Down-Regulation of Nuclear Factor κB Is Required for p53-dependent Apoptosis in X-Ray-irradiated Mouse Lymphoma Cells and Thymocytes

Hidehiko Kawai, Yukiko Yamada, Masaaki Tatsuka, Ohtsura Niwa, Ken-ichi Yamamoto, and Fumio Suzuki

Department of Regulatory Radiobiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8533 [H. K., Y. Y., M. T., F. S.]; Radiation Biology Center, Kyoto University, Kyoto 606-8501 [O. N.]; and Cancer Research Institute, Kanazawa University, Kanazawa 920-0934 [K-i. Y.], Japan

Abstract

Transcription factors p53 and nuclear factor κB (NF-κB) have been implicated in apoptosis induced by DNA-damaging agents, but the relationship between these two factors at the molecular level is largely unknown. We have isolated apoptosis-resistant mutant sublines from a radiosensitive mouse lymphoma 3SB cell line that undergoes p53-dependent apoptosis after X-ray irradiation, and we have analyzed the NF-κB activity. Two of these apoptosis-resistant sublines expressed mutant p53 protein and exhibited a defect in the induction of cyclin-dependent kinase inhibitor p21 after X-ray irradiation. A decrease in the DNA binding activity of NF-κB was observed in the parental 3SB cells after exposure to X-rays, whereas the same activity was unaffected by radiation in the two mutant sublines. A similar down-regulation of NF-κB activity by X-rays was observed in thymocytes derived from p53 wild-type and heterozygous mice, but not in thymocytes from p53 homozygous knockout mice. These results suggest that NF-κB inactivation is p53 dependent and is required for X-ray-induced apoptosis in thymic lymphoma cells and normal thymocytes.

Introduction

Apoptosis is an active process of selective cell killing in the thymus in which T and B cells respond to intercellular or intracellular signals. In the previous study (1), we found that 3SB, a thymic lymphoma cell line that expresses wild-type p53 protein, exhibited apoptosis shortly after exposure to X-rays. Five clonal sublines were isolated from the 3SB cells after mutagen treatment and repeated exposure to X-rays, and these sublines were refractory to X-ray-induced apoptosis. Interestingly, two of the five mutant sublines carried a mutation in the p53 gene. Studies of apoptotic response in thymocytes isolated from p53 knockout mice demonstrated that p53 is required for the induction of apoptosis after treatment with DNA-damaging agents such as γ-rays and etoposide but not after treatment with glucocorticoids or calcium (2, 3). This indicates the existence of p53-dependent and -independent apoptosis pathways in normal thymocytes, which are triggered by different external signals.

Miyashita et al. (4) exploited the temperature-sensitive p53 gene and demonstrated that p53 activates transcription of the proapoptotic factor bax but represses bel-2 in M1 leukemia cells. p53 has also been reported to enhance the expression of the apoptosis-inducing membrane receptor Fas (5) and to induce PAG608 (6) and p85 (7), genes that may play a role in mediating apoptotic cell death. Using a comprehensive technique that allows quantitative evaluation of gene expression, Polyak et al. (8) examined new p53-dependent transcripts and identified 14 genes, named p53-induced genes, that were directly trans-activated by p53 before}

Received 6/28/99; accepted 10/29/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and performed in part through the Radiation Carcinogenesis Research Foundation of Denkyo-ken in Japan.

To whom requests for reprints should be addressed, at the Department of Regulatory Radiobiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. Phone: 81-82-257-3824; Fax: 81-82-257-5825; E-mail: fmsuzuki@pc.hiroshima-u.ac.jp.

The abbreviations used are: NF-κB, nuclear factor κB; TNF, tumor necrosis factor; ES, embryonic stem; KO, knockout; EMSA, electrophoretic mobility shift assay; AT, ataxia telangiectasia; CREB, cAMP-responsive element-binding protein.

apoptosis. In addition to the transactivation-dependent pathway, p53 may participate in apoptosis by posttranslational modifications (9) that affect the stability and binding capability of p53 protein (10).

The function of transcription factor NF-κB is tightly controlled by the cytoplasmic factor IκBα (see Ref. 11 for a review) and can be modulated by various proinflammatory cytokines, such as TNF-α, and genotoxic agents, such as ionizing radiation and UV irradiation. It has recently been shown that inactivation of NF-κB by a variety of external stimuli leads to apoptotic cell death, indicating an antiapoptotic function of NF-κB (12–14). A similar protective function of NF-κB was reported in the E1A-mediated sensitization of radiation-induced apoptosis in human ovarian carcinoma cells (15) and in γ-ray-induced apoptosis in normal human fibroblasts, but not in radiosensitive fibroblasts derived from AT patients (16).

Although NF-κB and p53 have been implicated in radiation-induced apoptosis, functional interaction between these two factors is largely unknown. We therefore examined the p53 dependence of NF-κB inactivation during X-ray-induced apoptosis using a series of mouse lymphoma cell lines and mouse thymocytes derived from p53 knockout mice.

Materials and Methods

Cell Lines and Culture. In this study, we used the mouse thymic lymphoma 3SB cell line and five apoptosis-resistant sublines (1B1C4, 1A1-6, 1A3-4, 1D5-8, and 2A1-1) that had been isolated from 3SB cells through a round of extensive mutagenesis and selection by radiation (1). As described previously (1), the cells were grown in suspension in DMEM supplemented with 10% fetal bovine serum and irradiated with X-rays.

Preparation of Thymocytes from p53 Knockout Mice. The p53-deficient mouse was originally produced by the introduction of a neo gene fragment into the p53 gene locus in a ES line derived from a F1 mouse between the C57BL/6 and CBA strains (17). This knockout mouse was back-crossed to C57BL/6J mice for 25 generations. Male and female mice with the genotype of p53 (KO/+) were mated, and the offspring were genotyped as p53 (KO/KO), p53 (KO/+), and p53 (+/+). Thymocytes were isolated from the mice at the age of 4–6 weeks old and suspended in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were irradiated with X-rays and examined for apoptosis.

Apoptosis Assay. X-irradiated cells were incubated in growth medium for various intervals after irradiation and harvested by centrifugation at 600 × g. The cells were fixed in 1% glutaraldehyde, washed with PBS, and resuspended in PBS containing 1 mm Hoescht 33342. After incubation at room temperature for 15 min, at least 600 or more stained cells were counted under a Zeiss Axiovert 135M fluorescence microscope. In the case of thymocytes, we used the dye exclusion test, in which the cells were stained with erythrosin B to score nonviable cells, as described in our previous report (1).

Western Blot Analysis. Whole cell extract and cytoplasmic extract were prepared as described previously (1). These extracts were electrophoresed on 10% SDS-PAGE and transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). Western blotting was performed with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) according to the protocols.
represents the mean of two independent experiments.

The percentage of cells with abnormal nuclear morphology in 3SB cells (A) shows 3SB and 1B1C4 cells at after X-ray irradiation with 5 Gy, respectively. Neither the unirradiated 3SB and 1B1C4 cells nor X-irradiated 1B1C4 cells exhibit nuclear condensation. In contrast to these cells, almost all X-irradiated 3SB cells show extensive nuclear condensation and fragmentation (Fig. 1aC). The percentage of 3SB cells exhibiting nuclear change increased with incubation time and reached about 95.2% at 12 h after X-ray irradiation (Fig. 1b). However, the 1B1C4 cells and the other four radioresistant sublines (1A1-6, 1A3-4, 1D5-8, and 2A1-1) showed a low frequency of apoptosis, ranging from 4.8–10.3% at 12 h of postirradiation incubation.

In the previous study (1), we demonstrated that two of five radioresistant sublines, 1B1C4 and 1D5-8, carried a point mutation from TCC (serine) to TTC (phenylalanine) at codon 238 in the tumor suppressor p53 gene. In addition to the mutant allele, the 1D5-8 subline carried the wild-type p53 gene and showed normal p53 function when irradiated with X-rays. However, the 1B1C4 subline completely lacked normal p53 activities. To confirm the p53 function in X-ray responses, we examined the levels of the cyclin-dependent kinase inhibitor, p21 protein, expressed in 3SB cells and in three radioresistant sublines (1B1C4, 1A1-6, and 1D5-8) after irradiation with 5 Gy of X-rays. Fig. 2 shows the changes in p21 protein levels at 1–6 h after exposure. Under the experimental conditions described in “Materials and Methods,” we could not detect p21 protein in either cell line immediately after X-ray irradiation. In 3SB and 1A1-6 cells, the p21 protein levels increased with postirradiation incubation time, and a similar pattern was observed in X-irradiated 1A3-4 and 2A1-1 sublines (data not shown). In contrast, the 1B1C4 subline showed no production of p21 protein, indicating a complete defect in p53 function. Furthermore, the expression of p21 in the 1D5-8 subline, which was shown to express both wild-type and mutant-type p53 proteins (1), was not observed until 3 h after exposure and was detected at low levels thereafter. This may result from the dominant negative action of mutated p53 protein. At any rate, the Western blot analysis of p21 protein in X-irradiated cells indicates that the functional loss of p53 prevents apoptotic death in thymic lymphoma cells.

Transcription factor NF-κB has recently been shown to suppress apoptosis induced by TNF-α, ionizing radiation, or daunorubicin (12–14). NF-κB protein function was analyzed in 3SB cells and the five mutant sublines by an EMSA of nuclear extracts from X-irradiated cells. The oligomer used was a 36-bp DNA synthetic fragment containing NF-κB binding sequences. As seen in Fig. 3A, two NF-κB DNA binding activities were detected in nuclear extracts from 3SB cells and five apoptosis-resistant sublines immediately after irradiation with 5 Gy of X-rays. A similar NF-κB DNA binding pattern was also observed in the

Recommended by the manufacturer. Membranes were incubated with the appropriate antibody: (a) anti-p53 polyclonal antibody FL-393; (b) anti-p21 monoclonal antibody F-5; (c) anti-NF-κB p65 polyclonal antibody C-20; or (d) anti-IκBα polyclonal antibody C-15 (Santa Cruz Biotechnology, Santa Cruz, CA).

EMSA for NF-κB DNA Binding. Nuclear extracts were prepared as described previously (1). An EMSA for NF-κB DNA binding was performed using the IL-6κB probe (GGGATTTCCTCC) as described previously (18). The nuclear extracts (usually 6 μg/reaction) were incubated with the 32P-labeled-probe in a 20-μl reaction mixture for 15 min at 20°C, and the reactions were then terminated by the addition of EDTA, SDS, and bromphenol blue. Reaction products were electrophoresed on a 4% polyacrylamide gel.

Results and Discussion

To determine the frequency of apoptotic cells after X-rays, we stained the cells with Hoechst 33242 and examined the changes in nuclear morphology under a fluorescence microscope. Fig. 1a, A and B, shows unirradiated parental 3SB cells and the radioresistant 1B1C4 subline; Fig. 1a, C and D, shows 3SB and 1B1C4 cells at after X-ray irradiation with 5 Gy, respectively. Neither the unirradiated 3SB and 1B1C4 cells nor X-irradiated 1B1C4 cells exhibit nuclear condensation. In contrast to these cells, almost all X-irradiated 3SB cells show extensive nuclear condensation and fragmentation (Fig. 1aC). The percentage of 3SB cells exhibiting nuclear change increased with incubation time and reached about 95.2% at 12 h after X-ray irradiation (Fig. 1b). However, the 1B1C4 cells and the other four radioresistant sublines (1A1-6, 1A3-4, 1D5-8, and 2A1-1) showed a low frequency of apoptosis, ranging from 4.8–10.3% at 12 h of postirradiation incubation.

In the previous study (1), we demonstrated that two of five radioresistant sublines, 1B1C4 and 1D5-8, carried a point mutation from TCC (serine) to TTC (phenylalanine) at codon 238 in the tumor suppressor p53 gene. In addition to the mutant allele, the 1D5-8 subline carried the wild-type p53 gene and showed normal p53 function when irradiated with X-rays. However, the 1B1C4 subline completely lacked normal p53 activities. To confirm the p53 function in X-ray responses, we examined the levels of the cyclin-dependent kinase inhibitor, p21 protein, expressed in 3SB cells and in three radioresistant sublines (1B1C4, 1A1-6, and 1D5-8) after irradiation with 5 Gy of X-rays. Fig. 2 shows the changes in p21 protein levels at 1–6 h after exposure. Under the experimental conditions described in “Materials and Methods,” we could not detect p21 protein in either cell line immediately after X-ray irradiation. In 3SB and 1A1-6 cells, the p21 protein levels increased with postirradiation incubation time, and a similar pattern was observed in X-irradiated 1A3-4 and 2A1-1 sublines (data not shown). In contrast, the 1B1C4 subline showed no production of p21 protein, indicating a complete defect in p53 function. Furthermore, the expression of p21 in the 1D5-8 subline, which was shown to express both wild-type and mutant-type p53 proteins (1), was not observed until 3 h after exposure and was detected at low levels thereafter. This may result from the dominant negative action of mutated p53 protein. At any rate, the Western blot analysis of p21 protein in X-irradiated cells indicates that the functional loss of p53 prevents apoptotic death in thymic lymphoma cells.

Transcription factor NF-κB has recently been shown to suppress apoptosis induced by TNF-α, ionizing radiation, or daunorubicin (12–14). NF-κB protein function was analyzed in 3SB cells and the five mutant sublines by an EMSA of nuclear extracts from X-irradiated cells. The oligomer used was a 36-bp DNA synthetic fragment containing NF-κB binding sequences. As seen in Fig. 3A, two NF-κB DNA binding activities were detected in nuclear extracts from 3SB cells and five apoptosis-resistant sublines immediately after irradiation with 5 Gy of X-rays. A similar NF-κB DNA binding pattern was also observed in the

Fig. 2. Western blot analysis of the expression of p21 protein in 3SB, 1B1C4, 1A1-6, and 1D5-8 cells at various times after X-ray irradiation. The cells were unirradiated (0 h) or irradiated with 5 Gy of X-rays and harvested 1.5, 3.0, 4.5, and 6.0 h after exposure.
actively growing cells without X-ray irradiation (data not shown). These results indicate the existence of a constitutive activation of NF-κB in 3SB cells and five apoptosis-resistant sublines. As reported previously (14, 15, 18), the two bands indicated by arrows represent the DNA-protein complexes of the RelA/p65 and p50 heterodimer and the p50 homodimer, respectively. Interestingly, although p53 wild-type cell lines such as 3SB, 1A1-6, 1A3-4, and 2A1-1 showed a significant decrease in NF-κB DNA binding activity after exposure to X-rays, there was no such change in the 1B1C4 and 1D5-8 sublines. However, as seen in Fig. 3B, the levels of NF-κB protein in the cytoplasmic extracts were constant over the 3-h postirradiation periods in parental 3SB cells and the apoptosis-resistant 1B1C4 subline. Similarly, the IκBα protein was constantly expressed during a 1- or 3-h incubation after X-ray irradiation in 3SB cells or in the 1B1C4 subline, respectively (Fig. 3B). The decrease of IκBα in 3SB cells at 3 h after X-ray irradiation may result from a nonspecific protein degradation of dead cells accumulated in the X-irradiated cell population. A constant expression of both of the proteins was also observed in X-irradiated 1A1-6, 1A3-4, 1D5-8, and 2A1-1 sublines (data not shown).

Thus, the difference in the NF-κB DNA binding activity among these cell lines cannot be ascribed to either the different amount of NF-κB or the varied degradation of IκBα. As described above, the 1B1C4 subline expresses no p21 because of its complete defect in p53 function, and the 1D5-8 subline exhibits a delayed, low expression of p21, possibly due to the mutated p53. However, the other three sublines showed a transient increase in p53 protein (1) and a significant induction of p21 transcripts after X-ray irradiation. The expression patterns of both of the proteins in these three sublines are the same as those in their parental 3SB cells. The finding that there is a good correlation between the expression of functional p53 and the down-regulation of NF-κB in X-irradiated cells suggests that NF-κB plays a role in p53-dependent signaling pathways of X-ray-induced apoptosis in mouse thymic lymphoma cells.

To confirm the relationship between p53 and NF-κB in X-ray-induced apoptosis, p53-null thymocytes were examined. We isolated thymocytes from 4-6-week-old p53 (+/+), p53 (KO/KO), and p53 (KO/KO) mice and irradiated them with 5 Gy of X-rays, and cytoplasmic extracts were prepared immediately after irradiation or 0.5, 1.0, and 3.0 h after exposure.

mozygous null mice were extremely resistant to X-ray-induced apoptosis, whereas there was no difference in the induction of dye-stained cells between thymocytes from homozygous p53 (+/+) and heterozygous p53 (KO/+) mice. A similar p53 dependence in the induction of apoptosis was also observed when the changes in nuclear morphology were examined in X-irradiated thymocytes under the fluorescence microscope (data not shown). Using an EMSA, we analyzed the DNA binding ability of NF-κB protein in X-irradiated thymocytes. Nuclear extracts prepared immediately after irradiation from the thymocytes from p53 (+/+), p53 (KO/+) and p53 (KO/KO) mice showed two bands of NF-κB-DNA complexes (Fig. 4B). In addition, when unirradiated, these thymocytes exhibited a high intensity of NF-κB-DNA bands (data not shown), again indicating a constitutive activation of NF-κB in the mouse thymus. Two bands disappeared quickly during the incubation of p53 (+/+) and p53 (KO/+) thymocytes after 5 Gy of irradiation. Although a similar decrease in these two bands was noted in p53 (KO/KO), it was not as pronounced as that seen in p53 (+/+) and p53 (KO/+) thymocytes. These results confirm that p53 function is tightly coupled with down-regulation of NF-κB and further suggest that down-regulation of NF-κB is necessary for X-ray induction of p53-dependent apoptosis of normal thymocytes.

Inactivation of NF-κB has been shown to be required for the induction of apoptosis in a variety of cells by various genotoxic agents (12-16), but the mechanisms by which the presence of NF-κB enhances cell survival have not been fully analyzed. Chu et al. (19) reported that c-IAP2, one of the cellular homologues to baculovirus inhibitors of apoptosis, was critically involved in TNF-α-induced apoptosis and exerted positive feedback control on NF-κB. IEX-1L, a long type of gene product generated from immediate early response gene IEX-1, has also been shown to block cell killing by TNF-α treatment (20). Interestingly, Wang et al. (21) demonstrated that the activation of these apoptosis inhibitors and TNF receptor-associated factors 1 and 2, all of which are shown to be activated by NF-κB, functioned to suppress this type of apoptosis at the level of caspase 8. Recently, one prosurvival factor, Bcl-2 homologue Bfl-1/A1, was identified as a transcriptional target of NF-κB (22, 23). Because Bcl-2 family proteins are a key regulator of apoptotic response and the transcription of the Bfl-1/A1 gene is distinctly controlled by NF-κB, this transcription factor could be directly activated to inhibit apoptotic cell death.

ATM kinase, the gene product responsible for AT, is capable of phosphorylating p53 protein, and ATM kinase activity is enhanced in cells irradiated with ionizing radiation (21). It has been established that p53 is located downstream of ATM in DNA damage-induced
how activated p53 elicits the parallel inactivation of NF-κB would provide useful information to help understand the varying susceptibility among different tumor tissues to radiation-induced apoptosis.

Acknowledgments

We thank Drs. H. Hama-Inaba, M. Muto, and H. Ohyama for the kind gift of the 3SB cell line and Dr. H. Yajima for helpful suggestions.

References


27. Acknowledgments

We thank Drs. H. Hama-Inaba, M. Muto, and H. Ohyama for the kind gift of the 3SB cell line and Dr. H. Yajima for helpful suggestions.
Down-Regulation of Nuclear Factor κB Is Required for p53-dependent Apoptosis in X-Ray-irradiated Mouse Lymphoma Cells and Thymocytes

Hidehiko Kawai, Yukiko Yamada, Masaaki Tatsuka, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/59/24/6038

Cited articles  This article cites 26 articles, 18 of which you can access for free at: http://cancerres.aacrjournals.org/content/59/24/6038.full#ref-list-1

Citing articles  This article has been cited by 4 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/59/24/6038.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.