Increased Frequencies of Gene and Chromosome Mutations after X-Irradiation in Mouse Embryonal Carcinoma Cells Transfected with the bcl-2 Gene

Masataka Taga,1, 2 Kazunori Shiraishi,1 Tsutomu Shimura,1 Norio Uematsu,1 Mitsuo Oshimura2 and Ohtsura Niwa1, 3

1Department of Late Effect Studies, Radiation Biology Center, Kyoto University, Yoshida Konoe, Sakyo-ku, Kyoto 606-8501 and 2Department of Molecular and Cell Genetics, School of Life Sciences, Faculty of Medicine, Tottori University, Nishimachi 86, Yonago, Tottori 683-8503

Preimplantation stage mouse embryos are known to be highly sensitive to the killing effect of DNA-damaging agents such as radiation. Interestingly, however, this stage of development is well protected from radiation induction of malformation and carcinogenesis in postnatal life. In recent years, it has become clear that the stem cells of preimplantation stage embryos undergo extensive apoptosis after DNA damage. It has been postulated that this apoptosis is likely to be responsible for the resistance to malformation, by excluding cells carrying deleterious DNA damage. We have tested the possible role of apoptosis in elimination of gene and chromosome mutations in undifferentiated mouse embryonal carcinoma cell line, F9, transfected with human bcl-2 cDNA. The colony radiosensitivity of F9 cells was not affected by overexpression of the bcl-2 gene, but the apoptotic cell death was suppressed, as examined by DNA ladder assay and Hoechst staining. This suppression was accompanied by an increase in the frequencies of hprt mutation and micronucleus formation after X-irradiation. These results support the idea that maintenance of genomic integrity during early development is likely to be executed by apoptotic elimination of cells at risk.

Key words: Radiation — Mutation — Apoptosis — bcl-2 — Embryonal carcinoma cell

Radiation is known to induce mutation by directly damaging DNA. In addition, increasing evidence supports the idea that radiation induces genomic instability which leads to delayed mutation in the irradiated population.1, 2) Genomic instability has recently been proposed as a mechanism through which carcinogenic mutations are generated in somatic tissues.3) It has been well documented that preimplantation stage mouse embryos are highly sensitive to ionizing radiation.4–7) Irradiation of this stage frequently leads to unsuccessful pregnancy and prenatal death, but the live-born mice are devoid of malformation and other untoward outcomes of radiation, such as carcinogenesis at a later time.8, 9) The radiosensitivity decreases with continued development, but malformation would result from irradiation during organogenesis and carcinogenesis is the outcome of postnatal irradiation. It is possible that the cells of the preimplantation stage of development are endowed with a capacity to circumvent genomic instability. One possible mechanism might be the elimination of cells at risk by apoptosis.10) In other words, it is likely that apoptosis may selectively eliminate these cells carrying a damaged and unstable genome. This prediction was verified in a recent study in which p53-mediated apoptosis was found to suppress malformation after X-irradiation.11) However, selective elimination of cells at risk by apoptosis has not been investigated in cells of preimplantation stage origin.

Undifferentiated mouse embryonal carcinoma (EC) cells have been studied extensively as an in vitro model of preimplantation stage cells.12) They grow in vitro as well as in vivo and form colonies at a high efficiency in culture. In addition, they differentiate readily upon treatment with retinoic acid. These cells were shown to be highly sensitive to UV.13) Testicular tumors share many features in common with EC cells. These tumors are known to carry the wild-type p53 gene and respond well to conventional chemotherapy.14, 15) Similarly, F9 mouse EC cells exhibit characteristic features of apoptosis, such as morphological changes and internucleosomal DNA fragmentation after exposure to ionizing radiation.16) Therefore, undifferentiated mouse EC cells are a useful model to study the biological significance of apoptosis in preimplantation stage development.

The bcl-2 gene was originally identified at the break point of a translocation, t(14;18), associated with human follicular lymphomas.17) Bcl-2 is the best-known member of the family involved in regulation of apoptosis.18–21) Enforced expression of the bcl-2 gene has been shown to abrogate apoptosis in numerous experimental systems in response to diverse apoptotic stimuli, including ionizing radiation.22, 23) In addition, the bcl-2 gene is known to play a role in increasing the frequency of cancer by blocking apoptosis.24)
In this study, we have examined apoptotic elimination of gene and chromosome mutation in F9 cells. The results indicated that overexpression of the human bcl-2 gene and the subsequent suppression of radiation induction of apoptosis lead to elevated levels of hprt mutation and micronucleus formation.

MATERIALS AND METHODS

Cells and cell culture  F9 cells are an undifferentiated mouse EC cell line carrying the functional p53 gene.\(^\text{25, 26}\) F9 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin.

Plasmid construction and transfection  Human bcl-2 cDNA without a poly A signal was excised from pUC19-B4:human bcl-2 cDNA plasmid\(^\text{27}\) and introduced into the EcoRI site of pPyCAGIRESexeoconP vector (kindly supplied by Dr. H. Niwa). The structure of the resulting plasmid, designated as pPyCAGIRESexeocon-bcl2-pA, is shown in Fig. 1. The chicken β-actin promoter drives transcription of polycistronic mRNA consisting of the human bcl-2 cDNA, internal ribosome entry site (IRES), zeocin resistance gene (zeocin R) and the poly A signal. This plasmid was introduced into F9 cells by the use of LIPOFECTIN Reagent (Life Technologies, Inc., Rockville, MD). After selection in the presence of 100 µg/ml zeocin (Invitrogen, Groningen, The Netherlands), clones were isolated and tested for expression of the transgene.

Western analysis  Expression of bcl-2 protein was tested by western blotting. Cells were lysed in a buffer (150 mM NaCl, 1.0% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl pH 8.0), and 100 µg protein per lane was separated through 12% polyacrylamide gel. Protein was then transferred to Hybond-ECL membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Western blotting was blocked for non-specific protein binding by incubating it in 5% (w/v) skimmed milk for 1 h at 37 °C, the medium was removed and dishes were washed with phosphate-buffered saline (PBS), and lysed at 4°C for 10 min in lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% Triton X-100). After 13 000g centrifugation at 4°C for 30 min, the supernatant fluid of cell lysates was treated with RNase (400 µg/ml) and proteinase K (400 µg/ml) at 37°C for 2 h each. NaCl was added to 0.5 M and DNA was recovered by isopropanol precipitation at −20°C overnight. DNA was dissolved in 20 µl of TE buffer, a 3 µl DNA sample was electrophoresed on a 2.0% agarose gel and the gel was visualized by ethidium bromide staining.

Quantification of apoptosis by Hoechst staining  Cells were seeded 24 h prior to irradiation and were harvested at various times after irradiation. The cells were then fixed in 1% glutaraldehyde and washed with PBS. The fixed cells were stained with 0.25 µg/ml Hoechst 33342. Nuclear morphology was observed under a fluorescence microscope.

Analysis of hprt mutation  For HAT selection medium (0.1 mM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine) and HT selection medium (0.1 mM hypoxanthine, 16 µM thymidine), DMEM was added with 100× HAT Supplement (Life Technologies, Inc.) and 100× HT Sup-
plement (Life Technologies, Inc.), respectively. Prior to irradiation, cells were grown in HAT medium for 7 days to eliminate pre-existing hprt mutants and then in HT medium for 3 days for recuperation from the effect of HAT. Cells were then irradiated and maintained in non-selective medium for 7 days to allow fixation of mutations. Resistance to 6-thioguanine (6TG) was assayed by plating $4 \times 10^5$ cells onto 10 cm dishes in the medium containing $1 \mu g/ml$ 6TG. Ten and 30 dishes were used for the analyses of irradiated and unirradiated cells, respectively. After around 7 days, dishes were fixed, stained and colonies were scored.

Micronucleus assays For the micronucleus assays, $5 \times 10^4$ cells were plated onto a 6 cm dish 24 h prior to irradiation. Immediately after irradiation, cytochalasin B was added to the culture at $0.5 \mu g/ml$ and incubation was continued for another 48 h. Cells were harvested by trypsinization and fixed in a solution of methanol/acetic acid (3/1). Fixed cells were placed on a glass slide, air-dried, stained with 100 $\mu g/ml$ Acridine Orange for 1 min, washed with PBS for 3 min and observed under a fluorescence microscope. Under this condition, cytoplasm was stained red for RNA and the nucleus was stained yellowish green for DNA. Binucleated cells were examined for micronuclei (yellowish green staining).

RESULTS

Establishment of F9 clones overexpressing human bcl-2 protein The human bcl-2 cDNA expression plasmid, pPy-CAGIRESzoeacin-bcl2-pA, was transfected into F9 cells (Fig. 1). The chicken $\beta$-actin promoter efficiently functioned in EC cells, and polycistronic mRNA with the human bcl-2 cDNA and the zeocin $R$ gene was transcribed. With this transcript, synthesis of the human bcl-2 protein precedes that of the zeocin $R$ protein initiated at the IRES site, ensuring the expression of the former in the drug-resistant cells. The untransfected cells were completely blocked in the presence of zeocin, while transfected clones, S2 and S3, were unaffected (Fig. 2a). Strong expression of human bcl-2 protein was detected in S2 and S3 cells by western analysis (Fig. 2b). The doubling times were identical for F9, S2 and S3 cells, and were approximately 12 h, indicating that the overexpression of the human bcl-2 gene had no effect on cell growth under normal conditions.

Analysis of apoptosis One of the features of apoptosis is fragmentation of genomic DNA by endonuclease, which is detected by DNA ladder assay. F9, S2 and S3 cells were irradiated with 4 Gy of X-ray and the cells were harvested 48 h later. DNA was analyzed by electrophoresis through an agarose gel. X-Irradiation induced DNA ladder formation in F9 cells, but not in S2 or S3 cells (Fig. 3a). This result indicated that overexpressed human bcl-2 protein effectively suppressed DNA ladder formation after X-irradiation. A similar suppression of UV-induced apoptosis was observed (data not shown).

The frequency of cells undergoing apoptosis was quantified by scoring cells with irregular nuclear morphology after Hoechst staining (Fig. 3b). F9 cells exhibited spontaneous apoptosis at a frequency of around 1%. After irradiation with 4 Gy of X-rays, the frequency increased to 2.7% at 24 h and then gradually to 4.5% at 72 h. Spontaneous apoptosis was suppressed significantly in S2 and S3 cells ($P = 0.0005-0.02$ by $\chi^2$ test), and so was the X-ray-induced apoptosis ($P = 0.00002-0.001$ by $\chi^2$ test). At 48 h after irradiation, apoptosis was approximately four times less frequent in S2 and S3 cells than in F9 cells. These results indicated that overexpressed human bcl-2 protein inhibited apoptosis after X-irradiation in F9 cells.

Colony radiosensitivity of the cells The colony radiosensitivity was examined in F9, S2 and S3 cells after X-ray or UV exposure (Fig. 4, a and b). S2 and S3 cells were slightly more sensitive to X-rays and UV than parental F9 cells, but the difference was marginal. The experiments were repeated and almost identical results were obtained (data not shown). Thus, overexpression of human bcl-2 exerted no effect on the clonogenic survival of the cells.
even though it inhibited radiation-induced apoptosis quite efficiently.

**Gene mutation at the hprt locus** In order to test a possible role of apoptosis in selective removal of damaged and potentially deleterious cells, the frequency of X-ray- and UV-induced mutation at the hprt locus was examined in F9, S2 and S3 cells (Fig. 5). The spontaneous mutation frequencies at the hprt locus for F9, S2 and S3 cells were $7.3 \times 10^{-6}$, $2.6 \times 10^{-6}$ and $1.2 \times 10^{-6}$, respectively. The decreases in the frequency for S2 and S3 cells were not statistically significant. After irradiation with 4 Gy of X-rays, the mutation frequency increased to $18.7 \times 10^{-6}$ for

**Fig. 3.** Detection of apoptosis after X-irradiation. 

a. DNA ladder assay. DNA was isolated 48 h after X-irradiation from F9, S2 and S3 cells and electrophoresed on 2.0% gels. M is the 100 bp DNA ladder marker. Note that only X-irradiated F9 cells show the ladder.

b. Quantification of apoptosis by Hoechst staining. F9 (○), S2 (△) and S3 cells (□) were harvested at various times after X-irradiation (24, 48, 72 h). Harvested cells were washed, fixed, and stained with Hoechst 33342. Nuclear morphology was observed under a fluorescence microscope. Solid symbols show the 4 Gy X-irradiated cells (●, △, ■). The ordinate shows the percentage (%) of apoptotic cells and the abscissa shows time (h) after X-irradiation.

**Fig. 4.** Survival assay after X-irradiation. 

a. Colony survival after X-ray irradiation of F9 (○), S2 (△) and S3 cells (□).

b. Colony survival after UV irradiation of F9 (○), S2 (△) and S3 cells (□).
F9 cells, $6.43 \times 10^{-6}$ for S2 cells and $35.7 \times 10^{-6}$ for S3 cells. Similarly, after 16 J/m² UV, the mutation frequencies increased to $106.5 \times 10^{-6}$ for F9 cells, $148.4 \times 10^{-6}$ for S2 cells and $154.2 \times 10^{-6}$ for S3 cells. For both X-rays and UV, the frequencies were always higher for S2 and S3 cells than for F9 cells and the differences were statistically significant. Although the exact frequencies varied among independent experiments, the trend of higher mutation frequencies for S2 and S3 cells was consistently observed. In addition, examination of X-ray doses of 1 and 2 Gy also revealed that the mutation frequency was consistently higher for S2 cells than for F9 cells (data not shown).

**Micronucleus assays** Similar assessment of the effect of bcl-2 overexpression was performed on chromosome mutation. Acentric chromosome fragments induced by ionizing radiation form micronuclei (MN) in the subsequent cycle. MN frequencies were assessed at 48 h after X-irradiation in F9, S2 and S3 cells (Fig. 6). S2 and S3 cells exhibited a similar level of spontaneous MN frequencies to that in F9 cells. However, when the cells were irradiated, the slopes of the dose response for S2 and S3 cells were about twice that of F9 cells, indicating that MN frequencies after X-irradiation were consistently higher in cells overexpressing the bcl-2 gene and the differences were highly significant at the dose point of 4 Gy ($P=0.0002$–0.002).

**DISCUSSION**

Preimplantation stage mouse embryos are known to be highly sensitive to radiation, but surviving fetuses are devoid of malformations. Malformation was observed only after irradiation of the later stage fetus undergoing organogenesis. Radiation carcinogenesis requires still later stages of ontogeny. It is interesting to note that irradiation of newborn mice was less effective in induction of cancer than that of mice a few weeks/months old. Induction of genomic instability has been proposed to play an important role in carcinogenesis. During the post-implantation development and post natal life, tissues and cells of the body become less sensitive to radiation induction of apoptosis. Thus, lack of teratogenesis and carcinogenesis in preimplantation stage irradiation may be due to apoptotic elimination of genetically unstable cells.

Apoptotic response appears to occur in human embryos as early as in the blastocyst stage of development. Several laboratories have reported damage-induced apoptotic responses in embryos of *Xenopus* and zebrafish. These results suggest that the apoptotic surveillance mechanism is an integral part of the developmental program of early embryogenesis in a wide variety of vertebrate species.

In the present study, apoptotic surveillance of damaged and potentially mutagenic cells was tested in undifferentiated mouse EC cells. Radiation induction of apoptosis with formation of the characteristic DNA ladder is p53-dependent in EC cells. F9 cells used in the present study carry the wild-type p53 gene and readily undergo radiation-induced apoptosis. This DNA fragmentation was not observed in mouse fibroblastic lines such as NIH3T3 and m5S cells after the same dose of X-irradiation (Taga, unpublished observation).
It was shown that p53 acts to suppress tumor formation in vivo by preventing the accumulation of cells with chromosomal breakage.\(^5\) The importance of the p53 gene in induced mutagenesis is well recognized. The role of apoptosis in elimination of potentially mutagenic cells can be tested by the use of p53 null cells. However, the pleiotropy of p53 functions makes it difficult to clarify the role of apoptosis in mutation induction. Therefore, we utilized overexpression of the bcl-2 gene to suppress only the apoptotic pathway. A similar approach has been used to investigate the mutation frequency of human lymphoblastoid cell lines.\(^6\)

The colony radiosensitivity of EC cells was not affected by bcl-2 overexpression in S2 and S3 cells, which suppressed radiation-induced apoptosis with the characteristic DNA ladder formation. The dissociation of apoptotic radiosensitivity and colony radiosensitivity was noted for p53 null mouse EC cells after cisplatin treatment\(^7\) and for bcl-2 overexpressing human prostate cancer cells after X-irradiation.\(^8\) It was proposed that the major pathway of cell killing by radiation is mitosis-dependent and only a minor fraction of cell death is accounted for by apoptotic cell death.\(^9\)

The frequency of gene mutation at the hprt locus after X-rays and UV was consistently higher for S2 and S3 than for parental F9 cells. This indicated that parental F9 cells exploit apoptosis in elimination of potentially mutagenic cells. Thus, it appears that the surviving cells mutate more frequently when their apoptosis is suppressed by overexpression of the bcl-2 gene. These apparent paradoxes can be resolved if the mutagenic pathway shares the same inducible process with apoptosis. In this model, irradiated cells activate a pathway which either leads to genomic instability or apoptosis. Suppression of the latter leaves the former to function, resulting in higher frequencies of hprt mutation. Analyses of mutations at the hprt locus occurring as a result of radiation-induced genomic instability identified small deletions and point mutations, instead of the large mutations expected to be induced by ionizing radiation.\(^10\) Therefore, characterization of mutation types in EC cells with and without bcl-2 overexpression may reveal the mechanism of induced mutation and its elimination in preimplantation stage stem cells.

Elevated frequencies of X-ray-induced micronuclei formation in S2 and S3 cells suggest that apoptosis eliminates cells carrying unhealed double strand breaks which lead to acentric chromosome fragments. This elimination of large mutations may simply be attained by activation of the apoptotic pathway by unrepaired chromosome ends. Indeed, one double strand break in a nucleus was reported to activate p53 and cell cycle arrest.\(^11\) Our present study suggests that apoptosis is likely to be the mechanism of elimination of gene and chromosome mutations in preimplantation stage embryonal stem cells. Elucidation of the molecular pathway involved in the process, especially the inducible process which couples apoptosis and mutagenesis may throw light on a new mechanism regulating the frequency of mutation in somatic as well as in germ cells.

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