date have focused primarily on the effects of single doses. In a clinical setting, however, radiotherapy is delivered in multiple fractions. With respect to clonogenic cell death and multiple doses, each dose of radiation can be expected to result in at least the same proportional reduction in cell survival (9). Regarding induced gene expression, whether the effect of a subsequent radiation dose is the same as that resulting from the first dose has not, to our knowledge, been determined. A reasonable subject for such a study is the transcription factor nuclear factor κB (NFκB), which plays a major role in regulating genes coding for proteins involved in inflammation and immunity (10). In normal cells, ionizing radiation has been shown to activate NFκB in lymphoid cells (11, 12), fibroblasts (7), and, more recently, endothelial cells (13). Furthermore, NFκB activation has been found to contribute to TNF-α and interleukin 6 gene transcription induced by ionizing radiation (7, 14).

Considerable information is available concerning the biochemical regulation of NFκB activity (10). In unstimulated cells, NFκB is sequestered in the cytoplasm by complexing with the inhibitory protein IκBa. Upon stimulation, IκBa is degraded and NFκB translocates into the nucleus, where it binds to κB sites and regulates the transcription of specific genes. One of the genes that has a κB site in its promoter is IκBa. Thus the activation of NFκB increases the production of IκBa, which then binds to and deactivates free NFκB. Because NFκB activity is subject to this type of negative feedback control, it might be expected that the activation of NFκB by an initial dose of radiation would prevent activation by a second dose.

To test this hypothesis, we evaluated NFκB DNA binding in primary cultures of normal rat type 1 astrocytes after one and two doses of x-rays. Type 1 astrocytes are the most prevalent cell type within the central nervous system (CNS) and are a...
major source of cytokines, proteases, and other types of bioactive molecules (15). This in vitro model has been used extensively to investigate cytokine production by astrocytes under a variety of environmental and treatment conditions. Moreover, many of the signaling pathways regulating the synthesis of specific cytokines, neurotransmitters, and small molecular weight molecules such as nitric oxide defined in astrocyte cultures have been shown to be operative in vivo (16, 17). Of particular relevance to radiation studies, astrocytes are evaluated as confuent cultures, thus eliminating the complicating variables of cell cycle delay and redistribution. The data presented here indicate that, although the first dose of radiation increases NFkB activity in astrocyte cultures, the initial exposure desensitizes astrocytes to the NFkB-inducing actions of a second dose.

MATERIALS AND METHODS

Cell Culture—Primary cultures of type I astrocytes were generated from the cortex of 21-day-old Sprague-Dawley rat embryos as described by McCarthy and de Vellis (18) with some modifications (19). The cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 μg/ml gentamycin, and 2 mM glutamine in a humidified atmosphere of 5% CO₂, 95% air at 37 °C. After reaching confluence, contaminating oligodendrocytes and microglia were removed by overnight shaking followed by exposure to 20 μg/ml cytosine arabinoside for two periods of 48 h with an interval of 1 day between treatments. After the second cytosine arabinoside exposure, the cultures were maintained in DMEM containing 10% FBS for 1 day and then fed with DMEM containing 2% FBS. The cultures were maintained for at least 2 days in DMEM with 2% FBS before use in an experiment. The human glioma cell line U-373 MG (obtained from ATCC, Rockville, MD) was grown in DMEM containing 10% FBS until confluent and then maintained in 0.5% FBS for 1 day before use in an experiment.

Culture Treatment—Irradiation was performed using a Philips RT-250 x-ray unit with a 1-mm copper filter at the dose rate of 176 cGy/min. Cultures were returned to the incubator after irradiation, and cells were harvested at specified times. In experiments involving two x-ray exposures, astrocyte cultures were irradiated and returned to the incubator for a specific period of time; a second dose of radiation was then delivered, and cultures were again returned to the incubator for 2 h before analysis. In some experiments, cultures were treated with TNFα (1 ng/ml, 1 h), H2O2 (100 μM, 1 h), or 5,6-dichlorobenzimidazole riboside (DRB) (100 μM, 5 h).

Preparation of Nuclear Extracts—Cells were scraped from tissue culture flasks in 10 ml of ice-cold phosphate-buffered saline and pelleted by centrifugation at 1500 × g for 5 min. The cell pellet was resuspended in 400 μl of ice-cold lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiobalitone, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A). After incubation on ice for 15 min, 12 μl of 10% Nonidet P-40 were added. Samples were then vigorously vortexed for 10 s and centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in 30 μl of ice-cold extraction buffer (20 mM Hepes, pH 7.9, 0.4% NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiobalitone, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A), incubated with slow rocking at 4 °C for 30 min, and then centrifuged for 5 min. The supernatant (nuclear extract) was collected and frozen at −80 °C. Protein concentration was determined using a Bio-Rad DC kit (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed using the gel shift assay system from Promega (Madison, WI) as described in the protocol provided by the manufacturer with some modifications. Briefly, the oligonucleotide containing an NFkB consensus sequence (5′-αGT TGA GGC GAC TTT CCC AGG C-3′) was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP. The probe (2 μl) and the nuclear extracts (5 μg) were incubated with or without competitor in the presence of 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 50 mM NaCl, 2 mM EDTA, 0.5 mM dithiobalitone, 4% glycerol, and 50 ng/ml poly(dI-dC) in 16 μl total volume at 37 °C for 15 min. The reaction was stopped with 1 μl of gel loading buffer and resolved in a 5% native polyacrylamide gel. The gel was then dried, and the radioactivity of the bands was quantitated using a phosphorImager (Molecular Dynamics, Sunnyvale, CA). The supershift analysis was performed essentially the same as EMSA, except that antibodies were incubated with nuclear extracts before the probe was added. The antibodies to NFkB p50 (NLS), NFkB p65 (C-20), and c-Fos (K-25) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western Blotting—Astrocyte cultures were washed once with ice-cold PBS, scraped into 10 ml of PBS, and pelleted. The cell pellet was resuspended in 3 volumes of lysis buffer (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 20 mM NaF, and 1% Nonidet P-40) were added; the suspension was then incubated on ice for 20 min and vortexed for 10 s. The cell lysate was spun at 12,000 × g for 10 min at 4 °C. Aliquots of the resulting supernatant were stored at −80 °C. Protein concentration was determined using a Bio-Rad DC protein assay kit. Whole cell lysates (50 μg) were applied to a 6% stacking, 10% SDS-polyacrylamide gel and resolved at 60 mA constant current for 40 min. After electrophoresis, the gel was electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham Corp.). The nonspecific sites on the membrane were blocked at 4 °C overnight with 5% nonfat milk in PBS supplemented with 0.1% Tween 20 (PBS-T). The membrane was washed with PBS-T and probed with rabbit anti-rat IκB polyclonal antibody (C-21, Santa Cruz Biotechnol-
X-ray-induced NFκB Activation in Normal Astrocytes

The effect of a second radiation exposure on astrocyte NFκB activity was investigated using a split-dose protocol similar to those traditionally used in the analysis of clonogenic cell survival (9). In the study shown in Fig. 3, 15 Gy were divided into two 7.5-Gy doses delivered at an interval of 24 h, a time at which the induced NFκB DNA binding resulting from a single radiation exposure has returned to control levels (see Figs. 1A and 3, lane 6). Nuclear protein extracts were prepared 2 h after the second irradiation and NFκB DNA binding activity was determined. Irradiation of astrocytes with single doses of 7.5 and 15 Gy analyzed at 2 h resulted in approximately 2.5- and 4-fold increases in NFκB binding activity, respectively. However, when 7.5 Gy were followed 24 h later with an additional 7.5 Gy, no significant NFκB activation was detected after the second dose. This loss of activation cannot be attributed to merely a delay in the time course, because the same lack of induced NFκB DNA binding was observed when analyzed out to 8 h after the second irradiation (data not shown). These data suggest that after irradiation there is a refractory period during which the susceptibility of NFκB to activation by a subse-

FIG. 1. A, time-dependent induction of NFκB DNA-binding activity after x-irradiation. Astrocytes were exposed to a single dose of 15 Gy, and nuclear extracts were prepared at the specified times after treatment. DNA binding activity was analyzed by EMSA as described under “Materials and Methods.” Lane 1, untreated cells that had been incubated with a 50-fold excess of unlabeled probe oligonucleotide (competitor); lane 2, untreated cells; lanes 3–8, cells harvested 0.5, 1, 2, 4, 6, and 8 h after 15 Gy, respectively. C, unirradiated controls. Brackets indicate specific DNA-protein complexes; the asterisk (*) indicates a nonspecific complex. B, dose-dependent activation of NFκB DNA-binding by x-irradiation. Astrocyte cultures were exposed to the specified dose of x-rays and NFκB binding determined 2 h later. Nuclear protein extraction and EMSA were performed as described under “Materials and Methods,” and binding activity (upper band) was quantitated using a PhosphorImager. Results are presented as the fold stimulation as compared with unirradiated controls. Values represent the mean ± S.E. of three independent experiments.

FIG. 2. Supershift analysis of NFκB-DNA complexes. Astrocytes were exposed to a single dose of 15 Gy, and nuclear extracts were prepared 2 h after treatment. Supershift analysis was performed as described under “Materials and Methods” using the specified antibodies. Lanes 1–6, untreated cells, and lanes 7–12, irradiated cells. The anti-c-Fos antibody was included to control for nonspecific interactions. The amount of antibodies added was 1 or 5 μg as indicated for each lane. Brackets indicate specific DNA-protein complexes.

FIG. 3. Effect of two x-ray exposures on NFκB DNA binding activity. Astrocytes were irradiated with 7.5 Gy followed 24 h later by a second dose of 7.5 Gy (a total dose of 15 Gy). NFκB DNA binding activity was determined 2 h after the second dose except for lane 6, which was analyzed at 24 h after irradiation. Nuclear protein extraction and EMSA were performed as described under “Materials and Methods.” Lane 2, untreated cells; lanes 3, single dose of 7.5 Gy; lane 4, two doses of 7.5 Gy separated by an interval of 24 h; lane 5, single dose of 15 Gy; lane 6, single dose of 7.5 Gy assayed at 24 h. Brackets indicate specific DNA-protein complexes; the asterisk (*) indicates a nonspecific complex.
sequent x-ray exposure is significantly reduced.

To investigate the dose requirements for inducing this refractory period, astrocytes were exposed to 1.5, 3.8, or 7.5 Gy before specified intervals by a second dose of 15 Gy. Nuclear protein extracts were prepared 2 h after the second irradiation, and NFκB DNA binding activity was determined. A representative EMSA obtained after irradiation of astrocytes with 1.5 Gy followed by a subsequent dose of 15 Gy after specified intervals is shown in Fig. 4. As in previous experiments, a single dose of 15 Gy increased NFκB binding approximately 4-fold. However, when the 15 Gy were preceded by a 1.5-Gy exposure, the increase in NFκB activity was markedly reduced. This reduction was maximum when 15 Gy were delivered 2 h after the initial 1.5-Gy exposure, at which time no radiation-induced NFκB activity was detected. In contrast to the data shown in Fig. 3, indicating that after 7.5 Gy the refractory period lasted for at least 24 h, by 4 h after the initial dose of 1.5 Gy, the susceptibility of NFκB to activation by 15 Gy began to return to single exposure levels. The influence of radiation dose on the length of the refractory period is summarized in Fig. 5. After initial doses of 7.5, 3.8, or 1.5 Gy, the susceptibility of NFκB to activation by 15 Gy returned by approximately 72, 36, and 24 h, respectively. These data indicate that the ability of radiation to induce a refractory period or to desensitize astrocytes to NFκB activation by a subsequent irradiation is dose-dependent.

To determine whether gene transcription is necessary for this radiation-induced desensitization, the two radiation exposures protocol shown in Fig. 4 (1.5 Gy followed by 15 Gy) was performed in the presence of the transcription inhibitor DRB. In contrast to actinomycin D, DRB does not intercalate into nucleic acids, but inhibits RNA polymerase II (21–24). This results in a reversible and relatively rapid inhibition of RNA synthesis. Treatment of astrocytes with DRB (100 μM) for 1 h inhibits the incorporation of [3H]uridine into RNA by 90% (data not shown). Astrocytes were exposed to DRB for 1 h before irradiation with 1.5 Gy; 2 h later, the cultures received 15 Gy, and 2 h after that, the nuclear extract was examined for NFκB activity. DRB was present during the entire treatment protocol. Three independent experiments were performed, and a representative EMSA is shown in Fig. 6. DRB treatment of astrocytes alone (a total of 5 h) induced a slight increase in NFκB activity (2.3 ± 1.5-fold, mean ± S.E., n = 3), which is probably due to the inhibition of IκBα synthesis, as has been suggested in studies using cycloheximide (25). Pretreatment of astrocytes with DRB had no effect on NFκB activation induced by a single exposure to 15 Gy (4.4 ± 0.6 versus 4.5 ± 0.9 for 15 Gy without DRB). In the absence of DRB, 15 Gy failed to activate NFκB when administered after the initial radiation exposure to 1.5 Gy (lane 4), which is consistent with the data shown in Fig. 4. However, in the presence of DRB, the desensitization was eliminated, and the second radiation exposure induced NFκB activation (lane 5). The activation resulting from the combination of DRB and 1.5 Gy followed by 15 Gy is 9.4 ± 2.8-fold over untreated levels, which is greater than the activation induced by a single dose of 15 Gy (4.5 ± 0.9-fold). It is not possible at this time to determine whether the effect of the combined radiation protocol and DRB is additive or supraadditive. However, these data do indicate that induced gene expression resulting from the first x-ray exposure is required for the desensitization of NFκB activation to the subsequent irradiation.

The requirement for gene induction suggested that increases in IκBα levels may be involved in the refractory period after irradiation. It has recently been shown that, in HeLa and lymphoid cell cultures, glucocorticoids inhibit NFκB activity through the induction of IκBα synthesis (26, 27). To determine whether such a scenario might account for the failure of a second radiation dose to increase NFκB activity, IκBα protein levels were determined by Western blot analysis 24 h after irradiation of astrocyte cultures with 1.5 or 7.5 Gy. At this time after the first 7.5 Gy, NFκB activation by a second radiation exposure is essentially eliminated (see Fig. 4). If IκBα was
responsible for the ineffectiveness of the second radiation exposure, then the level of this protein would be expected to be elevated at this time. As shown in Fig. 7, the IκBα protein level in cultures irradiated with 1.5 or 7.5 Gy was the same as in the unirradiated cultures. Northern blot analysis showed that the IκBα mRNA level was also not affected under these conditions (data not shown). These results suggest that the synthesis of IκBα is unlikely to be involved in the desensitizing actions of the initial irradiation.

NFκB DNA binding is susceptible to induction by a wide variety of stimuli. To determine whether the desensitizing effect also occurs after other activators of NFκB, astrocyte cultures were treated with various combinations of x-rays, H_2O_2, and TNF-α. H_2O_2 (100 μM, 1 h) induced NFκB DNA binding with the maximum activation at 4 h post-treatment and returning to untreated levels by 24 h (data not shown). As shown in Fig. 8, exposure of astrocytes to 7.5 Gy 24 h before H_2O_2 treatment eliminated the NFκB activation by H_2O_2. The same desensitization was induced when H_2O_2 was followed by x-rays or by a second dose of H_2O_2 (Table I). Thus, H_2O_2 and ionizing radiation appear to have similar desensitizing effects. A different situation, however, existed for TNF-α. Treatment of astrocytes with TNF-α (1 nM, 1 h) induced NFκB DNA binding by 7-fold, which returned to untreated levels by 24 h post-treatment (data not shown), consistent with previous results (19). In contrast to H_2O_2, an initial exposure of astrocytes to 7.5 Gy 24 h before TNF-α treatment had no effect on the NFκB activating capability of TNF-α (Fig. 8). When cells were first treated with TNF-α and then exposed to 15 Gy 24 h later, a partial desensitization to radiation activated NFκB resulted (Table I). Interestingly, when TNF-α was followed 24 h later by a second TNF-α exposure, the NFκB activation was essentially the same as that induced by the first treatment. These data suggest that there is some specificity regarding the stimuli that are capable of inducing NFκB desensitization in astrocytes. On a technical note, the failure of radiation to alter NFκB activation by a subsequent TNF-α treatment indicates that the initial irradiation does not modify the solubility of the NFκB complex such that the efficiency of the extraction procedure used in

**Table I**

| First treatment | Second treatment | NFκB activity |
|----------------|-----------------|---------------|
| None           | 15 Gy           | 4.5 ± 0.3     |
| 7.5 Gy         | 15 Gy           | 1.2 ± 0.1     |
| None           | H_2O_2          | 2.8 ± 0.2     |
| 7.5 Gy         | H_2O_2          | 0.5 ± 0.2     |
| H_2O_2         | 15 Gy           | 0.8 ± 0.1     |
| H_2O_2         | H_2O_2          | 0.4 ± 0.1     |
| None           | TNF-α           | 7.1 ± 0.3     |
| 7.5 Gy         | TNF-α           | 7.3 ± 0.4     |
| TNF-α          | 15 Gy           | 3.0 ± 0.1     |
| TNF-α          | TNF-α           | 6.4 ± 0.6     |
exposed to two doses of x-rays (7.5 and 15 Gy) and NFκB binding in a number of cell types. To determine whether the formation may be involved in the radioresponse of the CNS, at least one protein complex; the excess of unlabeled probe oligonucleotide (competitor); lane 3, untreated culture; lane 4, 7.5-Gy exposed cells harvested 4 h later; lane 5, 7.5-Gy exposed cells followed by 15-Gy exposed cells harvested 4 h later. Brackets indicate specific DNA-protein complexes; the asterisk (*) indicates a nonspecific complex.

These studies are significantly affected.

Ionizing radiation has been reported to activate NFκB DNA binding in a number of cell types. To determine whether the radiation-induced refractory or desensitization period is unique to astrocyte cultures, the human glioma cell line U-373 MG was exposed to two doses of x-rays (7.5 and 15 Gy) and NFκB DNA binding activity was analyzed (Fig. 9). A single exposure to 15 Gy activated NFκB approximately 4-fold, with induction being highest 4 h after radiation and returning to control levels by 24 h. When the tumor cells were first exposed to 7.5 Gy followed by a second dose of 15 Gy 24 h later (the same protocol as for astrocyte cultures), the level of NFκB activation induced was the same as that observed with a single dose of 15 Gy. These data indicate that, in contrast to astrocytes, irradiation of the U-373 MG cells does not result in a refractory period.

**DISCUSSION**

Because of their involvement in a variety of CNS disease states and their increased expression after other types of CNS damage, cytokines have been suggested to play a role in the pathogenesis of radiation-induced CNS injury (1). Indeed, Hong et al. (6) recently showed that the expression of TNF-α, interleukin1β, and I-CAM-1 are increased in the mouse brain after a single dose of radiation. It was hypothesized that these inflammation/immunity-related gene products may also play a role in radiation-induced CNS damage either directly or through the initiation of a cellular and/or molecular cascade of events. A transcription factor critical to the regulation of genes coding for products participating in defense-type reactions such as inflammation and immunity, including those mentioned above, is NFκB (10). As shown herein, irradiation of astrocytes in vitro significantly increased NFκB DNA binding activity, which lends support to the concept that induced gene expression may be involved in the radioresponse of the CNS, at least after a single dose. The results of our study, however, also indicate that irradiation of normal astrocytes results in a refractory period during which the susceptibility of NFκB to activation by a subsequent x-ray exposure is significantly reduced.

Although the response of most normal tissues to multiple radiation treatments has been described in experimental models, investigations of the pathogenesis of radiation-induced normal tissue injury have focused primarily on the use of single doses. This also applies to studies specifically addressing radiation-induced gene expression. Experimental convenience and the necessity for a starting point for these relatively new investigations are certainly valid rationales for focusing on single radiation exposures. However, there is no a priori reason to assume that the changes in gene expression induced in cells and tissues after the first dose of radiation will be the same as those induced by a subsequent exposure. The data presented herein indicate that in the delivery of two radiation doses to normal astrocytes in vitro, using a clinically relevant interval of 24 h, the first dose markedly affects the response of the cells to the second x-ray dose. If a similar desensitization process occurs in vivo, then the spectrum of genes induced by the first radiation dose would not be expected to be induced by a subsequent dose. Thus, to understand the role of radiation-induced gene expression in the pathogenesis of normal tissue injury produced by radiotherapy, it will be necessary to define this event as it occurs after more than one radiation exposure.

Although the mechanism responsible for the desensitization induced by radiation has not been completely defined, our initial studies do provide some insight. As shown in Figs. 5 and 6, the length of the refractory period after irradiation of astrocytes is dose-dependent, and its induction requires gene transcription. These two observations raise the possibility that the desensitization involves the increased production of a protein that inhibits radiation-induced NFκB activation. Auphon et al. (26) and Scheinman et al. (27) have shown that the glucocorticoid-mediated inhibition of NFκB activity involves the induction of IκBα synthesis. In astrocytes, elevated IκBα levels, however, were not detected after irradiation, suggesting that an increased sequestration of free NFκB by IκBα is unlikely to be responsible for the ineffectiveness of a second radiation exposure. Although gene transcription may be required, the refractory period in astrocytes was induced by exposure to as little as 1.5 Gy, which does not induce a detectable level of NFκB DNA binding (Fig. 2). This low dose effect suggests that the desensitization may not require NFκB activation and, consequently, may not involve the induction of κB regulated genes. This possibility is consistent with the lack of a refractory period detected after TNF-α treatment (see below).

Additional information pertaining to the mechanism of radiation-induced desensitization is provided from the studies combining x-rays with other activators of NFκB. A critical regulatory step in NFκB activation is the dissociation of the NFκB/IκB complex (28). Brown et al. (29) showed that the mutation of IκBα at the Ser32 residue, a potential phosphorylation site, can prevent NFκB activation. Thus, the mechanism responsible for the radiation-mediated desensitization of astrocytes could involve some change in the NFκB/IκB complex that prevents its dissociation. Prior irradiation of astrocyte cultures, however, did not affect NFκB activation by TNF-α, indicating that the dissociation process of NFκB from IκBα is fundamentally intact in irradiated astrocytes. Furthermore, the TNF-α-mediated activation of NFκB in irradiated astrocytes and in cultures previously exposed to TNF-α suggests that an upstream event specific to a radiation-activated signal transduction pathway is responsible for the failure of a second x-ray dose to activate NFκB. H2O2 and x-rays are essentially interchangeable regard.
X-ray-induced NFκB Activation in Normal Astrocytes

It is well established that radiation can activate NFκB binding in a number of cell types; however, whether the desensitization effect detected in astrocytes is induced in other cells remains to be determined. As shown in Fig. 9, radiation activated NFκB binding in a human brain tumor cell line with similar kinetics as in astrocytes, and yet, exposure to a second dose of x-rays induced the same level of NFκB activation as the first dose. Thus, a refractory period was not induced in these brain tumor cells. Obviously, there are many caveats in attempting to compare the response of an established human brain tumor cell line to that of a primary culture of normal rat cells; other cell types need to be evaluated, especially those of brain tumor cell line to RB. Furthermore, if the lack of a desensitization process can be attributed to brain tumor cells in general, it may suggest that these tumor cells, in contrast to normal astrocytes, continue to produce NFκB-dependent cytokines during fractionated radiotherapy. Such cytokines may thus continue to influence the growth rate and invasion propensity of the tumor as well as the immune response of the host.

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