Inhibition of IkB-α phosphorylation at serine and tyrosine acts independently on sensitization to DNA damaging agents in human glioma cells

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Summary Molecular mechanisms and/or intrinsic factors controlling cellular radiosensitivity are not fully understood in mammalian cells. The recent studies have suggested that nuclear factor κB (NF-κB) is one of such factors. The activation and regulation of NF-κB are tightly controlled by IkB-α, a cellular inhibitory protein of NF-κB. Most importantly, phosphorylation regulates activity of the inhibitor IkB-α, which sequesters NF-κB in the cytosol. Two different pathways for the phosphorylation of IkB-α are demonstrated, such as serine (at residues 32 and 36) and tyrosine (at residue 42) phosphorylations. To assess a role of the transcription factor, NF-κB, on cellular sensitivity to DNA damaging agents, we constructed three different types of expression plasmids, i.e. S-IkB (mutations at residues 32 and 36), Y-IkB (mutation at residue 42) and SY-IkB (mutations at residues 32, 36 and 42). The cell clones expressing S-IkB and Y-IkB proteins became sensitive to X-rays as compared with the parental and vector-transfected cells. The cell clones expressing SY-IkB were further radiosensitive. By the treatment with herbimycin A, an inhibitor of phosphorylation, the X-ray sensitivity of cells expressing SY-IkB did not change, while that of the cells expressing S-IkB and Y-IkB and the parental cells was enhanced. Change in the sensitivity to adriamycin and UV in those clones was very similar to that in the X-ray sensitivity. The inhibition of IkB-α phosphorylation at serine and tyrosine acts independently on the sensitization to X-rays, adriamycin and UV. These findings suggest that the transcriptional activation induced by NF-κB may play a role in the DNA damage repair. The present study proposes a possibility that the inactivation of NF-κB by inhibition of both serine and tyrosine phosphorylations may be useful for the treatment of cancer in radio- and chemotherapies. © 2000 Cancer Research Campaign

Keywords: IkB-α, NF-κB; phosphorylation; DNA damaging agents; human glioma cells

Ionizing radiation activates immediately early genes, such as c-jun, c-fos and nuclear factor κB (NF-κB) (Hallahan et al, 1991; Wilson et al, 1993; Mohan and Meltz, 1994). The products of these genes are transcription factors involved in regulation of the gene expression associated with cell growth, healing of tissue injury and inflammation. Transfection with certain oncogenes has been found to cause cellular radiation resistance (Sklar, 1988; Kasid et al, 1989; Pirollo et al, 1993). These results suggest that related signalling pathways may be involved in acquisition of radiation resistance. It has been demonstrated that introduction of a truncated IkB-α corrected both hypersensitivity to ionizing radiation and the aberrant activation of NF-κB in ataxia telangectasia (AT) cells (Jung et al, 1995). Therefore, activation of transcription factor(s) may be involved in cellular radiosensitivity.

One of the proteins of the Rel/NF-κB family, NF-κB, is a transcription factor which binds specifically to κB motifs as homomeric or heterodimers and regulates the expression of many genes (reviewed in Baeuerle and Baltimore, 1988; Beg et al, 1992). Some of the stimuli including tumour necrosis factor-α (TNF-α), ultra-violet (UV) and ionizing radiation cause the degradation and disappearance of IkB-α (Alkalay et al, 1995; Scherer et al, 1995), and then the NF-κB translocates to the nucleus.

Phosphorylation is involved in the regulation of the activity of many transcription factors. The NF-κB is a critical regulator of cytokine-inducible gene expression (Baeuerle and Henkel, 1994). Most importantly, phosphorylation regulates activity of the inhibitor IkB-α, which sequesters NF-κB in the cytosol. For the activation of NF-κB, two different pathways for the phosphorylation of IkB-α are demonstrated. One is serine (at residues 32 and 36) phosphorylation, which induces subsequent ubiquitin-dependent degradation of IkB-α (Brockman et al, 1995; Brown et al, 1995; Traenckner et al, 1995). The other is tyrosine (at residue 42) phosphorylation, which has the potential to directly couple NF-κB to surface receptor associated tyrosine kinases, but not induce the degradation of IkB-α (Imbert et al, 1996).

We constructed three different types of expression plasmids, such as S-IkB containing Ser-to-Arg (AGC-CGC) mutations at residues 32 and 36, Y-IkB containing Tyr-to-Phe (TAC-TTC) mutation at residue 42, and SY-IkB containing all these mutations. To assess the role of NF-κB activation on cellular sensitivity to X-rays, adriamycin and UV, we examined the sensitivity to these DNA damaging agents in human malignant glioma cells expressing S-IkB, Y-IkB and SY-IkB proteins.
MATERIALS AND METHODS

Cell lines and culture conditions

The established human malignant glioma cell line, MO54, was used. The cells were kindly supplied from Dr RS Day, Cross Cancer Institute, Edmonton, AB, Canada. Cells were cultured in Dulbecco’s modified Eagle medium plus 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA) at 37°C with 95% air and 5% carbon dioxide.

Mutant constructions and transfections

For S-IkB plasmid, the IκB-α cDNA (a kind gift from Dr J Inoue, The Institute of Medical Science, University of Tokyo, Japan) inserted into the pBluescript II S/K plasmid was used as a template for further polymerase chain reaction (PCR) amplification. Mutagenic primers for 32Ser to 32Arg and 38Ser to 38Arg, such as MAD-LPU; 5’-CGACCCGACCAGGCGCCCTGGACGC-3’ and MAD-LPL; 5’-CGCTTTCATGGCGCTTTCATGCG-3’, were used in PCR reaction. The reaction mixture was as follows; 1 μl of pB-MAD3 plasmid (37 μg ml⁻¹), 1 μl of each primer (1 pmol μl⁻¹), 4 μl of 25 mM magnesium chloride, 5 μl of dNTP mix (2 mM each), 0.5 μl of KOD polymerase (5 units μl⁻¹), 5 μl of 10 × KOD buffer and 34 μl of distilled water. PCR condition was 15 cycles of 30 s at 98°C, 30 s at 55°C and 2 min at 74°C with a final extension at 72°C for 3 min. The mixture was treated with DpnI for the degradation of the template plasmid. The plasmid sample was introduced into Escherichia coli JM109 strain by electroporation. After 1 h incubation with SOC medium, the cells were plated on LB dishes containing ampicillin. The S-IκB-α cDNA inserted in the pBluescript II S/K plasmid were refined by using QIAprep Spin Miniprep kit (Qiagen, Germany) and checked by digestion with EcoRI. Then, sequencing of the S-IκB-α cDNA was done with a Prism Dye Primer Cycle Sequencing Kit (−21M13) (Applied Biosystems, Foster City, CA, USA). The pBluescript II S/K plasmid containing S-IκB-α cDNA was done with HindIII and XbaI. The S-IκB-α cDNA was ligated into the HindIII and XbaI sites of pcDNA3 as a template for further polymerase chain reaction using DNA ligation kit ver. 2 (Takara, Ohtsu, Shiga, Japan). This plasmid was named as pcDNA3-S-IκB. For the Y-IκB plasmid, we used the mutagenic primers for 42Tyr to 42Phe, such as MAD-LPU; 5’-GAAGAGA-CTGACAGGTCTCCCAGGACGACGC-3’ and MAD-LPL; 5’-CTTTCCTTTCTGCTCCAGG-3’ in the PCR reaction. For the SY-IκB plasmid, the S-IκB-α cDNA was used as a template and the mutagenic primers for 42Tyr to 42Phe were used in PCR reaction. The expression plasmids of Y-IκB and SY-IκB were made by similar methods as above-mentioned. A plasmid pcDNA3, without IκB-α cDNA, was used as a negative control.

The methods of transfection were reported previously (Miyakoshi et al, 1997; Yamagishi et al, 1997a). Approximately 10⁵ cells of MO54 were transfected with 10 μg of pcDNA3-S-IκB, pcDNA3-Y-IκB, pcDNA3-SY-IκB or pcDNA3 by the electroporation with the unit (model X-CELL 2000, pds Inc., Madison, WI, USA) operated at 800 V. The cells were then cultured in normal medium for 36–48 h and then cultured in the medium containing G418 (400–800 μg ml⁻¹). The cultures were maintained for 2–3 weeks until colonies were formed. The transfection efficiencies of these plasmids were 2–8×10⁻⁵.

Chemicals

Human TNF-α (Pepro Tech EC Ltd., London, UK) was dissolved in distilled water and adriamycin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in medium at a concentration of 20 μg ml⁻¹ and 500 μg ml⁻¹ respectively. Herbimycin A (Sigma, St Louis, MO, USA) and genistein (Fujikko Ltd, Kobe, Japan) were dissolved in dimethylsulphoxide (DMSO) at a concentration of 2 mg ml⁻¹ and 20 mg ml⁻¹ respectively. These solutions were diluted in the medium at a final concentration and used for the experiments.

Western immunoblots and immunoprecipitation

Details of Western immunoblotts were described elsewhere (Miyakoshi et al, 1997). The antibodies used in this experiment were as follows; anti-IκB-α/MAD3 antibody (C-21, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-β-actin antibody (Sigma) and anti-αNFκBp65 antibody (C-20, Santa Cruz Biotechnology). The immunoprecipitation was done by using Protein A Sepharose (Pharmacia Biotechnology). The nuclear extracts were prepared according to Schreiber et al (1989). Autoradiography with enhanced chemiluminescence was done according to the instructions by the manufacturer (Amersham International plc, Buckinghamshire, UK). The densitometric analysis was done by using NIH image 1.60.

X- and UV-irradiations and cell survival

Methods of X- and UV-irradiations and the analysis of cell survival were described previously (Miyakoshi et al, 1996; Yamagishi et al, 1997b). X-irradiation was performed using a Hitachi MBR-1520 at 150 kVp, 20 mA with 0.5 mm Al and 0.1 mm Cu filters with a dose-rate of 0.98–1.02 Gy min⁻¹. For UV exposure, a bank of two 15 W Toshiba germicidal UV lamps (predominantly 254 nm) was used with a dose rate of 1.2 J m⁻² s⁻¹. Control plating efficiencies for the parental MO54 cells and the transfected clones were determined by using NIH image 1.60.

Detection of apoptosis

Apoptosis Ladder Detection kit (Wako) was used. The cells were treated with TNF-α (20 ng ml⁻¹) for up to 12 h. DNA was extracted and electrophoresed in agarose gel at 100 V. The gel was stained with SYBR Green I (Molecular Probes, Eugene, OR, USA).

Flow cytometry

Analysis of cell cycle distribution was described previously (Miyakoshi et al, 1997). In brief, cells were suspended in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 after washing twice with PBS. RNAAse (1 mg ml⁻¹) (Sigma) was added to the cell suspension and the cells were incubated for 30 min at 37°C.
37°C. Propidium iodide (Molecular Probes) was added at a final concentration of 50 μg ml⁻¹ and the cell suspension was left at 4°C for 1 h. The cells were filtered through 50 μm nylon mesh before flow cytometry with a FACScan system (Becton Dickinson Co. Ltd, Franklin Lakes, NJ, USA).

RESULTS

X-ray sensitivity of cells expressing S-IκB, Y-IκB and SY-IκB proteins

The expressions of IκB-α in the parental MO54 cells and their S-IκB-, Y-IκB- and SY-IκB-introduced clones are shown in Figure 1A. The clones, MO54-S8, MO54-Y2 and MO54-SY4, were overexpressing S-IκB, Y-IκB and SY-IκB respectively, in which those proteins were non-phosphorylated type. The radiation-dose survival curves of these clones, other expression clones MO54-Y18 and MO54-SY6, the parental MO54 and the negative control of MO54-V are shown in Figure 1B. MO54-S8, MO54-Y2 and MO54-Y18 cells became radiosensitive as compared with the parental MO54 and the vector-transfected MO54-V cells. The cell clones expressing SY-IκB, MO54-SY4 and MO54-SY6, were further radiosensitive. The radiosensitization was estimated from the ratio of mean surviving fraction for the parental cells to that for cells expressing S-IκB, Y-IκB and SY-IκB (Table 1). The calculated additive radiosensitizing value was similar to the ratio of mean surviving fraction of cells expressing SY-IκB. The extent of the radiosensitization in cells expressing SY-IκB was almost additive at higher doses of 4 Gy and over, which means addition of the radiosensitization in each clone expressing S-IκB and Y-IκB. Changes in the amount of p65/RelA protein in nuclear fraction was examined in cells after X-irradiation. The relative amount of p65 protein in nuclear fraction from MO54, MO54-S8, MO54-Y2 and MO54-SY4 cells after X-irradiation is shown in Figure 2A. The constitutive level of p65 in MO54-S8 and MO54-Y2 cells was lower than that in MO54 cells, but this level in MO54-SY4 cells was still lower. After X-irradiation, the amount of p65 in nucleus in MO54 cells was increasing, while that in these clones did not change significantly. The amount of IκB-α/NF-κB complex was also examined after X-irradiation. The relative amount of IκB-α immunoprecipitated with anti-NF-κB/p65 after X-irradiation is shown in Figure 2B. The constitutive level of IκB-α/NF-κB complex observed in MO54-S8, MO54-Y2 and MO54-SY4 cells was higher than that in MO54 cells. After X-irradiation, the amount of the complex in MO54-SY4 cells did not change, but the amount in MO54, MO54-S8 and MO54-Y2 cells was decreased. The IκB-α/NF-κB complex was dissociated and the amount was very low in MO54 cells after X-irradiation.

Effect of herbimycin A on X-ray sensitivity

We examined whether a phosphorylation inhibitor, herbimycin A, can modify cellular X-ray sensitivity. MO54-S8, MO54-Y2, MO54-Y18, MO54-SY4 and the parental cells were treated with herbimycin A (1 μg ml⁻¹) for 2 h, and then irradiated with X-rays. Herbimycin A was removed at 1 h after X-irradiation. Figure 3 shows the X-ray dose–survival curves of these cells. The X-ray-survival of MO54 cells was decreasing by herbimycin A, but did not reach to the radiosensitivity level of those cells expressing SY-IκB protein. The X-ray sensitivity of MO54-S8, MO54-Y2 and MO54-Y18 cells became almost similar level to that of
MO54-SY4 cells. However, the radiosensitivity of MO54-SY4 cells did not change by the treatment with herbimycin A. Treatment with the other inhibitor, genistein (100 μg ml⁻¹), showed a similar trend to herbimycin A (data not shown).

**Effect of TNF-α on DNA degradation, cell cycle distribution and X-ray sensitivity**

We examined the effect of TNF-α, one of the phosphorylation stimuli, on DNA degradation, cell cycle distribution and X-ray sensitivity. MO54-S8, MO54-Y2, MO54-SY4 and the parental cells were treated with TNF-α (20 ng ml⁻¹) for up to 12 h. Figure 4A shows the photographs of DNA gels. TNF-α-induced DNA degradation was observed in MO54-S8, MO54-Y2 and MO54-SY4 cells. The parental MO54 cells did not occur such DNA degradation for up to a 12 h treatment with TNF-α. Patterns of cell cycle distribution after X-irradiation or TNF-α treatment are shown in Figure 4B. Typical G1 arrest and a decrease in S phase ratio were observed in MO54 but not in MO54-SY4 cells after X-irradiation. DNA degradations by the treatment with TNF-α for

![Figure 3](image_url)

**Figure 3** Effect of herbimycin A on the X-ray sensitivity of the cells expressing S-IκB, Y-IκB and SY-IκB proteins and the parental MO54 cells. Exponentially growing cells were treated with herbimycin A (1 μg ml⁻¹) for 2 h and then exposed to X-rays with various doses. Herbimycin A was removed at 1 h after X-irradiation. Each point represents the mean of the triplicate experiments with standard deviation.

![Figure 4](image_url)

**Figure 4** Ethidium bromide-stained gels, cell cycle distribution and the X-ray sensitivity of cells treated with TNF-α. (A) MO54-S8, MO54-Y2, MO54-SY4 and the parental MO54 cells were treated with TNF-α (20 ng ml⁻¹) for up to 12 h. DNA from those cells was extracted and electrophoresed. (B) Exponentially growing MO54 and MO54-SY4 cells were exposed to X-rays with 5 Gy followed by a 20 h incubation or treated with TNF-α (20 ng ml⁻¹) for 12 h, and then cell cycle distribution was analysed by quantitative flow cytometry. Each experiment was repeated twice. (C) Exponentially growing cells were treated with TNF-α (20 ng ml⁻¹) for 4 h and then exposed to X-rays with various doses. TNF-α was removed at 4 h after X-irradiation. Each point represents the mean of the triplicate experiments with standard deviation.

| X-ray dose | Ratio of mean surviving fraction [SF(parental cells)/SF(clones)] |
|-----------|---------------------------------------------------------------|
|            | Parental cells (MO54) | S-IκB clone (MO54-S8) | Y-IκB clones (MO54-Y2) (MO54-Y18) | SY-IκB clones (MO54-SY4) (MO54-SY6) | Calculated additive radiosensitizing value* |
| 4 Gy       | (1.00)              | 1.36                 | 2.52                                | 3.75                                | 3.43                                         |
| 6 Gy       | (1.00)              | 2.15                 | 4.70                                | 10.66                               | 10.11                                        |
| 8 Gy       | (1.00)              | 7.11                 | 8.50                                | 70.59                               | 60.44                                        |

The radiosensitizing value was estimated from the ratio of mean surviving fraction for the parental cells (MO54) to that for clones expressing S-IκB (MO54-S8), Y-IκB (MO54-Y2 and MO54-Y18) and SY-IκB (MO54-SY4 and MO54-SY6). *The additive radiosensitizing value for SY-IκB was calculated as a multiplication of the surviving fraction ratio for S-IκB and that for Y-IκB.

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The phosphorylation of IκB-α is almost additive (Table 1). Changes in the nuclear fraction of p65/Rel protein and the amount of NF-κB/IκB-α complex after X-irradiation also depended on the status of IκB-α phosphorylation (Figure 2). Therefore, the inhibition of phosphorylation at serine and tyrosine of IκB-α acts independently on the radiosensitization of the cells.

Phosphorylation pathways are important for NF-κB activation. Treatment with a phosphorylation inhibitor, herbimycin A or genistein, inhibits radiation-induced NF-κB activation (Iwasaki et al, 1992; Uckun et al, 1993). The X-ray sensitivity of MO54-SY4 cells did not change by the treatment with herbimycin A, while that of the parental MO54 cells was enhanced by herbimycin A (Figure 3). Genistein had a similar effect of herbimycin A on the X-ray sensitivity of those cells (data not shown). Inhibition of phosphorylations by herbimycin A sensitized cells to X-rays, supports that the radiosensitization of cells expressing S-IκB, Y-IκB and SY-IκB proteins could be, in part, due to the inhibition of IκB-α phosphorylation.

The phosphorylation of IκB-α is induced by a variety of activators, such as TNF-α, 12-O-tetradecanoylphorbol-13-acetate (TPA) and interleukin (IL)-1, resulting in the induction of NF-κB activation (Beg et al, 1993; Brown et al, 1993; Cordle et al, 1993; Mellits et al, 1993). The expression of S-IκB protein potentiately enhanced the ability of TNF-α to initiate apoptosis in a variety of cells that are normally resistant to this cytokine, suggests that the activation of NF-κB by TNF-α is protective (Wang et al, 1996). Treatment with TNF-α induced DNA degradation in MO54-S8, MO54-Y2 and MO54-SY4 cells, but not in the parental MO54 cells (Figure 4A). The results of cell cycle distribution of MO54-SY4 and MO54 cells supported these observations (Figure 4B). By the treatment with TNF-α, X-ray survivals of MO54-SY4 cells did not change significantly, but those of MO54 cells increased slightly (Figure 4C). Thus, the absence of NF-κB/p65 sensitized cells to TNF-α, but an increase in cell killing induced by X-irradiation in MO54-SY4 might be independent of the toxic action of TNF-α, such as an induction of DNA degradation. In addition, TNF-α-induced activation of NF-κB in MO54 cells may provide protection against cell killing by X-rays.

For the response of cells to other DNA damaging agents, such as adriamycin and UV, MO54-S8, MO54-Y2, MO54-Y18 and MO54-SY4 cells became sensitive as compared with the parental MO54 cells (Figure 5). In addition, changes in the sensitivity to adriamycin and UV in those clones showed a very similar trend to that in the X-ray sensitivity.

**DISCUSSION**

For the activation of NF-κB, two different pathways for the phosphorylation of IκB-α are demonstrated, one for serine (at residues 32 and 36) phosphorylation and the other for tyrosine (at residue 42) phosphorylation. Wang et al (1996) reported that inhibition of NF-κB nuclear translocation by the introduction of S-IκB plasmid into human fibrosarcoma cell line HT 1080 enhanced cell killing by ionizing radiation. Our finding using MO54-S8 cells in this study is consistent with their data (Figure 1B). In addition, the clones expressing Y-IκB protein became radiosensitive and the clones expressing SY-IκB protein were further radiosensitive (Figure 1B). The extent of radiosensitization in cells expressing
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