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c-IAP2 is induced by ionizing radiation through NF-κB binding sites

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Abstract Transcriptional promoters responsive to low doses of X-irradiation may be useful in developing a new strategy in gene therapy combined with conventional radiotherapy. The retrovirus-mediated gene trap screening identified c-IAP2 as one of genes possessing such promoters. The analysis of the *cis*-elements responsive to X-irradiation in c-IAP2 promoter revealed that the NF-κB binding sites were necessary and sufficient for the X-ray-responsiveness. We constructed the plasmid p4NFB-BAX, which had four tandem repeats of the NF-κB binding sites of c-IAP2 promoter (4NFB) and a suicide gene *BAX* under the control of 4NFB. The human tumor cells transfected with p4NFB-BAX significantly reduced the number of cells that survived 2 Gy irradiation.

Key words: Gene trap; c-IAP2/HIAP-1/hMIHC; Radiation; Nuclear factor-κB; BAX; Apoptosis

1. Introduction

c-IAP2/HIAP-1/hMIHC is a cellular protein homologous to baculovirus inhibitors of apoptosis (IAPs) and has been identified to interfere with the transmission of intracellular death signals [1,2]. c-IAP2 can prevent the proteolytic processing of pro-caspase-3, -6, and -7 by blocking the cytochrome *c*-induced activation of pro-caspase-9 and also inhibit active caspase-3 directly, thus blocking downstream apoptotic events such as further activation of caspases [3]. Recent studies with a variety of cell types suggest that c-IAP2 is one of the target genes of the transcription factor nuclear factor-κB (NF-κB) [4–7] and mediates the anti-apoptotic function of NF-κB by blocking the activation of caspase-8 [7].

We have recently established a method for screening the genes responsive to various external stimuli in cultured cells based on the retrovirus-mediated gene trap approach [8]. Our method is sensitive enough to detect genes whose levels of mRNA are induced as little as twofold. In this study, we applied this method to identify genes responsive to 2 Gy of X-irradiation and identified c-IAP2 as one such gene. We also demonstrate that NF-κB binding sites (NFB) in the c-IAP2 promoter is necessary and sufficient for the X-ray-responsiveness and that the apoptotic protein BAX under the regulation of the NFB promoter increased the cell death in human cancer cell lines after 2 Gy irradiation.

2. Materials and methods

2.1. Cell culture and β-galactosidase (β-Gal) assay

A549, a human lung adenocarcinoma cell line, and HEK293, a human embryo kidney cell line, were obtained from ATCC and maintained in DMEM supplemented with 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Bleomycin hydrochloride (BLM, Japan Chemical Co.) was added to the culture at the final concentration of 0.5 μg/ml. 48 h after irradiation or the addition of BLM, a β-Gal assay was done as described previously [8].

2.2. Gene trap screening and cDNA isolation

Trap lines were isolated by the similar method as described previously [8]. Briefly, a total of 2.6×10^6 A549 cells (2×10^5 cells per 100 mm dish) were infected with ROSA-nGBT virus and BLA-S (2 μg/ml) was added 3 days later. The selection medium was renewed once every 3–4 days. Colonies that survived were isolated and expanded, and their aliquots were plated in duplicates onto 24 well plates (one well without BLM and the other with BLM), and after 48 h incubation, the cells were stained with X-gal. Trap lines stained deeper in the presence of BLM than in its absence were subjected to the secondary screening using 2 Gy of X-irradiation. The trap lines stained deeper after 2 Gy irradiation as compared to the untreated counterparts and were analyzed further. The trapped genes were identified by 5′-RACE and 3′-RACE as described previously [8].

2.3. X-ray irradiation and colony assay

Irradiation was performed at room temperature using an X-ray machine (MI-201, Shimadzu Co., Kyoto, Japan) set at 250 kV, 16 mA with a 0.5 mm Cu filter. The dose rate was 184.40 cGy/min. For colony assay, 1×10^3 cells were seeded in a 100 mm dish and the following day, the cells were irradiated at doses of 2, 5, 7.5, 10 or 20 Gy. 10 days later, the cultures were fixed and stained with Giemsa's staining solution (Merck) and the colonies composed of more than 200 cells were counted.

2.4. Cloning of c-IAP2 promoter and preparation of deleted or modified constructs of the c-IAP2 promoter with luciferase reporter gene and bax gene

The 3.5 kb c-IAP promoter region (3.5K promoter) was isolated with Human Genome Walker kit (Clontech) from HDL-3 *Sca*I Library by using two c-IAP2 specific primers, IAP1 (CCA GAG GCC GAC GCA TGC ACC) and IAP2 (AGC CCA GTC TTT TCA AGC GAC ACC). To prepare the 5′-flanking region of 3.5K promoter, 2K, 1K, 0.5K, NFB(A+B), NFB(B) and NFB(0), 3.5 kb c-IAP promoter region was treated with DNase III and 2, 1, 0.5, 0.242, 0.191, and 0.141 kb respectively were inserted to a luciferase reporter plasmid, pGL3-basic vector (Promega). 3′-flanking region of c-IAP2 promoter (1.5 kb) was prepared by inserting a 1.5 kb *Pst*I fragment of 2 kb c-IAP2 promoter to pGL3-promoter vector (Promega). NF-κB binding sequence NFB was prepared by PCR using the 0.5 kb c-IAP2 promoter fragment as a template. Primers used for amplification of NFB

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Abbreviations: NF-κB, nuclear factor-κB; NFB, NF-κB binding sites; IAP, inhibitors of apoptosis; BLM, bleomycin hydrochloride

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were (+) CTG AAG TCG ACG GGT CAT GGA AAT CCC CGA GTG and (−) GTC AAC TCG AGG GGA ACT CCA GCG GTA ATA ACC. The amplified fragments were digested with *Sal*I and *Xho*I, ligated to each other and then digested with *Sal*I and *Xho*I again. To construct pGL3-4NFB, the four tandem repeat products were extracted from the agarose gel and inserted to the *Xho*I site of pGL3-promoter vector. To construct pGL3-5A and pGL3-5B, NF-κB binding sites 5A and 5B were prepared by annealing the oligonucleotides NFB-A (+) C(GGA AA T CCC CGA G)$_4$GGA AAT CCC C and NFB-A (−) TCG AGG GGA TTT CC(CTC GGG GAT TTC C)$_4$GGTAC for 5A, NFB-B (+) C(TGG AGT TCC CCT A)$_4$TGG AGT TCC C, and NFB-B (−) TCG AGG GAA CTC CA(TAG GGG AAC TCC A)$_4$GGT AC for 5B. 5A and 5B were inserted to the *Xho*I/KpnI site of the pGL3-promoter vector. The *BAX* cDNA fragment (650 bp) was amplified by RT-PCR from A549 cDNA library. The primers used for the bax amplification were Bax-FW1:ATG GAC GGG TCC GGG GAG CAG CCC A and Bax-REV2:TCA GAC GTA AGG AAA ACG CAT TA. To construct p4NFB-bax, the *BAX* cDNA fragment amplified was substituted for the luciferase gene of pGL3-4NFB.

2.5. Plasmids transfection and luciferase assays

Transfection was carried out as described [9]. Briefly, HEK293 cells (1×10^4) were seeded into a 24 well microtiter plate and on the following day 1.8 μg of each reporter luciferase construct was transfected along with 0.2 μg of standard plasmid pRL-SV40 (Promega). The following day, the cells were irradiated at doses of 2, 5 or 10 Gy or cultured with the medium containing BLM. Then 6, 12, 24 or 48 h later transfectants were lysed in 0.1 ml of passive lysis buffer (Promega) and 5 μl of the lysates were subjected to luciferase assay using the Dual-luciferase reporter assay system (Promega).

2.6. Western blot analysis

5×10^5 cells were seeded into a 60 mm dish. The following day, the cells were irradiated at a dose of 2 or 5 Gy. The cell lysates were prepared 24 h after irradiation, electrophoresed on a 15% polyacrylamide gel, blotted onto a nitrocellulose filter, and detected with an anti-human BAXα monoclonal antibody (Transduction Lab.).

3. Results

3.1. Isolation of trap lines which responded to X-irradiation

To isolate the genes involved in the cellular responses to clinically relevant doses of X-irradiation, we applied the gene trap method to the human lung adenocarcinoma cell line A549, which retains a wild-type p53 gene. First, the sensitivity of A549 to X-rays was examined (Fig. 1A). Almost all the cells irradiated at a dose of 2 Gy were eventually recovered, and approximately 30 and 10% of cells irradiated at a dose of 5 and 7.5 Gy, respectively survived. Almost all the cells, however, irradiated with higher doses eventually died. We found previously that cytotoxic treatments induced endogenous β-Gal-like enzyme activity [8]. Thus we selected the blasticidine-S-resistant colonies (trap lines) without any other selective pressure after infecting ROSA-nGBT virus. Since radiosensitivity of cells is based on their progression through the cell cycle, we assumed that it should be difficult to screen trap lines that response to 2 Gy of X-irradiation without cell synchronization. In the primary screening, instead of exposing to X-irradiation, the trap lines were cultured for 48 h with the medium containing BLM, a radiomimetic drug known to induce DNA damages and cellular responses similar to those induced by X-irradiation. The final concentration of BLM (0.5 μg/ml) was determined so as to yield no apparent damage to the cells after 48 h treatment (data not shown).

After screening about 1450 trap lines with X-gal staining, we obtained four trap lines stained deeper in the presence of BLM, which were isolated for further analysis. The cDNA fragment (50–150 bp) obtained by 5′-RACE from four trap lines were sequenced, and searched for homology in the GenBank and SwissProt databases using the BLASTN or BLASTX algorithm, respectively. No homology sequences was found for three trapped genes and a cDNA fragment (150 bp) obtained from one trap line (B112) was identical to the 5′-non-coding region of c-IAP2 cDNA starting at the putative transcription initiation site (data not shown). The β-Gal activities in B112 cells at 48 h after addition of BLM or X-irradiation (2 Gy) were 8 and 2.5 fold higher than their untreated counterpart, respectively (Fig. 1B).

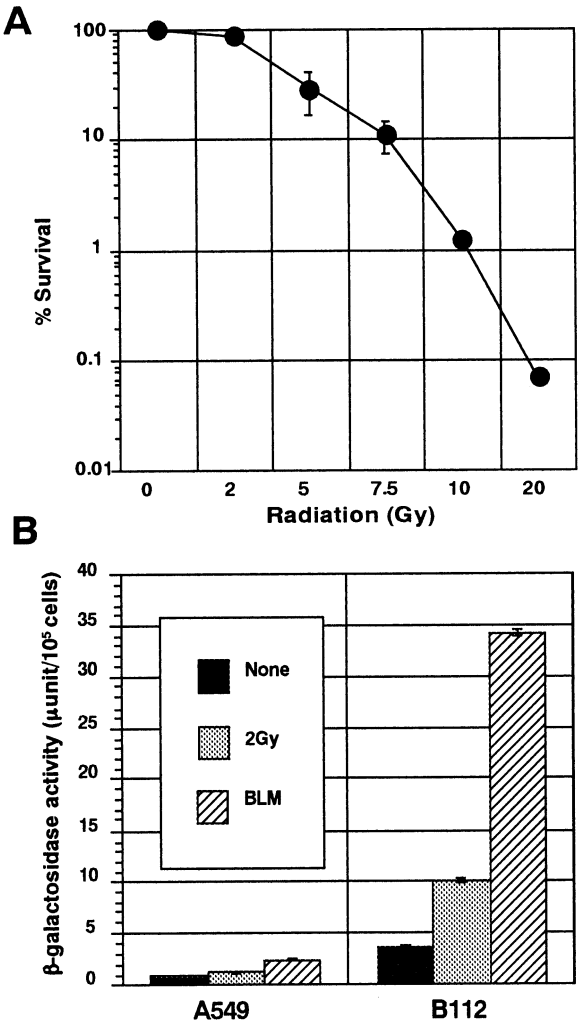

Fig. 1. The response of A549 cells and B112 trap line to X-rays and BLM. A: 1×10^3 A549 cells were seeded into a 100 mm dish and irradiated at doses of 2, 5, 7.5, 10 and 20 Gy on the following day. The colonies composed of more than 200 cells were counted 10 days after irradiation. Each experiment was done in duplicate. B: β-Gal activity in parental A549 cells and B112 trap line were examined 24 h after irradiation or BLM addition. Each assay was done in triplicate.

3.2. Analysis of c-IAP2 promoter

To understand the regulation of c-IAP2 response to X-irradiation, we next analyzed the promoter of c-IAP2. The 3.5 kb upstream region from the c-IAP2 transcription initiation site was isolated and linked to the luciferase reporter gene (3.5K). Since HEK293 cells have a much higher transfection

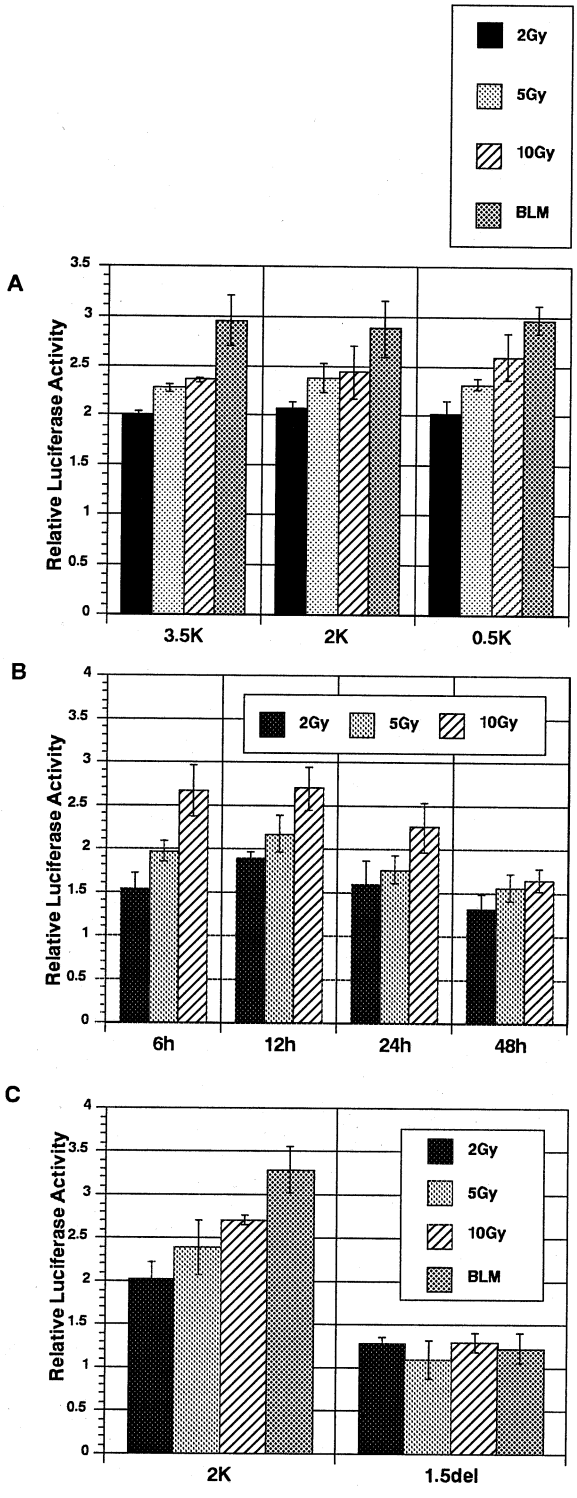

Fig. 2. Analysis of c-IAP2 promoters. A: The response of c-IAP2 promoters to X-rays and BLM. The HEK293 cells transfected with 3.5K, 2K or 0.5K of c-IAP2 promoter-luciferase reporter genes were irradiated at doses of 2, 5 and 10 Gy, or cultured with BLM. The cell lysates were prepared 12 h later and luciferase activities were measured with Dual-luciferase assay. B: The response of 0.5K c-IAP promoter to X-rays. The HEK293 cells transfected with 0.5 kb c-IAP2 promoter-luciferase reporter gene were irradiated at doses of 2, 5, or 10 Gy. Dual-luciferase assay was carried out 12 h after irradiation. C: The response of 5′-deletion mutants of 2K promoter to X-rays and BLM. The HEK293 cells transfected with 1.5del were irradiated at doses of 2, 5 and 10 Gy, or cultured with BLM. The cell lysates were prepared 12 h later and luciferase activities were measured with Dual-luciferase assay. Relative luciferase activity was indicated by the ratios of the irradiated cells M1/M2 to unirradiated cells M1/M2 (M1, net count of firefly luciferase activity; M2, net count of renira luciferase activity). Standard error is indicated by an error bar.
←

efficiency than A549 cells, the activation of c-IAP2 promoters was examined in HEK293 cells to detect the subtle response. First, to narrow the region containing a *cis*-element for X-ray-response in c-IAP2 promoter, 5′-deleted mutants of the 3.5 kb promoter fragment (2K and 0.5K) were constructed and their activities were examined 12 h after irradiation or incubation with BLM (Fig. 2A). The relative luciferase activities of these promoters were comparable each other. Although the induction of c-IAP2 promoters by 2 Gy irradiation was at most twofold, the activation of 0.5K promoter by X-rays was reproducibly detected with the basically same pattern as the 3.5K promoter (Fig. 2B). These data suggested that the *cis*-elements for X-ray-response should be locate in 0.5K promoter. Time course of c-IAP promoter activation after irradiation indicates that c-IAP2 was quickly induced after irradiation (by 6 h), peaked at 12 h and gradually decreased by 48 h after irradiation (Fig. 2B). When the 0.5K promoter region was removed from the 2K promoter (1.5del), the response to X-rays was abrogated (Fig. 2C), confirming that the 0.5K promoter contained a *cis*-element for X-ray-response.

3.3. X-ray and BLM activated c-IAP2 promoter through NF-κB binding sites

The promoter analysis so far indicated that the responses of c-IAP2 promoter to X-rays and BLM were basically the same. Since the condition of BLM-treatment was more constant than the one of irradiation and the data were more reproducible, the further analysis of c-IAP promoter was done with BLM-treatment. To identify the *cis*-element for X-ray-response, next we constructed three 5′-deletion mutants of the 0.5K reporter plasmid, NFB(A+B), NFB(B) and NFB(0) (Fig. 3A). In this region, there are two NFB, A and B (Fig. 3A). The analysis of the promoter of NFB(A+B), NFB(B) and NFB(0) revealed that NFB are necessary for the response to DNA damage since NFB(0), lacking the first NFB, significantly reduced the response to BLM (Fig. 3A).

To improve the response of NFB promoters to X-rays, tandem repeats of NFB(A+B), NFB(A) or NFB(B) were constructed. As we expected, the tandem repeat of NFB(B) was almost the same as the single component (Fig. 3B). The interval sequence of NFB(A) and NFB(B) did not have any enhancer activity (data not shown). The tandem repeats of NFB(A+B) and NFB(A) increased their response to BLM (Fig. 3B).

3.4. Bax expression under the regulation of NFB promoters increases death after 2 Gy irradiation

To examine whether the NFB promoters make applicable vectors to gene therapy combined with conventional radiotherapy, we constructed the tandem repeats of four units of NFB (4NFB) and placed the *bax*-coding region under the control of 4NFB. The resulting plasmid, p4NFB-bax, was transfected to A549 cells and 24 stable clones were isolated

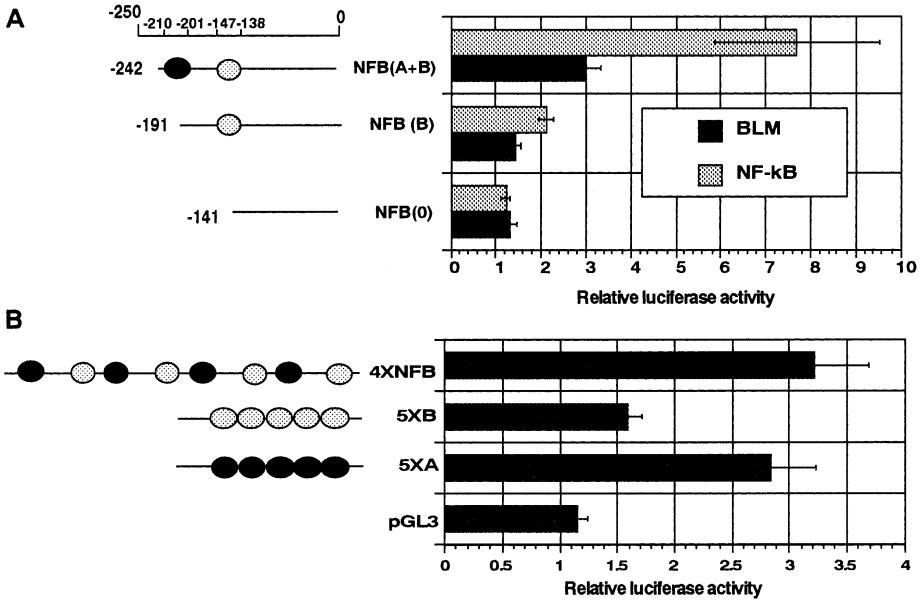

Fig. 3. Analysis of NF-κB binding sites in c-IAP2 promoter. A: The promoter regions indicated in the figure were inserted to a luciferase reporter plasmid as described in Section 2. Two NFB located at −210 to −201 and −147 to −138 are indicated as a solid and a gray cycle, respectively. The numbers indicated in the figure are the relative position to the transcription initiation site. The HEK293 transfectants of the luciferase-reporter plasmids with indicated promoters were cultured with BLM or cotransfected with pCG-p65, a plasmid encoding NF-κB (p65). The cell lysates were prepared 12 h later and luciferase activities were measured with Dual-luciferase assay. B: The HEK293 cells transfected with the luciferase-reporter plasmids with indicated promoters were cultured with BLM. The cell lysates were prepared 12 h later and luciferase activities were measured and indicated as in the legend of Fig. 2.

to examine their response to X-rays. Among them two representative clones were examined by colony assay (Fig. 4). In the colony assay, the number of colonies formed by p4NFB-bax transfectants was significantly reduced after 2 Gy irradiation. Such a reduction was not observed in the cells transfected with the empty vector (Fig. 4A). The increased BAX expression was detected in the p4NFB-bax transfectants 24 h after irradiation (Fig. 4A). More flatter and enlarged cells, which are typically seen in the growth-arrested cells after X-irradiation, were observed in both empty vector and p4NFB-bax transfectants from several days after irradiation although the number of such colonies were few (Fig. 4B(b) and data not shown). In the p4NFB-bax transfectants, typical apoptotic cells were observed from the second day through several days after irradiation (Fig. 4B(d)).

4. Discussion

The results reported here provide evidence that X-rays induce c-IAP2 expression in human cancer cell lines through NFB. The expression of c-IAP2 is induced by various apoptotic stimuli such as tumor necrosis factor (TNF), Fas, menadione, staurosporine, etoposide, Taxol, and growth factor withdrawal [10]. Recently, NF-κB has been reported as a primary regulator of the response to various stresses including DNA damage [11,12]. Our data suggest that X-ray-induced stress activates a similar regulatory mechanism of apoptosis and anti-apoptosis in the irradiated cells as the stimuli above.

Since the stimulation by X-rays is instantaneous and the radiosensitivity of cells is based on their progression through the cell cycle [13], it is difficult to adjust all the cell populations in the best timing for the screening assay. Thus for the primary screening for trap lines, we used BLM as a radiomimetic drug. BLM causes similar changes in cells as X-rays, such as generation of reactive oxygen radicals, induction of DNA double-strand breaks, and the following activation of p53-dependent transduction pathways [14–16]. All four trap lines isolated after primary screening with BLM also responded to X-rays (Fig. 1B and data not shown). Although it has been suggested that BLM and X-rays provoke different modes of DNA damage and repair [17], our results might indicate that the molecules induced by X-rays are largely overlapped with those induced by BLM.

We identified the c-IAP2 promoter as one of the X-ray-responsive one by the gene trap screening. Based on the transcriptional initiation sequences obtained by the 5′RACE, we cloned the 3.5 kb c-IAP2 promoter region which contained the *cis*-element for response to X-rays (Fig. 2). Our sequence analysis of c-IAP2 promoter confirmed the previous report that the c-IAP2 promoter region contains NFB, which can be transcriptionally activated by NF-κB (Fig. 3A and [6]). NF-κB is a dimeric transcriptional factor composed of two proteins belonging to the Rel family, RelA (p65) and NF-κB1 (p50) [18]. NFB(A) should be a RelA binding site since the coexpressed RelA increased NFB(A+B)-regulated luciferase activity but did not increase the NFB(B)-regulated activity (Fig. 3A). Tandem repeats of NFB- or NFB(A)-regulated luciferase activities were increased about three times by 2 Gy X-irradiation and BLM (Fig. 3B and data not shown). The degrees of increased luciferase activities did not change when the number of repeated components were varied from three to five. These data may suggest that the limited number of RalA was activated to bind NFB promoters by the relatively mild stresses of 2 Gy X-irradiation and 0.5 μg/ml BLM.

Although a drastic increase in the relative luciferase activity was not obtained by NFB promoters, a considerable increase

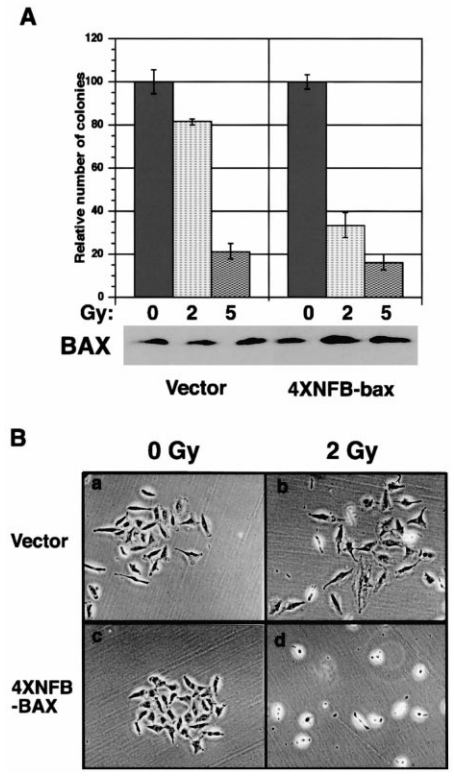

Fig. 4. Analysis of NFB-regulated BAX expression. A: A549 cells transfected with 4NFB-bax were isolated and their BAX expression and colony forming efficiency after 2 or 5 Gy irradiation was examined. The average number of colonies formed by unirradiated vector-transfectants and p4NFB-bax transfectants were 976.5 and 1465, respectively. The cell lysates for Western blot analysis were prepared 24 h after irradiation and the colonies were counted 10 days after irradiation. B: Photographs were taken 3 days after irradiation and each of untreated (a) or 2 Gy-irradiated (b) empty vector-transfectant colonies and untreated (c) or 2 Gy-irradiated (d) pNFB-bax transfectants colonies are shown.

of BAX expression was detected in the p4NFB-bax transfectants 24 h after irradiation and was enough to decrease the number of colonies formed after irradiation (Fig. 4A). Typical apoptotic morphology could be observed on the following day through several days after irradiation (Fig. 4B). The prolonged apoptosis period might be explained by the synergistic effect of the prolonged expression of NFB-regulated BAX and the delayed apoptosis. X-rays are known to induce biphasic apoptotic response; an immediate early response (within 24 h of exposure to radiation) and a delayed response, which occurs usually after cell division and often after multiple cell divisions [19–21].

The other X-ray-responsive genes identified by the gene trap method have been cloned and their promoters are being studied (Akiyama et al., in preparation). This study and the analysis of other X-ray-responsive gene promoters will provide the information to construct promoters highly responsive to a clinically relevant dose of X-irradiation. The combination of gene therapy using such promoters and conventional radiotherapy will be useful in developing improved cancer therapy.

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