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Levels of Superoxide Dismutases, Glutathione, and Poly(ADP-ribose) Polymerase in Radioresistant Human KB Carcinoma Cell Line

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In order to investigate the radioresistance mechanism of human carcinoma cells, we measured intracellular manganese- (Mn-) and copper/zinc- (Cu/Zn-) superoxide dismutases (SODs), glutathione (GSH) and poly(ADP-ribose) polymerase (PARP) in radioresistant N10 and its parental KB cell lines. The Mn-SOD level was 1.3-fold less in N10 than in KB, but Mn-SOD was induced at 1.3 to 1.5-fold higher level in N10 than in KB by X-irradiation (4 Gy). Cu/Zn-SOD in N10 showed a higher level than that in KB both without and with irradiation. In addition, N10 had a 1.65-fold higher GSH level than did KB and became radiosensitive on treatment with buthionine sulfoximine, an inhibitor of GSH. Furthermore, PARP mRNA was highly expressed in N10 as compared to KB under unirradiated conditions. X-Irradiation reduced the PARP mRNA level in KB in a time-dependent manner, whereas the PARP mRNA level in N10 was still high at 6 h postirradiation. Assay for PARP activity demonstrated an approximately 3-fold higher activity in N10 than in KB under unirradiated conditions. X-Irradiation caused a rapid induction of PARP activity within 1 h in both cell lines, but treatment of cells with nicotinamide, a PARP inhibitor, markedly reduced the enzyme induction in N10, but not in KB, and potentiated the radiosensitivity in N10. These factors may all contribute to the radioresistance of the N10 cell line.

Key words: KB carcinoma cell line — Radioresistance — Superoxide dismutase — Glutathione — Poly(ADP-ribose) polymerase

DNA damage in irradiated cells is considered to be induced by direct and indirect interactions of electrons secondarily excited by radiation energy.[1] Indirect effects, due to free radicals, hydrated electrons and hydrogen peroxide produced by the reaction of secondary electrons with water molecules, are particularly important. Many factors influence the radiosensitivity in tumor cells: inherent radiosensitivity,[2] intracellular SH content[3,4] and related enzyme activities,[5] cell proliferation kinetics,[6] chromosome polyploidy,[7] cell cycle,[8] activation of oncogenes[9] and expression of mutant tumor suppressor genes.[10,11] However, the precise mechanism of radioresistance in tumor cells is still unclear. In order to investigate this, we isolated and established radioresistant human KB carcinoma cell lines by treatment with mutagens, followed by X-irradiation. Among several cell lines isolated, we preliminarily characterized a cell line, N10, that was isolated after pretreatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and selection with bleomycin and X-irradiation, and showed stable growth with a 3.1-fold radioresistance. This cell line exhibited a similar growth capability, cell proliferation kinetics and chromosome polyploidy to those of the parental KB cell line. Thus, it should be a suitable model for analyzing other factors involved in the radioresistance.

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In the present study, we compared levels of superoxide dismutase (SOD), glutathione (GSH) and poly (ADP-ribose) polymerase (PARP) in the radioresistant N10 with those in the parental KB. Their contribution to the radioresistance of N10 is discussed.

MATERIALS AND METHODS

Cells and cell culture KB[12] and the radioresistant N10 cell line,[13] which was isolated from KB cells by pretreatment with MNNG (Aldrich Chemical Co., Inc., Milwaukee, WI) and subsequent treatment with 5 μg/ml of bleomycin (BLM; Nippon Kayaku Co., Tokyo) and 10 Gy of X-rays, were used in this study. The N10 cell line showed a 3.1-fold resistance to X-irradiation as compared to the parental KB cell line. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 4 m*M* L-glutamine as the growth medium in a 5% CO_2 incubator at 37°C, except that growth medium containing 2.5 μg/ml of BLM was used for N10 cell line for maintenance of radioresistance. The cells were subcultured routinely by dissociation with 0.15% ethylenediaminetetraacetic acid (EDTA) and 0.08% trypsin dissolved in Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline [PBS(−)]. N10 cell line was

cultured in growth medium without BLM for 48 h before experiments.

Assay for SOD SOD was determined by an enzyme-linked immunosorbent assay (ELISA) using the Cu/Zn-SOD and Mn-SOD ELISA System (Amersham International plc, Bucks, UK). Briefly, $3\text{–}5\times10^6$ unirradiated or X-irradiated cells were collected by trypsinization and suspended in 0.5 ml of PBS. After sonication on ice, the homogenates were centrifuged at 2,500*g* for 5 min at 4°C. The supernatants were diluted to the protein concentration of 100 μg/ml with PBS containing 0.1% bovine serum albumin and then 100 μl aliquots were added to anti-human Cu/Zn- or Mn-SOD mouse monoclonal antibody-coated wells.[14, 15] After incubation for 1 h at room temperature, unbound antigen was removed by washing with PBS containing 0.05% Tween 20, and then 100 μl of secondary anti-mouse IgG goat antibody conjugated with horseradish peroxidase was added to each well. After 1 h at room temperature, wells were washed with PBS containing 0.05% Tween 20. The substrate for horseradish peroxidase (100 μl of 0.003% H_2O_2 in 0.1 *M* sodium citrate buffer, pH 5.0, containing 0.6 mg/ml of *o*-phenylenediamine) was then added to the wells. After 15 min at room temperature, the enzyme reaction was stopped by the addition of 50 μl of 2 *N* sulfuric acid. Absorbance was measured at 492 nm with a Microplate-Reader (Model 550, BIO RAD Laboratories, Hercules, CA). Protein concentration in the sample was measured by the Bradford method[16] using Coomassie Plus Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard. SOD levels are given as ng/100 μg protein.

Assay for GSH Exponentially growing cells were washed, suspended at a density of 1×10^6 per ml, and centrifuged at 2,500*g* for 5 min at 4°C. The pellet was resuspended in 4 volumes of 6% metaphosphoric acid, and mixed thoroughly. The pellet suspension was centrifuged at 3,000*g* for 10 min at 4°C and the clear supernatant was collected as a sample. The glutathione was measured colorimetrically by the GSH-400 method (Bioxytech GSH-400; OXIS International S. A., Bonneuil/Marne Cedex, France). The sample was diluted to a final volume of 900 μl with a buffer containing 200 m*M* potassium phosphate (pH 7.8), 0.2 m*M* diethylenetriaminepentaacetic acid, and 0.025% Lubrol. The diluted sample was mixed with 50 μl of 12 m*M* chromogenic reagent in 0.2 *N* HCl and then further mixed with 50 μl of 30% NaOH. After incubation at 25°C for 10 min in the dark, the absorbance at 400 nm of the reaction mixture was measured. The amount of GSH in the sample was calculated as μM per 100 μg protein.

Assay for radiosensitivity Cell survival after X-irradiation was measured by clonogenic assay. The cells grown at 37°C for 48 h were irradiated at various doses. Immediately after irradiation, the cells were harvested with an EDTA-trypsin mixture, and plated in 60 mm tissue culture dishes (Iwaki Glass, Chiba). The cells were incubated at 37°C for 10–14 days to form colonies and stained with 0.1% crystal violet. Colonies consisting of 32 cells or more were counted as surviving cells. The surviving fraction was determined as the ratio of colonies formed after irradiation to those formed by unirradiated controls. Conventionally, D_0 (the mean inactivation dose) and D_q (quasi-threshold dose) have been used as parameters of radiation survival. The D_0 is the dose of radiation required to reduce the surviving fraction to approximately 0.37, where the biological activity declines exponentially as a function of dose. The degree of radioresistance was expressed as the ratio of D_0 of the radioresistant cell line to D_0 of the parental cell line. The D_q is a measure of the width of the shoulder of the survival curve calculated by extrapolating the straight portion of the survival curve back to the X-ray dose axis. It indicates the ability to recover from sublethal damage induced by irradiation.

DNA probes Human PARP cDNA,[17] which was generously provided by Dr. T. Ogura (National Cancer Center Institute, Tokyo) was used as a DNA probe in this study. It is a 3.8 kb cDNA clone derived from human fibroblasts and contains an open reading frame for a 1,014-amino-acid polypeptide. This cDNA probe (*Eco*R I-*Eco*R I, 2,074 base) was labeled with ^{32}P by a random priming method.[18, 19] The specific activity was 1×10^9 cpm/μg DNA.

RNA isolation Total cellular RNA was isolated by the guanidine isothiocyanate/buffer-saturated phenol extraction method described by Sambrook *et al.*[20] with some modifications. Briefly, the culture medium was discarded, the cells were washed with PBS(−), and then 4 *M* guanidine isothiocyanate, 50 m*M* Tris-HCl (pH 7.5), and 25 m*M* EDTA were added. The cells were scraped off the plates with a rubber policeman, and homogenized with a sterile hypodermic syringe fitted with a 23-gauge needle. The homogenate was laid on a cushion of 5.7 *M* cesium chloride containing 0.01 *M* EDTA in an ultracentrifuge tube and centrifuged at 32,000 rpm for 21 h at 22°C (Rotor type SW41Ti, Optima, Beckman Instrument, Palo Alto, CA). The fluid was carefully withdrawn from the ultracentrifuge tube. The RNA pellet was dissolved in 300 μl of TE (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA). The RNA solution was added to an equal volume of buffer-saturated phenol. After mixing, the sample was centrifuged at 11,000 rpm for 5 min. The supernatant was added to 30 μl of 3 *M* sodium acetate (pH 7.0), and 700 μl of 99.5% ethanol. The sample was mixed well and stored for at least 1 h at −20°C. After centrifugation at 11,000 rpm for 5 min, the RNA pellet was washed with 99.5% ethanol, vacuum-dried, and

dissolved in an appropriate volume of TE. The RNA concentration was measured in terms of the absorbance at 260 nm.

Northern blot analysis Total RNA (15 μg per lane) was denatured in 44% formamide containing 5.8% formaldehyde at 65°C for 5 min and subjected to agarose gel electrophoresis (1% agarose/5.4% formaldehyde) in 1× MOPS buffer (20 m*M* 3-(*N*-molpholino)propanesulfonic acid, 5 m*M* sodium acetate, 1 m*M* EDTA). The RNA was transferred to a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) in 10×SSC (1×SSC; 150 m*M* NaCl, 15 m*M* sodium citrate). The filter was baked for 2 h at 80°C in a vacuum oven and prehybridized in 50% formamide, 25 m*M* KH_2PO_4 (pH 7.2), 5×SSC, 5× Denhardt's solution, 0.5% SDS and 50 μg/ml of salmon sperm DNA at 42°C for 3 h. The RNA was hybridized with ^{32}P-labeled DNA probe (25 ng) for 16 h at 42°C in 50% formamide, 25 m*M* KH_2PO_4 (pH 7.2), 5×SSC, 5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate, and 50 μg/ml of salmon sperm DNA. Filters were washed once in 2×SSC, twice in 0.2×SSC and once in 0.1×SSC at room temperature. Autoradiography was performed at −80°C for 3–7 days. RNA gels were stained with ethidium bromide before northern blotting to ascertain that equivalent amounts of RNA had been loaded in each lane.

Southern blot analysis Genomic DNA was isolated by the modified method of Blin and Stafford, as described by Sambrook *et al.*[21] The DNA (10 μg per lane) was digested with *Eco*R I, subjected to 1% agarose gel electrophoresis in 1×TBE (90 m*M* Tris base, 90 m*M* boric acid, 1 m*M* EDTA) buffer and blotted onto a nitrocellulose filter. The DNA was hybridized with ^{32}P-labeled DNA probe (25 ng) and washed under the same conditions as those used for northern blot analysis.

Assay for PARP activity PARP activity was measured by the TCA assay technique, using a modification of the method of Tanuma and Kanai.[22] Briefly, the cells were incubated at 37°C for 48 h in a 5% CO_2 incubator. After irradiation with various doses of X-rays, cells were washed once with buffer A (10 m*M* Tris-HCl, pH 7.8, 3 m*M* $MgCl_2$, 3.3 m*M* 2-mercaptoethanol, 0.3 m*M* phenylmethylsulfonyl fluoride). The cells were suspended at a density of 2×10^7 cells/ml in buffer A containing 0.1% Nonidet P-40 and allowed to swell for 3 min at 0°C. The cell suspension was then homogenized in a Teflon-glass homogenizer, and the homogenate was centrifuged at 400*g* for 5 min at 4°C. Chromatin was prepared from this pellet by ultrasonication (UR-20P, Tomy Seiko, Tokyo) at 0°C in 1 ml of buffer A. The reaction mixture (100 μl) contained 74.2 m*M* Tris-HCl (pH 8.0), 45 m*M* KCl, 9 m*M* $MgCl_2$, 3 m*M* 2-mercaptoethanol, 0.1 m*M* nico-

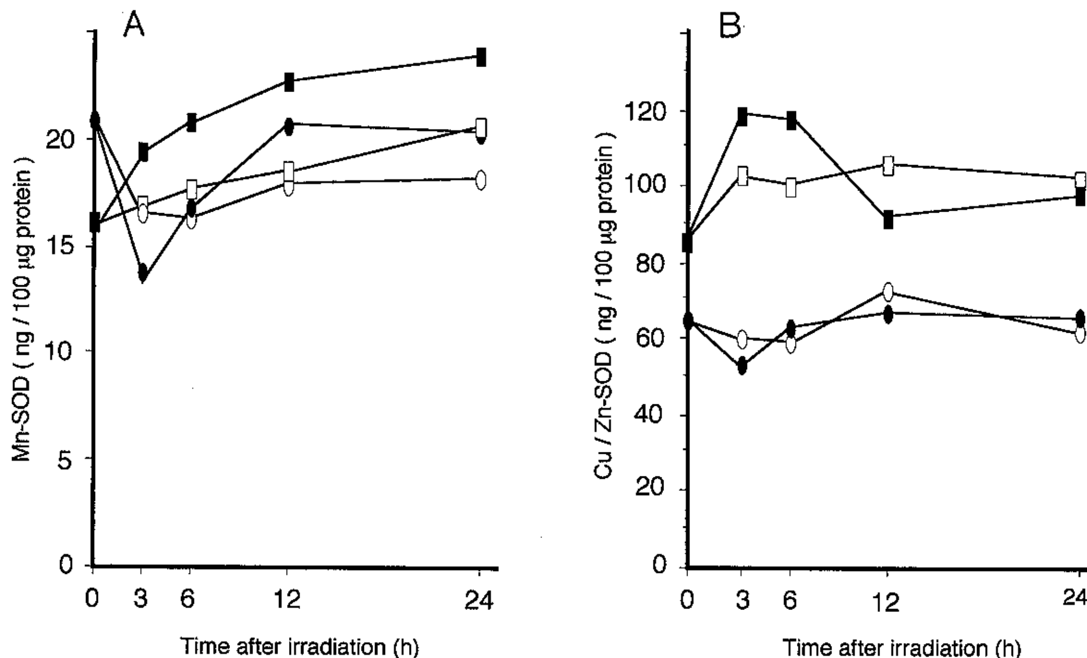

Fig. 1. Changes in the SOD level in KB and N10 cells after irradiation. Cells were assayed for Mn-SOD (A) and Cu/Zn-SOD (B) at intervals (0–24 h) after X-irradiation with 10 or 20 Gy. ○, KB irradiated with 10 Gy; □, N10 irradiated with 10 Gy; ●, KB irradiated with 20 Gy; ■, N10 irradiated with 20 Gy.

tinamide dinucleotide (NAD^+), [^{3}H]NAD^+ (0.03 μCi/reaction; 0.1 mCi/ml; specific activity 4.0 Ci/mmol; DuPont/NEN Research Products, Boston, MA), and chromatin equivalent to 1×10^6 cells. Incubation was carried out at 25°C for 15 min. The reaction was terminated by adding 1 ml of 5% trichloroacetic acid and the mixture was kept at 4°C for 2 h. The acid-precipitable fraction was collected on a nitrocellulose membrane filter and the radioactivity was counted.

RESULTS

Cellular contents of SOD and GSH It has been reported that SOD and GSH protect cells from DNA damage due to oxidative stress, such as free radicals and hydrogen peroxide produced by X-irradiation.[23] Therefore, we first compared the cellular SOD and GSH contents in the radioresistant N10 with those in the parental KB cell line.

As shown in Fig. 1, cellular Mn-SOD content was 1.3-fold higher in parental KB than in N10. When both cell lines were irradiated at the dose of 10 or 20 Gy, however, the Mn-SOD content in KB was decreased by 3 to 6 h postirradiation and then increased to the unirradiated level or less by 24 h postirradiation. In contrast, Mn-SOD in N10 was increased 1.3- to 1.5-fold by 24 h postirradiation in a time- and dose-dependent manner. On the other hand, Cu/Zn-SOD content was 1.3-fold higher in N10 than in KB under unirradiated conditions, but the enzyme level was increased in N10 by 6 h postirradiation and was almost unchanged in KB.

Table I. Glutathione (GSH) Contents in Radioresistant N10 and Parental KB Cell Lines

Cell line	D_0[a] (Gy)	Resistance (fold)	GSH Content (μM/100 μg protein)	GSH Increase (fold)
KB	1.35	1	8.88±0.55[b]	1
N10	3.90	3.11	14.68±1.78	1.65[c]

a) The mean inactivation dose necessary to reduce the surviving fraction to 0.37.
b) Data represent means±SD ($n=5$).
c) Significantly different from control value; $P<0.05$.

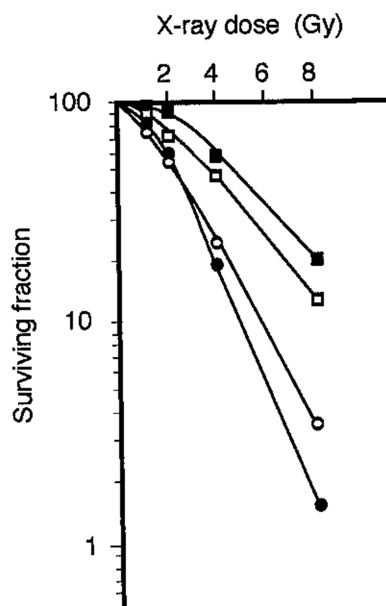

Fig. 2. Radiation survival curves of KB and N10 cells untreated or treated with 25 μM BSO for 24 h prior to irradiation. Each point represents the mean of duplicate experiments (triplicate dishes in an experiment). ●, KB; ○, KB with BSO; ■, N10; □, N10 with BSO.

Table II. GSH Levels and Radiation Survival Parameters without and with BSO Treatment

Cell line	GSH level (μM/100 μg protein) −BSO	GSH level +BSO	D_0[a] (Gy) −BSO	D_0[a] (Gy) +BSO	D_q[b] (Gy) −BSO	D_q[b] (Gy) +BSO
KB	8.88±0.55 (1)	3.13±0.59 (0.35)	1.53±0.16 (1)	1.72±0.26 (1.12)	1.15±0.03 (1)	1.13±0.02 (0.98)
	*		***	**	*	
N10	14.68±1.78 (1)	3.99±0.66 (0.27)	3.70±0.17 (1)	3.37±0.25 (0.91)	2.00±0.06 (1)	1.05±0.03 (0.52)
					* (N10 −BSO vs +BSO)	

KB and N10 cells were treated with 25 μM buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, for 24 h prior to X-irradiation and then the radiosensitivity was determined. Data represent means±SD of 3 to 5 determinations and values in parentheses indicate the ratio of radiation dose with BSO to that without BSO.
a) The mean inactivation dose necessary to reduce the surviving fraction to 0.37.
b) Quasi-threshold dose, which is a measure of the width of the shoulder of the survival curve.
Significantly different from the control value; * $P<0.05$, ** $P<0.005$, *** $P<0.0001$.

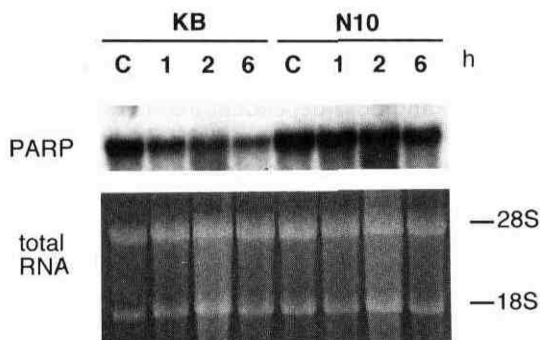

Fig. 3. PARP mRNA levels of KB and N10 cells in unirradiated and irradiated conditions. Cells were cultured for various times (0–6 h) after irradiation (4 Gy). Northern blot analysis of mRNA was performed by blotting 15 μg of total RNA per lane. The positions of 28S and 18S ribosomal RNA are indicated. C, unirradiated control.

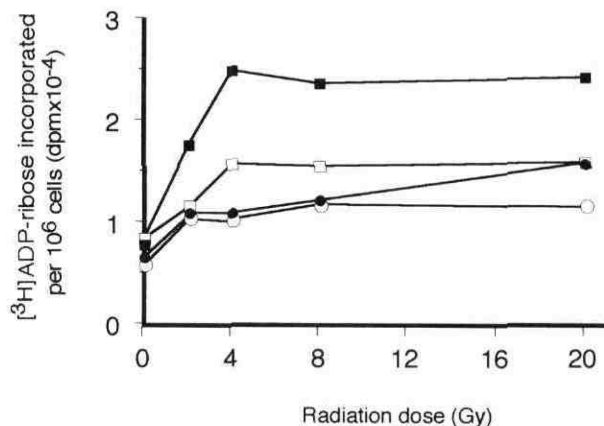

Fig. 5. PARP activity after treatment with various radiation doses in the presence or absence of 1 mM NA. ●, KB; ○, KB with NA; ■, N10; □, N10 with NA.

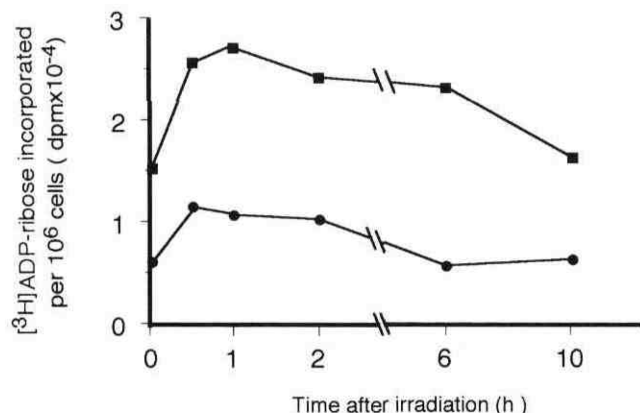

Fig. 4. Time course of PARP activity in KB and N10 after irradiation with 4 Gy. ●, KB; ■, N10.

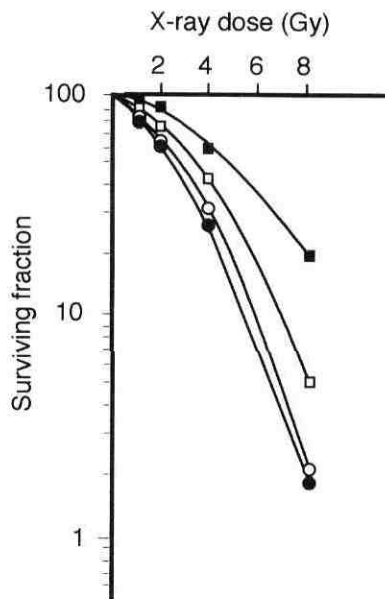

Fig. 6. Radiation survival curves of KB and N10 untreated or treated with 1 mM NA before and after irradiation. Each point represents the mean of duplicate experiments. ●, KB; ○, KB with NA; ■, N10; □, N10 with NA. D_0 ratio: ●, 1.00; ○, 0.97; ■, 2.40; □, 1.33.

As shown in Table I, cellular GSH content in radioresistant N10 was 1.65-fold higher than that in parental KB and this difference was statistically significant. When both cell lines were treated with 25 μM buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase in the glutathione synthetic pathway, for 24 h prior to X-irradiation, the radiosensitivity was slightly increased in N10, but not in KB. The increased sensitivity in N10 was significant in terms of D_q ratio, but not D_0 ratio (Fig. 2 and Table II). A BSO concentration of 25 μM was noncytotoxic and caused approximately 70% reduction in cellular GSH content of both cell lines.

Expression of PARP mRNA It has been considered that PARP existing in the nucleus of eukaryotic cells is involved in DNA repair, DNA replication and cell differentiation by catalyzing the formation of poly (ADP-ribose) from NAD^+ covalently linked to acceptor proteins such as histone and nonhistone chromosomal proteins.[24, 25] Therefore, we examined the expression of this enzyme mRNA in N10 and KB cell lines by northern blot analysis.

As shown in Fig. 3, PARP mRNA was highly expressed in N10 as compared to KB. When both cell lines were irradiated with 4 Gy of X-rays and the expression of PARP mRNA was examined at intervals, the level of expression in KB gradually decreased up to 6 h postirradiation, whereas that in N10 was unchanged. There was no significant difference in the copy number of the PARP gene in Southern blot analysis of *Eco*R I-digested genomic DNA of the two cell lines (data not shown).

Elevated PARP activity Since the cell lines showed different expression patterns of PARP mRNA, the PARP activity was examined. N10 had a 3-fold higher enzyme activity than did KB. When cells were irradiated with 4 Gy of X-rays, the enzyme activity increased, reaching a plateau at 30 to 60 min in both cells, but it was 2.3-fold higher in N10 than in KB. Thereafter, enzyme activity gradually decreased toward unirradiated levels by 10 h postirradiation (Fig. 4). When cells were irradiated with various doses of X-rays and assayed 1 h postirradiation, the enzyme activity increased with radiation dose up to 4 Gy and reached a plateau. The treatment of cells with 1 m*M* nicotinamide (NA), an inhibitor of this enzyme, for 24 h before and 1 h after X-irradiation resulted in a 40% inhibition of the enzyme activity in N10, but no change in KB (Fig. 5). This concentration of NA was chosen as a noncytotoxic level.

Effect of NA on cell survival following X-irradiation Since treatment of the radioresistant N10 cell line with NA markedly inhibited the PARP induction by X-irradiation, the effect of NA on cell survival following X-irradiation was examined. When cells were treated with 1 m*M* NA for 24 h before X-irradiation and incubated for 3 h in growth medium containing NA after X-irradiation, the radiosensitivity of N10 was significantly increased by approximately two times ($P<0.005$), whereas that of KB was unchanged (Fig. 6).

DISCUSSION

Most of the damaging effects of ionizing radiation are mediated by reactive free radicals such as hydroxyl radicals and superoxides.[23] The interaction of these free radicals with DNA causes DNA strand breaks, leading to cell death and radiation-induced carcinogenesis.[26] Thus, we first examined SOD, which is a scavenger of superoxides, in the radioresistant N10 and the parental KB cell lines. SODs are a class of metalloproteins which catalyze the dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2).[27] In eukaryotes, there are three types of isoenzymes; Mn-SOD in the mitochondrial matrix, Cu/Zn-SOD in the cytoplasm and extracellular SOD.[28] It is known that Cu/Zn-SOD is a constitutive enzyme, Mn-SOD is an enzyme inducible by various stimuli[29, 30] and extracellular SOD is a secreted enzyme with a heparin-binding domain.[28] The previous studies revealed that X-irradiation increased Mn-SOD activity in the mouse heart,[31] while treatment with a SOD inhibitor decreased the radioresistance of Chinese hamster cells.[32, 33] Recently, it has been reported that transfection of Mn-SOD cDNA into Chinese hamster ovary cells resulted in increased survival after treatment with doxorubicin, mitomycin C and γ-irradiation *in vitro*.[30] These results indicate a relationship between cellular content of SOD and radiation resistance. In our system using highly reproducible ELISA assay with monoclonal antibodies to SOD,[14, 15] the radioresistant N10 cells showed a higher content of constitutive Cu/Zn-SOD than did the parental KB, and Mn-SOD was inducible more efficiently in N10 than in KB after X-irradiation, although it was at a lower level in N10 than in KB in the unirradiated condition. Furthermore, treatment with diethyldithiocarbamate, an inhibitor of Cu/Zn-SOD, decreased the radioresistance of N10 by 40% as compared with untreated N10, while it was unchanged in KB (data not shown).

GSH also functions in the protection of cells against the effects of free radicals and reactive oxygen intermediates.[34] Intracellular GSH removes the free radicals and hydrated electrons formed by radiation via enzymatic reactions catalyzed by peroxidase and transhydrogenase and oxidation-reduction reactions with hydrogen transfer.[35] Several studies have suggested that GSH is involved in the resistance to radiation and DNA-reactive drugs such as platinum analogs and alkylating agents.[36–39] Thus, we examined the GSH content in N10 and KB cell lines. The GSH content in N10 was 1.65-fold higher than that in KB. Although this increased level is still low as compared with that reported in cisplatin-resistant human ovarian cancer cell lines,[39] the difference is statistically significant. The treatment of N10 cells with BSO resulted in a significant increase of radiosensitivity in terms of D_q ratio, but not D_0 ratio, indicating that glutathione in N10 may be involved in the resistance to low-dose irradiation. Although the contents of SOD and GSH were increased in N10 cells, these changes may not be sufficient to account for all of the radioresistance.

In our previous study, we found that bleomycin-resistant HeLa cells induced DNA repair-related PARP activity at a high level after exposure to BLM.[40] Recently, it has been reported that mutant V79 hamster cell lines deficient in PARP activity were hypersensitive to UV- and X-irradiation, as well as a variety of antitumor agents including BLM, melpharan, BCNU and mitomycin C.[41] Therefore, it is conceivable that PARP plays an important role in DNA repair and radiation resistance. Northern blot analysis showed that the radioresistant N10 cells not only showed high expression of PARP mRNA in unirradiated conditions, but also maintained a

high level of expression after X-irradiation (4 Gy). In contrast, expression of PARP mRNA in the parental KB cells was low and decreased gradually after X-irradiation. Concomitant with the expression of PARP mRNA, the radioresistant N10 cells showed higher enzyme activity than the parental KB cells, and the enzyme activity was further induced to a high level in a time- and dose-dependent manner after X-irradiation. Elevation of enzyme activity was slight in KB cells. Furthermore, treatment of cells with NA efficiently inhibited the induction of enzyme activity and significantly increased the radiosensitivity in N10, but not in KB.

In conclusion, the radioresistance mechanism in N10 cells is considered to be multifactorial, and PARP, SOD and GSH all contribute in part. Further investigations using newly isolated human DNA repair genes are under way in our laboratory.

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REFERENCES

1) Hutchinson, F. Chemical changes induced in DNA by ionizing radiation. *Prog. Nucleic Acid Res. Mol. Biol.*, **32**, 115–154 (1985).
2) McMillan, T. J., Cassoni, A. M., Edwards, S., Hoimes, A. and Peacock, J. H. The relationship of DNA double-strand break induction to radiosensitivity in human tumor cell lines. *Int. J. Radiat. Biol.*, **58**, 427–438 (1990).
3) Louie, K. G., Behrens, B. C., Kinsella, T. J., Hamilton, T. C., Grotzinger, K. R., McKoy, W. M., Winker, M. A. and Ozols, R. F. Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. *Cancer Res.*, **45**, 2110–2115 (1985).
4) Miura, M. and Sasaki, T. Role of glutathione in the intrinsic radioresistance of cell lines from a mouse squamous cell carcinoma. *Radiat. Res.*, **126**, 229–236 (1991).
5) Moore, W. R., Anderson, M. E., Meister, A., Murata, K. and Kimura, A. Increased capacity for glutathione synthesis enhances resistance to radiation in *Escherichia coli*: a possible model for mammalian cell protection. *Proc. Natl. Acad. Sci. USA*, **86**, 1461–1464 (1989).
6) Peters, L. J., Withers, H. R., Thames, H. D., Jr. and Fletcher, G. H. Tumor radioresistance in clinical radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.*, **8**, 101–108 (1982).
7) Cowan, J. M., Beckett, M. A. and Weichselbaum, R. R. Chromosome changes characterizing *in vitro* response to radiation in human squamous cell carcinoma lines. *Cancer Res.*, **53**, 5542–5547 (1993).
8) Sinclair, W. K. Cyclic x-ray responses in mammalian cells *in vitro*. *Radiat. Res.*, **33**, 620–643 (1968).
9) McKenna, W. G., Iliakis, G., Weiss, M. C., Bernhard, E. J. and Muschel, R. J. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-*ras* and V-*myc*. *Radiat. Res.*, **125**, 283–287 (1991).
10) Lee, J. M. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. *Proc. Natl. Acad. Sci. USA*, **90**, 5742–5746 (1993).
11) Fan, S., El-Deiry, W. S., Bae, I., Freeman, J., Jondle, D., Bhatia, K., Fornace, A. J., Magrath, J. I., Kohn, K. W. and O'Connor, P. M. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res.*, **54**, 5824–5830 (1994).
12) Eagle, H. Propagation in a fluid medium of human epidermoid carcinoma, strain KB. *Proc. Soc. Exp. Biol. Med.*, **89**, 362–364 (1955).
13) Yanagisawa, T., Urade, M., Takahashi, Y., Kishimoto, H. and Sakurai, K. Isolation and characterisation of radioresistant human KB carcinoma cells *in vitro*. *Oral Oncol. Eur. J. Cancer Part B*, **34** (1998), in press.
14) Ogino, K., Oka, S., Sakaida, I., Ando, K., Okita, K., Takemoto, T., Uda, T. and Noji, S. Characterization of a monoclonal antibody to copper,zinc-superoxide dismutase. *Jpn. J. Inflam.*, **7**, 259–263 (1987).
15) Kawaguchi, T., Noji, S., Uda, T., Nakashima, Y., Takeyasu, A., Kawai, Y., Takagi, H., Tohyama, M. and Taniguchi, N. A monoclonal antibody against COOH-terminal peptide of human liver manganese superoxide dismutase. *J. Biol. Chem.*, **264**, 5762–5767 (1989).
16) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
17) Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M. and Sugimura, T. Nucleotide sequence of a full-length cDNA for human fibroblast poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.*, **148**, 617–622 (1987).
18) Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132**, 6–13 (1983).
19) Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. *Anal. Biochem.*, **137**, 266–267 (1984).
20) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning: A Laboratory Manual," 2nd Ed., pp. 7.19–7.22 (1989). Cold Spring Harbor Laboratory, New York.
21) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning: A Laboratory Manual," 2nd Ed., pp. 9.14–9.58 (1989). Cold Spring Harbor Laboratory, New York.
22) Tanuma, S. and Kanai, Y. Poly(ADP-ribosyl)ation of chromosomal proteins in the HeLa S3 cell cycle. *J. Biol. Chem.*, **257**, 6565–6570 (1982).

23) Clark, I. A., Cowden, W. B. and Hunt, N. H. Free radical-induced pathology. *Med. Res. Rev.*, **5**, 297–332 (1985).
24) Ueda, K. and Hayaishi, O. ADP-ribosylation. *Annu. Rev. Biochem.*, **54**, 73–100 (1985).
25) Cleaver, J. E. and Morgan, W. F. Poly(ADP-ribose) polymerase: a perplexing participant in cellular responses to DNA breakage. *Mutat. Res.*, **257**, 1–18 (1991).
26) Akashi, M., Hachiya, M., Paquette, R. L., Osawa, Y., Shimizu, S. and Suzuki, G. Irradiation increases manganese superoxide dismutase mRNA levels in human fibroblasts: possible mechanisms for its accumulation. *J. Biol. Chem.*, **270**, 15864–15869 (1995).
27) Fridovich, I. Superoxide dismutases: an adaptation to a paramagnetic gas. *J. Biol. Chem.*, **264**, 7761–7764 (1989).
28) Marklund, S. L. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem. J.*, **222**, 649–655 (1984).
29) Wong, G. H. W. and Goeddel, D. V. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science*, **242**, 941–944 (1988).
30) Hirose, K., Longo, D. L., Oppenheim, J. J. and Matsushima, K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB J.*, **7**, 361–368 (1993).
31) Oberley, L. W., Clair, DK-ST., Autor, A. P. and Oberley, T. D. Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. *Arch. Biochem. Biophys.*, **254**, 69–80 (1987).
32) Lin, P. S., Kwock, L. and Butterfield, C. E. Diethyldithiocarbamate enhancement of radiation and hyperthermic effects on Chinese hamster cells *in vitro*. *Radiat. Res.*, **77**, 501–511 (1979).
33) Westman, G. and Marklund, S. L. Diethyldithiocarbamate, a superoxide dismutase inhibitor, decreases the radioresistance of Chinese hamster cells. *Radiat. Res.*, **83**, 303–311 (1980).
34) Meister, A. Selective modification of glutathione metabolism. *Science*, **220**, 472–477 (1983).
35) Arrick, B. A. and Nathan, C. F. Glutathione metabolism as a determinant of therapeutic efficacy. A Review. *Cancer Res.*, **44**, 4224–4232 (1984).
36) Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C. and Ozols, R. F. Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem. Pharmacol.*, **34**, 2583–2586 (1985).
37) Hosking, L. K., Whelan, R. D., Shellard, S. A., Bedford, P. and Hill, B. T. An evaluation of the role of glutathione and its associated enzymes in the expression of differential sensitivities to antitumour agents shown by a range of human tumour cell lines. *Biochem. Pharmacol.*, **40**, 1833–1842 (1990).
38) Mistry, P., Kelland, L. R., Abel, G., Sidhar, S. and Harrap, K. R. The relationships between glutathione, glutathione-*S*-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br. J. Cancer*, **64**, 215–220 (1991).
39) Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C. and Anderson, M. E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc. Natl. Acad. Sci. USA*, **89**, 3070–3074 (1992).
40) Urade, M., Sugi, M., Mima, T., Ogura, T. and Matsuya, T. High induction of poly(ADP-ribose) polymerase activity in bleomycin-resistant HeLa cells. *Jpn. J. Cancer Res.*, **80**, 464–468 (1989).
41) Chatterjee, S., Cheng, M. F. and Berger, N. A. Hypersensitivity to clinically useful alkylating agents and radiation in poly(ADP-ribose) polymerase-deficient cell lines. *Cancer Commun.*, **2**, 401–407 (1990).