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Exogenous Expression of Exonuclease Domain-deleted WRN Interferes with the Repair of Radiation-induced DNA Damages

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WRN exonuclease/Radiosensitivity/DNA double-strand breaks/DNA repair/X-rays.

Werner syndrome (WS) is an autosomal recessive disease characterized by multiple progeroid features. The gene responsible for WS, *WRN*, is a member of the human RecQ helicase family. WRN is unique among this family, associated with an exonuclease activity. In the present study, we established the human 293-derived cell lines, which expressed exogenously truncated WRN protein, lacking the N-terminal exonuclease domain but having normal helicase activity, and found that they were slightly, but nonetheless significantly, radiosensitive than control cell lines, into which the empty vector had been introduced. The truncated WRN-expressing cells also exhibited increased numbers of micronuclei, chromosome aberrations, and the foci of phosphorylated histone H2AX with X-rays. These results suggested a function of WRN exonuclease activity that is separable from helicase activity and is essential for the repair of radiation-induced DNA damages.

INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive disease. Affected individuals are characterized by multiple progeroid features such as premature graying of the hair, cataracts, osteoporosis, diabetes and accelerated atherosclerosis accompanied by a high incidence of cancers.[1,2] Studies using cultured cells demonstrate that chromosome aberrations and large deletion mutations are frequently engendered in WS cells.[3–5] These facts suggest that the WRN protein act as a caretaker for maintaining genomic integrity.

The gene responsible for WS (*WRN*) was identified by positional cloning in 1996 and turned out to encode a 1,432 amino acid protein.[6] The WRN protein possesses both 3'→5' helicase and 3'→5' exonuclease activities and is one of human five RecQ DNA helicases.[7–9] Although the cellular function of the WRN protein is not well understood, several reports indicate its role in maintaining genomic integrity by being involved in the repair of DNA double-strand breaks (DSBs). For example, *in vitro* studies demonstrate that the WRN exonuclease activity is enhanced by interacting with the Ku70/80 complex, which is essential for nonhomologous end joining (NHEJ) for DSB repair.[10–15] However, the WRN helicase activity is increased by interacting with replication protein A (RPA), which is involved in homologous recombination (HR).[16] These findings have evoked the hypothesis that the WRN exonuclease and the WRN helicase play a role in the NHEJ and HR repair pathways, respectively, by independent functions. However, this hypothesis has never been examined in experiments.

In the present study, we established immortalized human kidney 293 cell lines overexpressing the N-terminal truncated WRN protein, lacking the exonuclease domain. We measured the radiosensitivity of these cells and demonstrated that the overexpression of N-terminal truncated WRN protein leads to increased radiosensitivity in cell killing, and the induction for micronuclei, chromosome aberrations, and the foci of phosphorylated histone H2AX. These results suggest that the overexpression of the N-truncated WRN protein attenuates the endogenous WRN exonuclease activity in DNA repair and further that the WRN exonuclease participates in a DSB repair pathway by an independent function

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from the WRN helicase.

MATERIALS AND METHODS

Cell culture

Cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml hygromycin, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Introduction of a truncated mutant WRN gene into a human cell line

A truncated mutant *WRN* gene whose exonuclease encoding region was deleted was prepared by PCR using *pfu* DNA polymerase (Stratagene, La Jolla, CA) as previously described.[17] An expression vector, into which the mutant WRN gene was inserted, was introduced to an immortalized human 293 cell line using the Flap-In system (Invitrogen) according to the manufacture's instruction, and hygromycin (100 μg/ml)-resistant cells were used for further analyses.

Western blotting

Cells were lysed in a radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 1% sodium deoxychloride, and 0.1% SDS) containing 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride as described previously.[18] The cell lysate was cleared by centrifugation at 15,000 rpm for 10 min at 4°C, and the supernatant was used as total cellular protein. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Protein samples (20 μg) were electrophoresed on a SDS-polyacrylamide gel. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane in transfer buffer (100 mM Tris, 192 mM glycine). After an overnight incubation with blocking solution (10% skim milk), the membrane was sequentially incubated with an anti-WRN antibody (Abcam, Cambridge, UK), a biotinylated secondary antibody, and streptavidin-alkaline phosphatase. The bands were visualized after the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

X-irradiation and hydroxyurea treatment

For X-irradiation, exponentially growing cells were irradiated with X-rays using an X-ray generator (M-150 WE; Softex, Osaka) operating at 150 kVp and 5 mA with a 0.1 mm Cu filter at a dose rate of 0.425 Gy / min. For hydroxyurea (HU) treatment, exponentially growing cells were treated with 2 mM HU for 3, 6, 12 and 24 hr.

Assay for cell survival

The killing effect of X-rays or hydroxyurea (HU) (Wako, Osaka) was determined by a colony formation assay. X-irradiated or HU treated cells were immediately seeded into 100 mm dishes and incubated for 10–14 days, and colonies containing more than 50 cells were scored as survivors.

Micronuclei assay

To investigate the induction of micronuclei by X-irradiation, cells were irradiated with 4 Gy of X-rays and then treated with 2 μg/ml cytocharacin B for 24 h in a 100 mm dish. The cells were then harvested, treated with 3 ml of hypotonic (0.1 M) KCl for 20 min, and fixed with 3 ml of fixative (methanol: acetic acid, 5:1). The cell suspensions were then centrifuged at 1,200 rpm for 5 min. After removal of the supernatant, the cells were resuspended in 5 ml fixative, and then incubated on ice for 5 min. The centrifugation was repeated followed by fixation with 5 ml of fixative. After the second removal of the supernatant, the cells were resuspended in 0.5–1 ml fixative and dropped onto glass slides. The slides were stained with 4% Giemsa and the yield of micronuclei per 1000 binucleate cells was scored.

Chromosome analysis

Chromosome aberrations induced with X-rays were examined by the premature chromosome condensation (PCC) method. Cells were irradiated with 1 or 2 Gy of X-rays and treated with 500 nM Okadaic acid (Wako, Osaka) for 2 h in a 25 cm^2 flask. The cells were then harvested and subjected to chromosome preparation, as previously described.[19,20] Fifty G_2 PCC spreads were examined for each sample, and the number of chromatid breaks was scored.

Immunostaining for phosphorylated histone H2AX

Cells cultured on cover slips (22 × 22 mm) were fixed with 4% formaldehyde for 10 min at room temperature, and treated with 0.1% Triton X for 5 min on ice. Then, the cells were sequentially treated with a monoclonal antibody against phosphorylated histone H2AX (γ-H2AX) (Upstate-Inc., Lake Placid, NY) for 2 h at 37°C, and an FITC-labeled anti-mouse Ig G (Amersham Pharmacia Biotech, Tokyo) for 1 h at 37°C. Finally, the cover slips were mounted on glass slides with a PI solution (10 μg/ml propidium iodide, 200 μg/ml RNase A). The foci of γ-H2AX were scored using a fluorescence microscopy (AX70, Olympus, Tokyo).

RESULTS

Establishment of cell lines overexpressing the N-terminal truncated WRN protein

Figure. 1A shows the structure of the wild-type and truncated WRN proteins. The truncated WRN protein was deleted for 231 amino acids including the N-terminal exonuclease domain. By transfecting a vector including a null gene or a truncated mutant *WRN* gene into an immortalized human 293 cell line, we established three control cell lines

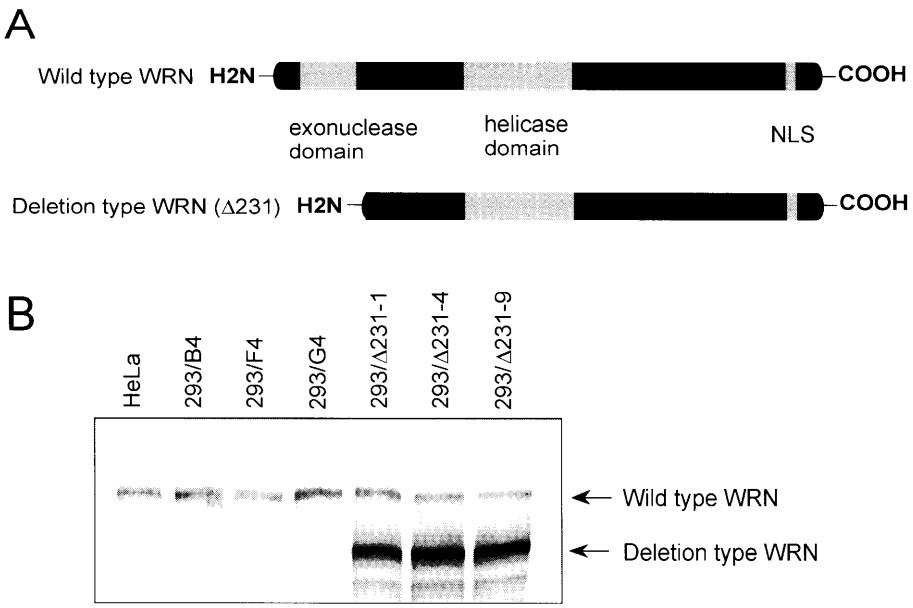

Fig. 1. (A) Schematic structures of wild-type and deletion-type (N-terminal truncated) WRN proteins. The wild-type WRN protein possesses an exonuclease domain at the N-terminal, a helicase domain at the center, and a nuclear localizing signal (NLS) at the C-terminal. The N-terminal truncated WRN protein, which is constructed using a Flap-In System (Invitrogen), does not possess an exonuclease domain due to the loss of 231 amino acids. (B) Western blotting analysis of the wild-type and deletion-type (N-terminal truncated) WRN proteins. After transfection with the N-terminal truncated *WRN* gene into an immortalized human 293 cell line, three cell lines (293/Δ231-1, 293/Δ231-4 and 293/Δ231-9) were isolated. These cell lines were cultured under the same conditions as three control 293 cell lines (293/B4, 293/F4 and 293/G4). HeLa cells were used as a control that expressed the wild-type WRN protein.

expressing the endogeous wild-type WRN protein and three mutant cell lines expressing the N-terminal truncated WRN protein, respectively. Western blotting analysis revealed that three mutant WRN cell lines, i.e., Δ231-1, Δ231-4 and Δ231-9, expressed the excess level of the truncated WRN protein as shown in Fig. 1B. In contrast, the expression level of the endogenous wild-type WRN protein was equivalent in all cell lines examined (Fig. 1B). These results indicate that the mutant WRN cell lines provide the opportunity to examine the phenotypic change in DNA repair by the overexpression of the exonuclease-deficient WRN protein.

Radiosensitivity of the mutant WRN cells

To gain an understanding of the biological effects of the overexpression of the N-terminal truncated WRN protein, we examined the radiosensitivity of three mutant WRN cell lines that overexpressed the truncated WRN protein and three control cell lines that expressed the endogeous wild-type WRN protein. Figure. 2A shows cell survival of each cell line that was exposed to graded doses of X-rays. To compare the radiosensitivity of the mutant WRN cell lines and that of the control 293 cell lines, the average surviving fractions of each cell line were calculated and plotted in Fig. 2B. As shown, the mutant WRN cell lines were found slightly but significantly more radiosensitive than the control 293 cell lines. For example, the D_0 values for the mutant WRN cell lines and the control 293 cell lines were 0.60 Gy and 0.77 Gy, respectively, and the surviving fraction of the former was 2.2-fold lower than that of the latter ($p < 0.05$ by Student's *t*-test) when they were exposed to 4 Gy of X-rays.

Cytogenetic damage by X-irradiation

To confirm the enhanced radiosensitivity of the mutant WRN cell lines, we examined the induction for micronuclei and chromosome aberrations by X-irradiation. As shown in Table 1, three mutant WRN cell lines (Δ231-1, Δ231-4, Δ231-9) were more susceptible to ionizing radiation than three control 293 cell lines (B4, F4, G4) in terms of micronucleus induction. The average rate of micronuclei in the mutant WRN cell lines (0.153) was 1.8-fold higher than that in the control 293 cell lines (0.084) ($p < 0.01$ by Student's *t*-test). In contrast, no significant difference was observed in the spontaneous yields of the micronuclei between the mutant and control 293 cells.

Since DNA double-strand breaks induced by X-irradiation lead to the formation of chromosome aberrations, we studied the induction of chromatid breaks at 2 h after X-irradiation by the PCC method. As shown in Table 2, the level of chromatid breaks increased in a dose-dependent manner in all cell lines examined. The yields of chromatid breaks by 1 Gy and 2 Gy irradiations were higher in two mutant WRN cell

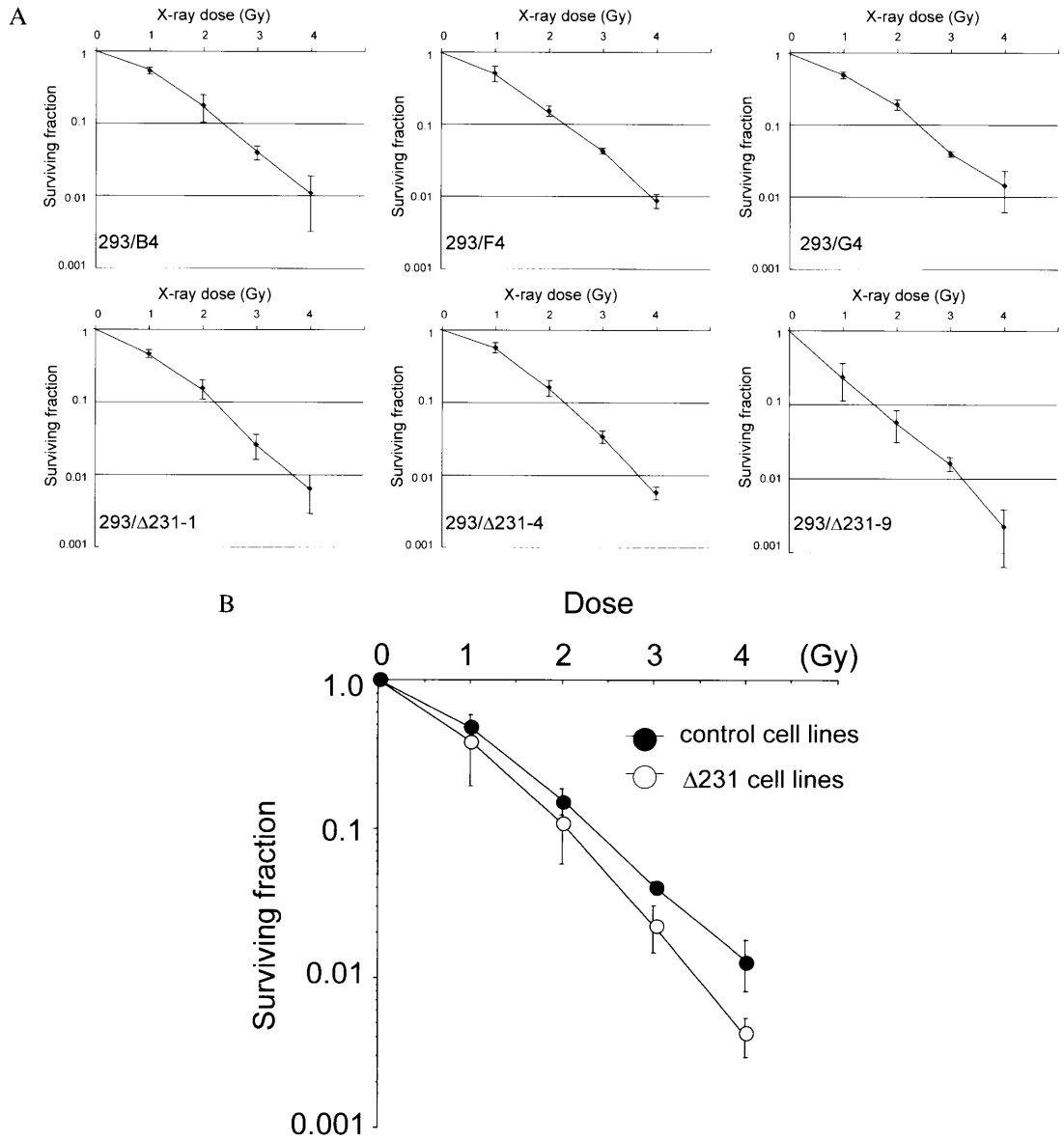

Fig. 2. Cell killing effect by X-irradiation in the mutant WRN cell lines and the control 293 cell lines. (A) Three mutant WRN cell lines (293/Δ231-1, 293/Δ231-4 and 293/Δ231-9) that overexpressed the exonuclease-deficient WRN protein (lower) and three control cell lines (293/B4, 293/F4 and 293/G4) that expressed the endogenous wild-type WRN protein (upper) were examined for cell survival by exposure to graded doses of X-rays. The average values calculated from three independent trials were plotted in each cell line. (B) The average values calculated from three control cell lines (293/B4, 293/F4 and 293/G4) and from three mutant WRN cell lines (293/Δ231-1, 293/Δ231-4 and 293/Δ231-9) were plotted. Closed and open symbols represent the control cell lines and the mutant WRN cell lines, respectively.

lines (Δ231-4, Δ231-9) than two control 293 cell lines (B4, F4). Significant differences were observed in the average yields of the mutant WRN cell lines (0.164 for 1 Gy and 0.213 for 2 Gy) and the control 293 cell lines (0.052 for 1 Gy and 0.141 for 2 Gy)($p < 0.05$ by Student's *t*-test for each dose). These results, together with the data for cell survival, indicate that the overexpression of the exonuclease-deficient WRN protein enhances cellular radiosensitivity.

Repair kinetics for DNA double-strand breaks

To determine the repair kinetics for DNA double-strand breaks (DSBs), we examined the focus formation of phosphorylated histone H2AX (γ-H2AX) at 0.25, 2, 4, 6, and 24 hr after X-irradiation with 3 Gy in a mutant WRN cell line (Δ231-4) and a control 293 cell line (F4) (Fig. 3). Accumulated evidence showed that the focus formation of γ-H2AX was a sensitive indicator responsible for the repair process

Table 1. Rate of micronuclei after 4Gy-irradiation

Cell line	X-ray dose (Gy)	No.of binucleated cells scored	No. of micronuclei	Rate of micronuclei (per binucleated cell ± SE)
293/B4	0	1000	40	0.040 ± 0.006
	4	1000	92	0.092 ± 0.010
293/F4	0	1000	45	0.045 ± 0.007
	4	1000	76	0.076 ± 0.009
293/G4	0	1000	30	0.030 ± 0.006
	4	1000	85	0.085 ± 0.009
293/Δ231-1	0	1000	50	0.050 ± 0.007
	4	1000	159	0.159 ± 0.013
293/Δ231-4	0	1000	60	0.060 ± 0.008
	4	1000	172	0.172 ± 0.013
293/Δ231-9	0	1000	55	0.055 ± 0.007
	4	1000	129	0.129 ± 0.011

Table 2. Rate of chromatid breaks after X-irradiation

Cell line	X-ray dose (Gy)	No. of cells scored	Total no. of chromosome	Rate of chromatid breaks (per chromosome ± SE)
293/B4	0	50	2855	0.005 ± 0.001
	1	50	2830	0.056 ± 0.001
	2	50	2871	0.119 ± 0.002
293/F4	0	50	2791	0.004 ± 0.001
	1	50	2660	0.047 ± 0.001
	2	50	2859	0.162 ± 0.003
293/Δ231-4	0	50	2745	0.002 ± 0.001
	1	50	2796	0.116 ± 0.002
	2	50	2822	0.244 ± 0.005
293/Δ231-9	0	50	2737	0.002 ± 0.001
	1	50	2732	0.096 ± 0.006
	2	50	2778	0.181 ± 0.008

of DSBs.[21,22] The number of γ-H2AX foci increased in a dose-dependent manner at doses from 1 Gy to 4 Gy at 15 min after X-irradiation in both cell lines (data not shown). As shown in Fig. 3, the disappearance rate of the foci after X-irradiation was much slower in the mutant WRN cell line (Δ231-4) than in the control 293 cell line (F4). Differences in the number of foci between the mutant Δ231-4 cells and the control F4 cells were significant (Mann-Whitney's test, $p < 0.01$) at all time points except 15 min. This observation suggested that the WRN exonuclease plays an important role in DSB repair process.

Sensitivity to HU in the mutant WRN cells

To know an effect of the overexpression of the exonuclease-deficient WRN protein on the recovery from DNA replication block, we examined the sensitivity to hydroxyurea (HU), an inhibitor of DNA replication, in a mutant WRN cell line (Δ231-4) and a control 293 cell line (F4). As shown in Fig. 4, the mutant Δ231-4 cells were more resistant to HU than the control F4 cells when they were treated with

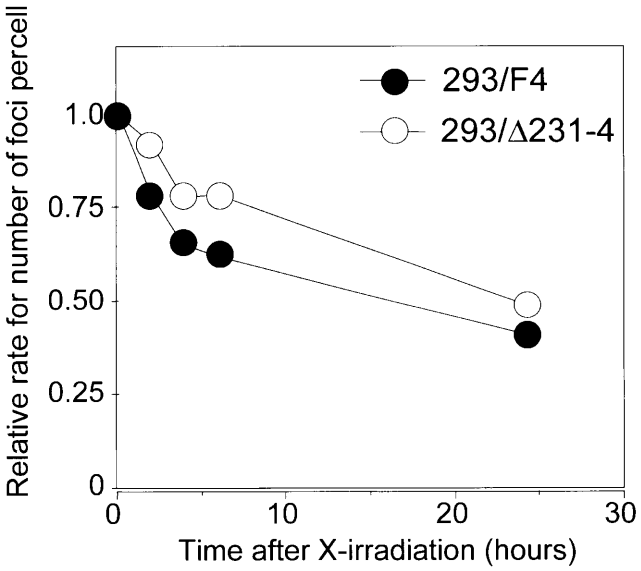

Fig. 3. Disappearance of phosphorylated histone H2AX after X-irradiation. The number of foci for phosphorylated histone H2AX per 100 cells were scored at 0.25, 2, 4, 6 and 24 hr after X-irradiation with 3 Gy in the control cell line (293/F4) and the mutant WRN cell line (293/Δ231-4). Differences were significant between two cell lines at time points for 2, 4, 6, and 24 hr (Mann-Whitney test, $p < 0.05$) in each experiment.

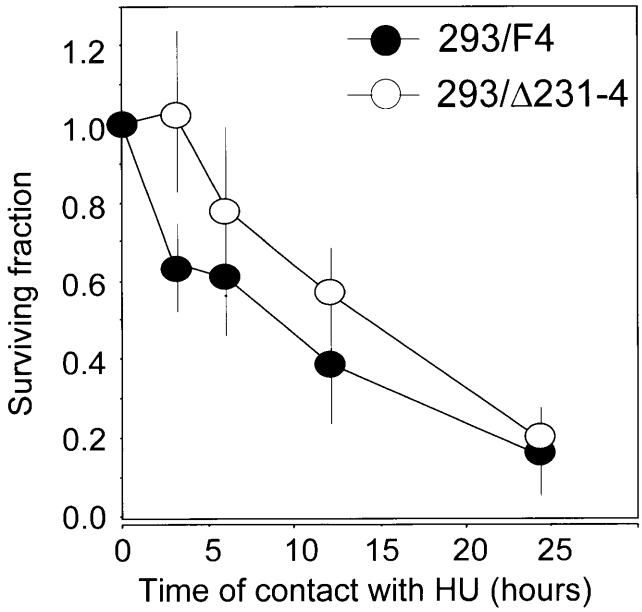

Fig. 4. Cell killing effect by hydroxyurea (HU) treatment in the control cell line (293/F4) and the mutant WRN cell line (293/Δ231-4). The cells were treated with 2 mM hydroxyurea for 3, 6, 12 and 24 hr. Three independent experiments were performed, and the average values were calculated in each cell line. Differences were significant between two cell lines when they were treated with HU for 3, 6 and 12 hr (Student's *t*-test, $p < 0.05$).

HU for 3, 6 and 12 hr ($p < 0.05$ by Student's *t*-test), suggesting an enhanced WRN helicase activity by overexpressing the mutant WRN protein in the Δ231-4 cells. However, the difference in HU sensitivity was no longer observed when both cell lines were exposed to HU for 24 hr, indicating that the resistance to HU by overexpressing the mutant WRN protein was limited in the exposed condition less than 24 hr.

DISCUSSION

In the present study, we established human cell lines overexpressing the N-terminal truncated WRN protein, which possessed the helicase activity but not the exonuclease activity *in vitro*,[17] and demonstrated that the cell lines expressing exogenously the N-terminal deleted WRN protein were slightly but, nonetheless, significantly more radiosensitive in terms of cell killing and cytogenetic damage than the control cell lines expressing only endogenous WRN protein.

We speculate that the reason for enhanced radiosensitivity in the mutant WRN cells is that the overexpression of the exonuclease-deficient WRN protein disturbs the endogenous WRN exonuclease by diluting the endogeous protein that localizes in a nucleus. This putative dominant-negative effect of the overexpressing mutant WRN protein on exonuclease activity is suggested by the fact that the cell line overexpressing the mutant WRN protein (Δ231-4) becomes temporarily resistant to HU (Fig. 4), implying that the cells possess the substantial WRN helicase activity possibly by a high level of the mutant WRN protein that retains the helicase activity.

A dominant-negative effect caused by a mutant WRN protein is reported in the former studies. For example, down-regulation of the endogeous wild-type WRN protein was observed in transgenic mice expressing a mutant WRN protein, which abolished the ATPase and helicase activities, but not the exonuclease activity, by a single amino acid substitution at position 577 (K577M).[23] In addition, an exogenous expression of the K577M-WRN protein resulted in telomeric instability in a human cancer cell line, while no such instability was caused in a cell line without expression of the mutant WRN protein.[24] These results suggest that the exogenous expression of the mutant WRN protein that defects in an enzymatic activity disturbs the endogeous WRN function by a dominant-negative effect.

Although WS has not been categorized as a repair-deficient genetic disease, there are several lines of evidence showing the defective repair capacity in WS cells. Grigorova *et al.* demonstrated an enhanced susceptibility to chromosome aberrations induced by X-irradiation in WS cells compared to the control cells.[25] Similarly, we examined X-ray-induced micronuclei in a SV40-transformed WS cell line (WS780) and found that WS780 cells were more sensitive to the induction of micronuclei by X-irradiation compared to a control cell line (GM638) (data not shown). In addition,

Yannone *et al.* reported that h*TERT* immortalized WS cells were radiosensitive, and that this hypersensitivity was complemented by introduction of the *WRN* gene.[13] These findings suggest that a defective WRN function leads to increased radiosensitivity. Moreover, Oshima *et al.* demonstrated that the loss of WRN function caused deletion mutations in plasmid DNA in an assay for nonhomologous end joining (NHEJ), suggesting the possibility that the WRN protein plays a role in NHEJ.[26] Previous studies using *in vitro* assays, which indicated that the WRN exonuclease is activated by interacting with Ku proteins, strongly supports this possibility.[10–15] These results indicate that WS cells have characteristics that would cause a deficient repair of DSBs, and that the WRN protein participates in a repair pathway for DSBs.

The evidence has been accumulated to suggest that WRN protein plays a role in the recovery process from replication block in view of hypersensitivity to some chemicals, such as hydroxyurea (HU) and 4-nitroquinoline-1-oxide (4NQO), in WS cells.[27–29] Although the reason for high susceptibility to those chemicals in WS cells is not well understood, recent studies suggest a putative role of the WRN helicase activity to confer resistance to those chemicals. For example, primary tail fibroblasts from transgenic mice overexpressing K577M-WRN protein, defective in the helicase activity but not in the exonuclease activity, exhibited hypersensitivity to 4NQO.

Similar hypersensitivity to 4NQO was also observed in a human cell line expressing the K577M-WRN protein. These results support the assumption that the helicase activity but not the exonuclease activity of WRN protein is involved in the recovery from the damage, which is supposed to be replication block,[26–28] induced by HU or 4NQO.

In summary, the present study indicates that a defect in the WRN exonuclease enhances cellular radiosensitivity, possibly due to the reduced repair fidelity of DSBs, despite retaining the WRN helicase activity. This suggests that each enzyme behaves independently in their function for genome stabilization.

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