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Werner and Bloom helicases are involved in DNA repair in a complementary fashion

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Werner syndrome (WS) is a recessive disorder characterized by premature senescence. Bloom syndrome (BS) is a recessive disorder characterized by short stature and immunodeficiency. A common characteristic of both syndromes is genomic instability leading to tumorigenesis. *WRN* and *BLM* genes causing WS and BS, encode proteins that are closely related to the RecQ helicase. We produced $WRN^{-/-}$, $BLM^{-/-}$ and $WRN^{-/-}/BLM^{-/-}$ mutants in the chicken B-cell line DT40. $WRN^{-/-}$ cells showed hypersensitivities to genotoxic agents, such as 4-nitroquinoline 1-oxide, camptothecin and methyl methanesulfonate. They also showed a threefold increase in targeted integration rate of exogenous DNAs, but not in sister chromatid exchange (SCE) frequency. $BLM^{-/-}$ cells showed hypersensitivities to the genotoxic agents as well as ultraviolet (UV) light, in addition to a 10-fold increase in targeted integration rate and an 11-fold increase in SCE frequency. In $WRN^{-/-}/BLM^{-/-}$ cells, synergistically increased hypersensitivities to the genotoxic agents were observed whereas both SCE frequencies and targeted integration rates were partially diminished compared to the single mutants. Chromosomal aberrations were also synergistically increased in $WRN^{-/-}/BLM^{-/-}$ cells when irradiated with UV light in late S to G_2 phases. These results suggest that both WRN and BLM may be involved in DNA repair in a complementary fashion.
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Introduction

Werner syndrome (WS) is a rare autosomal recessive genetic disorder causing premature aging, including short stature, juvenile cataracts, atrophy of the skin, graying and loss of hair, diabetes, arteriosclerosis, and osteoporosis, accompanied by rare cancers (Epstein *et al*., 1966). *In vitro* studies of fibroblast growth characteristics also suggest that WS may be related to normal aging since the life span of WS fibroblasts as expressed by population doubling levels is much shorter than that of normal fibroblasts (Faragher *et al*., 1993; Salk *et al*., 1985) and they have a prolonged S phase of the cell cycle. In addition to replication, WS cells are genetically unstable, as described initially by the finding of nonclonal chromosomal translocations (Salk *et al*., 1985) and extensive genomic deletions (Fukuchi *et al*., 1989).

On the other hand, Bloom syndrome (BS) is a rare autosomal recessive disorder, too. BS is characterized by proportional dwarfism, immunodeficiency, sun-sensitive facial erythema, genomic instability and the early development of a wide variety of cancers (German, 1993). Cultured fibroblasts and B-lymphoblastoid cells from BS patients are hypermutable and have chromosomal aberrations, such as an excessive number of locus-specific mutations and a high frequency of microscopically visible chromatid gaps, breaks, and rearrangements (Rosin and German, 1985). BS cells also show 10–15-fold higher levels of sister chromatid exchanges (SCEs) than normal control cells (Chaganti *et al*., 1974; German, 1993; Heartlein *et al*., 1987). The increase in SCE frequency in BS cells is further enhanced by exposure to ultraviolet (UV) light (Kurihara *et al*., 1987) and treatment with DNA damaging agents, such as ethyl methanesulfonate, N-ethyl-N-nitrosourea and 5-bromodeoxyuridine (BrdU) (Heartlein *et al*., 1987; Krepinsky *et al*., 1980).

WRN and *BLM* genes causing WS and BS, respectively, encode proteins WRN and BLM, respectively. Both proteins have seven signature motifs conserved in a wide range of DNA and RNA helicases (Ellis *et al*., 1995; Yu *et al*., 1996). Both WRN and BLM have greatest similarity to the RecQ subfamily of DNA helicases that include *Escherichia coli* RecQ (Nakayama *et al*., 1985), *Saccharomyces cerevisiae* Sgs1

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(Gangloff *et al*., 1994; Watt *et al*., 1995), *Schizosaccharomyces pombe* Rqh1 (Stewart *et al*., 1997), human RecQL4 that is defective in some cases of Rothmund-Thomson syndrome (Kitao *et al*., 1999), human RecQL1 (Puranam and Blackshear, 1994; Seki *et al*., 1994), and human RecQL5 (Shimamoto *et al*., 2000). Purified recombinant WRN and BLM proteins unwind duplex DNA in a 3′-to-5′ direction and require a 3′ single strand tail. (Gray *et al*., 1997; Karow *et al*., 1997; Suzuki *et al*., 1997). Both enzymes interact directly with human replication protein A (RPA) that facilitates their DNA-unwinding activities (Brosh *et al*., 1999; 2001).

Advanced sequence alignment analysis showed a putative exonuclease domain near the N-terminus of WRN (Mushegian *et al*., 1997). This domain contains three conserved motifs that resemble the conserved motifs in the proofreading exonuclease domain of *E. coli* DNA polymerase I and in *E. coli* RNaseD. An exonuclease activity of purified WRN protein has been described, although there is some disagreement on its directionality and dependency on the helicase activity (Huang *et al*., 1998; Kamath-Loeb *et al*., 1998; Shen *et al*., 1998; Suzuki *et al*., 1999).

Hypersensitivities of WS cells to 4-nitroquinoline 1-oxide (4-NQO) and camptothecin (CPT) have been reported (Gebhart *et al*., 1988; Ogburn *et al*., 1997; Poot *et al*., 1999). However, WS cells do not exhibit a hypersensitivity to other DNA-damaging agents such as most alkylating agents, and X-rays, bleomycin, or H_2O_2 that produce reactive oxygen species (Fujiwara *et al*., 1977; Gebhart *et al*., 1988; Higashikawa and Fujiwara, 1978; Okada *et al*., 1998), as well as to UV irradiation (Krepinsky *et al*., 1979). On the other hand, BS cells show hypersensitivities to N-ethyl-N-nitrosourea, ethyl methanesulfonate, methyl methanesulfonate (MMS) and 4-NQO, as well as UV irradiation (Krepinsky *et al*., 1979; Kurihara *et al*., 1987; Shiraishi *et al*., 1985). These observations suggest that both WRN and BLM may be directly or indirectly involved in DNA repair and may prefer certain types of DNA damage, respectively.

A possible role in recombination is implicated for WRN and BLM as their homologs in yeast, Sgs1 and Rqh1+, and in *E. coli* RecQ, negatively regulate recombination (Gangloff *et al*., 1994; Hanada *et al*., 1997; Stewart *et al*., 1997; Watt *et al*., 1995). Notably human WRN and BLM can suppress increased homologous and illegitimate recombination in the *sgs1* mutant (Yamagata *et al*., 1998). WRN and BLM have abilities to recognize Holliday junctions and to efficiently accelerate its ATP-dependent branch migration *in vitro*, suggesting that WRN and BLM may suppress homologous recombination (HR) by disrupting recombinogenic molecules that arise at sites of stalled replication forks *in vivo* (Constantinou *et al*., 2000; Karow *et al*., 2000). We (Imamura *et al*., 2001) and Wang *et al*. (2000) developed $BLM^{-/-}$ chicken B cell DT40 lines, and showed that the $BLM^{-/-}$ DT40 cells have a higher sensitivity to several genotoxic agents, increases in the levels of SCE and targeted integration of exogenous DNAs. We also showed that the chicken BLM is involved in early S phase-specific surveillance of damaged adducts of DNA (Imamura *et al*., 2001). An experiment system using DT40 cell lines enabled us to target several genes in a single cell line for analysing complementary functions of these genes. In this study, we produced $WRN^{-/-}$ and $WRN^{-/-}/BLM^{-/-}$ cells from DT40 in addition to $BLM^{-/-}$ DT40 cells and studied their phenotypes to further investigate the roles of WRN and BLM on DNA repair. Our study demonstrated that both WRN and BLM are involved in DNA repair in a complementary fashion.

Results

WRN targeting constructs and production of WRN-deficient DT40 clones

A full-length cDNA encoding GdWRN was isolated from a cDNA library prepared from the chicken DT40 cell line. The helicase domain of chicken WRN shares a 75% identity with the helicase domain of human WRN at the amino acid level, and a 38, 42, 29 and 36% identity with the helicase domains of human BLM, RecQL1, RecQL4 and RecQL5, respectively (Figure 1). Further analysis of the sequence of the N-terminal region shows that chicken WRN shares a 73% identity with the exonuclease domain of human WRN at the amino acid level providing evidence that chicken WRN must be an ortholog of human WRN.

Next, we isolated genomic clones of the *WRN* locus using long-range PCR amplification with primers designed from the chicken *WRN* cDNA. The genomic clones were partially sequenced to determine the positions of exons and introns. To produce *WRN* deletion constructs, approximately 0.8 kb of genomic sequence of the *WRN* locus was replaced at the helicase motif regions with either the histidinol- or blasticidin-resistance gene (His^R or Bsr^R) (Figure 2a). We expected the targeted integration of these constructs to delete amino acids 615–654. To isolate heterozygous $WRN^{+/-}$ mutant clones, the *WRN*-His^R construct was transfected into wild-type DT40 cells. After Southern blot analysis of *Bam*H I/*Sca*I-digested genomic DNA, drug-resistant clones that had a 3.8-kb band were selected (Figure 2a,b). One $WRN^{+/-}$ mutant clone was then transfected with the *WRN*-Bsr^R construct to isolate homozygous $WRN^{-/-}$ mutant clones. The disruption of the *WRN* gene was assessed by northern blot analysis and RT–PCR (Figure 2c,d).

$BLM^{-/-}$ clones were produced by replacing approximately 1 kb of genomic sequence in the series of helicase motifs of the *BLM* locus with either the His^R or Bsr^R as described by Imamura *et al*. (2001). To produce $WRN^{-/-}/BLM^{-/-}$ clones, the two *BLM* targeting constructs containing a selection marker of either neomycin or puromycin were sequentially transfected into a $WRN^{-/-}$ clone. The disruption of the *BLM* gene was also verified using Southern blot analysis (data not shown).

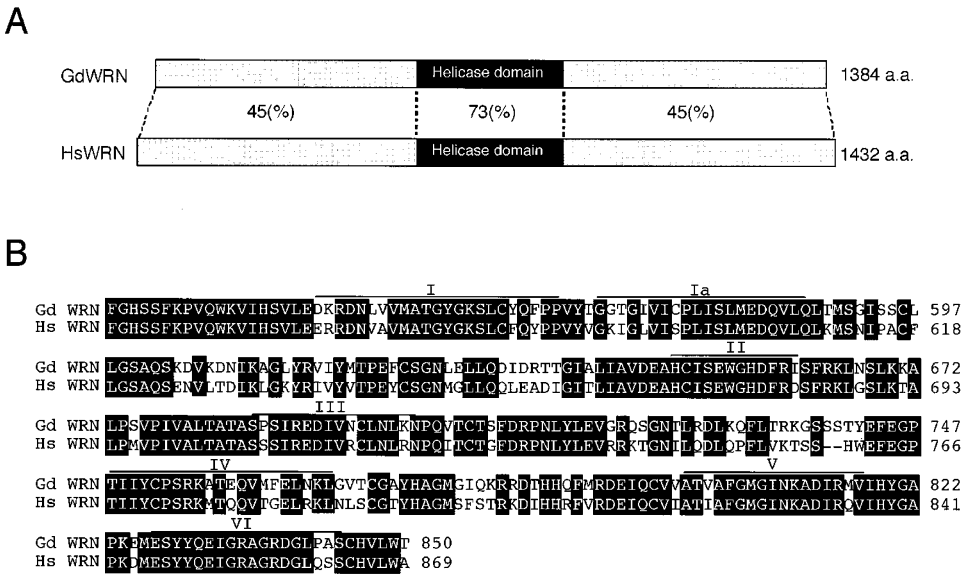

Figure 1 (**a**) Schematic representation of the homology between chicken WRN (GdWRN) and human (Hs) WRN. The darkened areas indicate the locations of the helicase domains shared by the RecQ helicase family. The hatched areas are putative nuclease domains homologous to bacterial RNase D and DNA polymerase I. The light grayish areas are acidic regions contained in most of RecQ helicase family members. The numbers in brackets indicate each area of GdWRN homologous to the areas of HsWRN calculated by the GeneWorks (IntelliGenetics). (**b**) Amino acid alignments in the helicase domains of GdWRN and HsWRN. Thick lines shown above the sequence indicate helicase motifs. The darkened areas represent identical amino acid species. For convenience of alignment, spaces are inserted

Proliferative properties of mutant cells

The proliferative properties of $WRN^{-/-}$, $BLM^{-/-}$ and $WRN^{-/-}/BLM^{-/-}$ mutant clones were monitored by growth curves and cell cycle analyses. $WRN^{-/-}$ and $BLM^{-/-}$ cells proliferated at slightly lower rates than wild-type cells, while $WRN^{-/-}/BLM^{-/-}$ cells proliferated at a considerably lower rate than either single mutant cells (Figure 3). The approximate doubling times were 9, 10, 12 and 13 h for wild-type cells, $WRN^{-/-}$, $BLM^{-/-}$ and $WRN^{-/-}/BLM^{-/-}$ mutant cells, respectively. Although our previous study showed a slight retardation of cell cycle from G_1 to S phase in asynchronously cultured $BLM^{-/-}$ cells (Imamura *et al*., 2001), neither the cell cycle profile of $WRN^{-/-}$ cells nor that of $WRN^{-/-}/BLM^{-/-}$ cells analysed by flow cytometry showed obvious differences from the cell cycle profile of wild-type cells in this study (data not shown).

Increases in sensitivities of mutant cells to genotoxic agents

To examine the sensitivities to various genotoxic agents in a colony survival assay, the mutant cells were grown in medium containing various concentrations of DNA-damaging chemicals or were grown after UV irradiation. Our previous study (Imamura *et al*., 2001) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that $BLM^{-/-}$ cells were hypersensitive to DNA genotoxic agents, such as methyl methanesulfonate (MMS), etoposide and 4-NQO and with UV irradiation. In addition to these genotoxic agents, $BLM^{-/-}$ cells also showed a hypersensitivity to CPT when compared with wild-type cells in the present study (Figure 4).

$WRN^{-/-}$ cells showed markedly increased sensitivities to 4-NQO, MMS and CPT, and a slightly increased sensitivity to etoposide, compared with wild-type cells. However, these cells showed no marked difference in sensitivity to UV irradiation in the range of 1 to 10 J/m^2, which markedly affects on the growth of $BLM^{-/-}$ cells compared with wild-type cells (Figure 4).

$WRN^{-/-}/BLM^{-/-}$ cells were more sensitive to 4-NQO, MMS, CPT, etoposide and UV irradiation than either $WRN^{-/-}$ or $BLM^{-/-}$ cells (Figure 4). These results suggest that separate DNA repair pathways involving WRN and BLM exist and that both pathways might partially complement each other in repairing DNA damages in DT40 cells.

Chromosomal aberrations associated with DNA damage during progression from late S to G_2 phase in $WRN^{-/-}/BLM^{-/-}$ cells

DT40 cells have a stable karyotype with a modal chromosome number of 80 and show no obvious abnormalities except for a trisomy of chromosome 2 and one additional microchromosome (Sonoda *et al*., 1998). Chromosomal aberrations were hardly detectable in asynchronously grown wild-type, $BLM^{-/-}$, and $WRN^{-/-}$ cells, whereas they were more frequent in $WRN^{-/-}/BLM^{-/-}$ cells (Table 1). When cells were irradiated with 1 J/m^2 of UV light, wild-type cells at the late S to G_2 phase were expected to enter mitosis

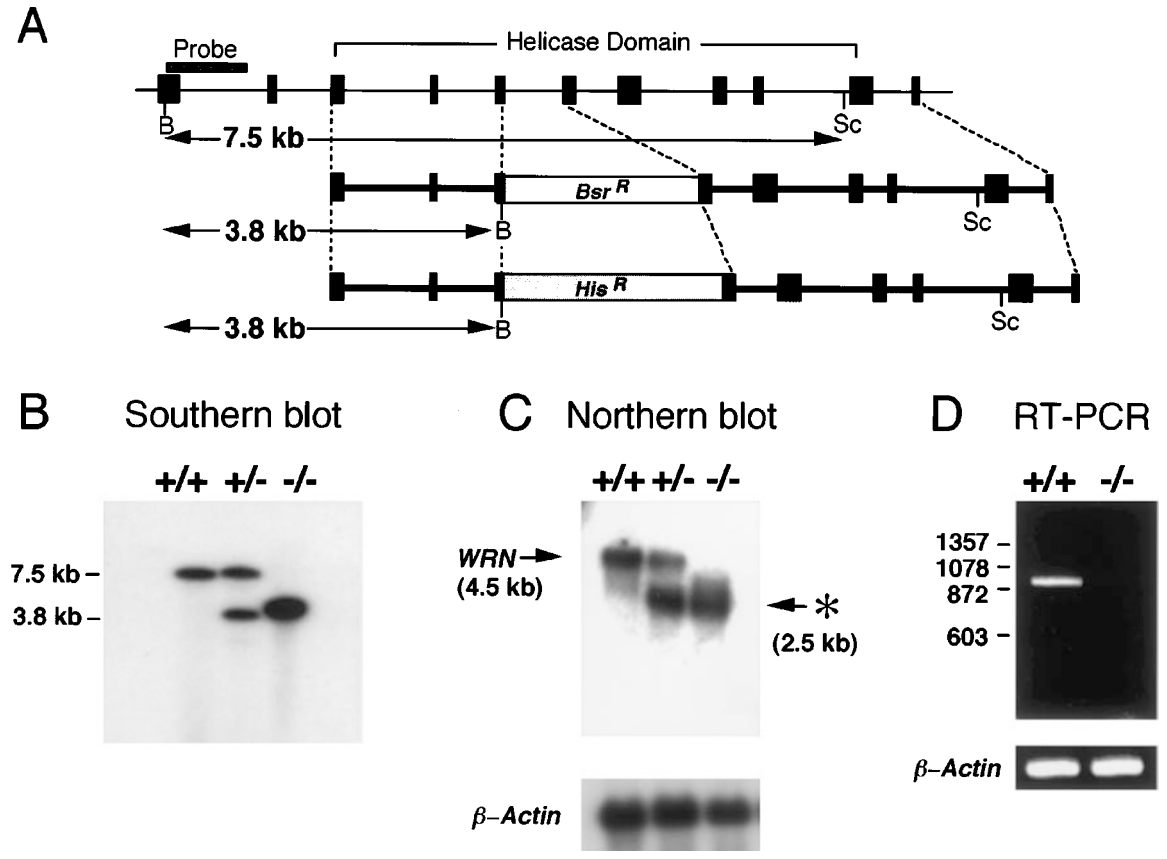

Figure 2 Generation of $WRN^{-/-}$ clones. (**a**) Schematic representation of the partial restriction map of the chicken *WRN* locus, the two gene disruption constructs and the configuration of the targeted loci. Black boxes are the positions of exons. Relevant restriction enzyme sites are shown as follows: B, *Bam*HI; Sc, *Sca*I. (**b**) Southern blot analysis of wild-type (+/+), heterozygous mutant (+/−) and homozygous mutant (−/−) clones. Genomic DNA digested with *Bam*HI and *Sca*I was hybridized with the probe DNA shown in (**a**). (**c**) Northern blot analysis of total RNA of the indicated genotype after hybridization with a chicken *WRN* cDNA probe and chicken β-actin probe. An asterisk indicates shortened transcription products resulted from the genome disruption. (**d**) RT–PCR analysis of purified total RNA from wild-type (+/+) and homozygous mutant (−/−) cells. cDNA was synthesized and was used as a template for amplification with primers that were designed based on the sequence of a coding region containing the area lost by targeting

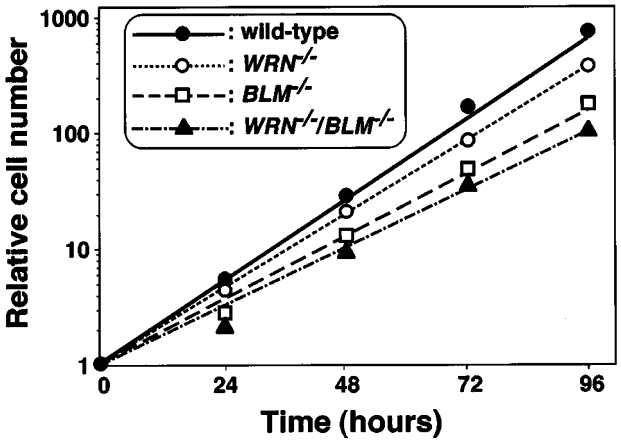

Figure 3 Proliferative characteristics of wild-type, $BLM^{-/-}$, $WRN^{-/-}$ and $BLM^{-/-}/WRN^{-/-}$ cells. Growth curves corresponding to the indicated cell cultures in the absence of histidiol, blasticidin, neomycin and puromycin. Shown data are the mean values for three experiments

within 0–6 h after UV irradiation. As reported by our group (Imamura *et al*., 2001), chromatid-type gaps and breaks were frequent in these cells (Table 1), reflecting the formation of double-stranded DNA (dsDNA) breaks after DNA replication.

$BLM^{-/-}$ cells that had been in the G_1 to early S phase on UV irradiation and entered into mitosis 6–12 h after irradiation also showed increased frequencies of chromatid-type gaps and breaks in addition to a marked increase in frequency of chromatid exchanges, i.e., chromosomal quadriradials (Table 1), suggesting that Holliday junctions persisting during the S phase lead to the induction of dsDNA breaks in *BLM*-deficient cells (Imamura *et al*., 2001).

In contrast to $BLM^{-/-}$ cells, $WRN^{-/-}$ cells showed no obvious change in frequency of chromosomal aberrations after UV irradiation (Table 1), being consistent with the observation that $WRN^{-/-}$ cells have almost the same susceptibility to UV irradiation as wild-type cells (Figure 4). However, $WRN^{-/-}$/

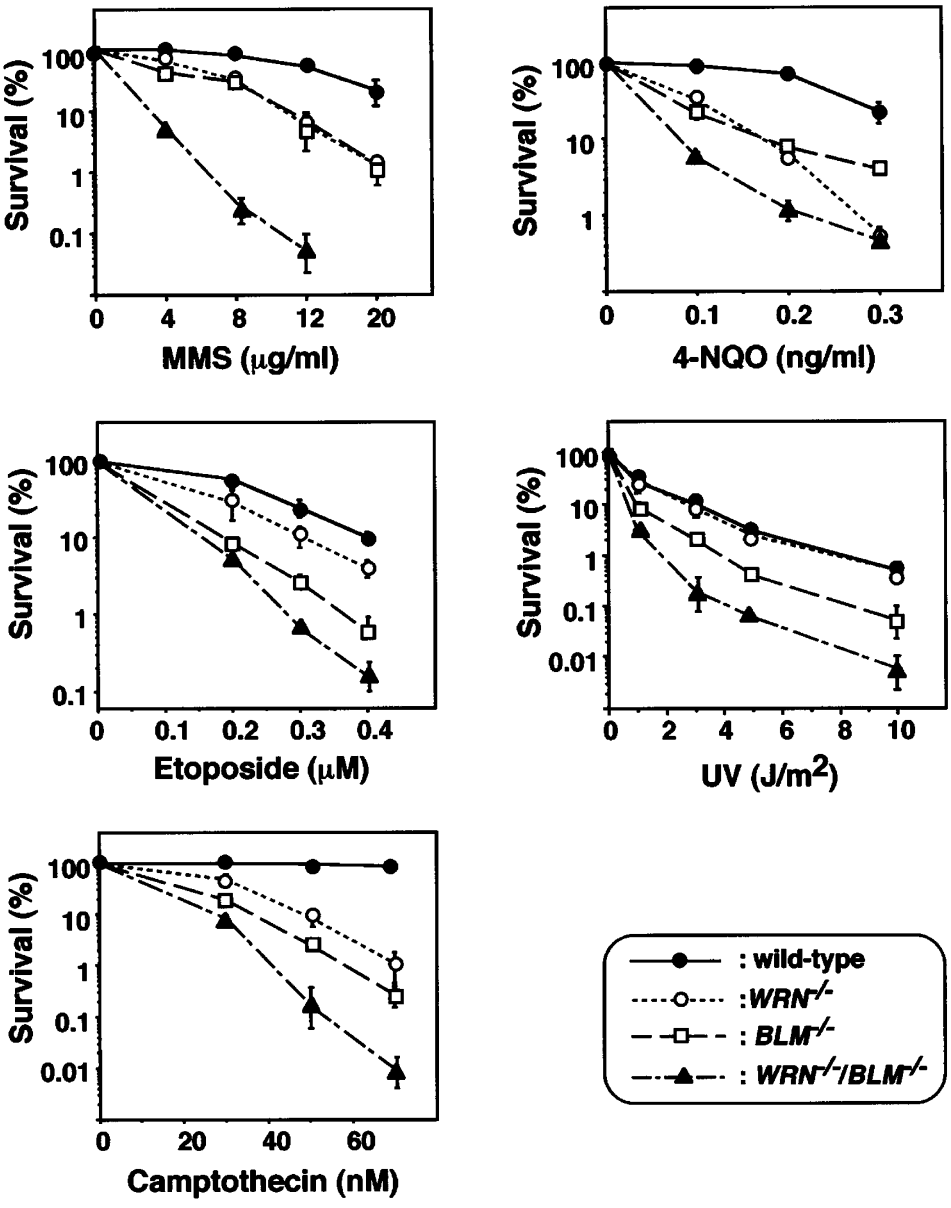

Figure 4 Dose-dependent growth inhibition curves showing the sensitivities of asynchronous cells to various genotoxic agents. The *y* and *x* axes mean rates of the surviving cells and the doses of each genotoxic agents, respectively. The genotypes of cells are at the right bottom panel. Each value represents the mean of survival rates from three independent experiments. Error bars show the standard deviation of the mean

$BLM^{-/-}$ cells that were in the late S to G_2 phase on UV irradiation and entered into mitosis 0–6 h after irradiation showed a marked increase in frequency of chromatid-type breaks and gaps with no increase in frequencies of chromosome-type breaks and gaps and chromatid exchanges. This result implies a possible involvement of WRN in the repair of dsDNA breaks produced at the sites of stalled replication forks.

Targeted integration of exogenous genomic DNA in mutant cells

We examined targeted integration frequencies at the hypoxanthine-guanine phosphoribosyl transferase gene (*HPRT*) locus by comparing wild-type cells with mutant cells. As we had previously measured the targeting integration frequencies at the *β-actin* and *Ovalbumin* loci in $BLM^{-/-}$ cells (Imamura *et al*., 2001), the disruption of *BLM* markedly increased the frequency of targeted integration of the exogenous *HPRT* without a marked increase in transfection efficiency (Table 2). $WRN^{-/-}$ cells showed a 2–3-fold increase in the frequency of targeted integration compared with wild-type cells without a marked increase in transfection efficiency (Table 2), suggesting that WRN may suppress spontaneous HR. However, additional disruption of *WRN* in $BLM^{-/-}$ cells caused a sixfold increase, but lower, in the frequency of targeted integration in

Table 1 Frequencies of chromosomal aberrations in *BLM*-, *WRN*- and *WRN*/*BLM*-deficient DT40 cells after irradiation with UV light

Cells	*Time after irradiation*[a] (h)	*Chromatid breaks/gaps*	*Chromosome breaks/gaps*	*Chromatid exchanges*	*No. of abberrations* (per cell)
Wild-type	spontaneous	4	0	0	0.02
	3	67	13	1	0.41
	6	61	6	0	0.34
	9	17	7	7	0.16
	12	22	6	8	0.18
$BLM^{-/-}$	spontaneous	4	3	0	0.04
	3	73	10	1	0.42
	6	54	12	4	0.35
	9	44	4	22	0.35
	12	19	3	36	0.29
$WRN^{-/-}$	spontaneous	3	2	0	0.03
	3	65	13	0	0.39
	6	30	11	0	0.21
	9	7	14	10	0.16
	12	6	15	13	0.17
$WRN^{-/-}/BLM^{-/-}$	spontaneous	25	5	1	0.16
	3	210	2	1	1.07
	6	104	8	6	0.59
	9	55	7	31	0.47
	12	16	19	24	0.30
	15	19	8	24	0.26

[a]Chromosome aberrations were scored at every 3 h following irradiation with 1 J/m^2 of UV light. All samples were treated with colcemid for the last 3 h.

Table 2 Targeted integration frequencies in $WRN^{-/-}/BLM^{-/-}$ cells

Locus	Wild-type	*Targeted integration frequency in:* $WRN^{-/-}$	$BLM^{-/-}$	$WRN^{-/-}/BLM^{-/-}$
HPRT				
Expt 1	7.1% (2/28)	20% (6/30)	73% (24/33)	46% (11/24)
Exp 2	6.6% (2/30)	13% (4/29)	71% (25/35)	41% (12/29)
Total	6.9% (4/58)	17% (10/59)	72% (49/68)	43% (23/53)
Transfection efficiency	2.1×10^{-5}	2.2×10^{-5}	2.8×10^{-5}	2.5×10^{-5}

contrast to an approximately 10-fold increase in $BLM^{-/-}$ cells, suggesting that WRN may accelerate the HR that arises from *BLM*-deficiency.

Increased SCE frequency in BLM$^{-/-}$ *cells was reduced by disruption of the WRN gene*

$BLM^{-/-}$ DT40 cells show a 10-fold higher number of SCEs than wild-type cells (Imamura *et al*., 2001; Wang *et al*., 2000), suggesting that the defect in chicken BLM is sufficient to cause the high SCE phenotype characteristic of BS cells (Chaganti *et al*., 1974; German, 1993; Heartlein *et al*., 1987). A considerable number of the SCEs observed in chicken $BLM^{-/-}$ cells are formed depending on the Rad54 function, i.e., they are formed by HR (Wang *et al*., 2000). In this study, wild-type cells had as few as 1.8 SCEs/cell (Figure 5). By contrast, $BLM^{-/-}$ cells had a high 20.7 SCEs/cell on average as reported by Wang *et al*. (2000) and Imamura *et al*. (2001). In contrast to $BLM^{-/-}$ cells, $WRN^{-/-}$ cells showed no marked increase in SCE frequency (2.3 ± 1.4 SCEs/cell) compared with wild-type cells. However, additional disruption of *WRN* in $BLM^{-/-}$ cells caused a slight reduction of the SCE frequency (14.6 ± 3.9 SCEs/cell), suggesting that WRN may be partially involved in HR increased in $BLM^{-/-}$ cells.

Discussion

Complementary involvement of WRN and BLM in DNA repair

As DT40 cell lines, like most transformed chicken cell lines, do not express p53 (Takao *et al*., 1999), they may proliferate well throughout the cell cycle and are killed by a genotoxic agent without a checkpoint control downstream of p53. The present study demonstrates that $BLM^{-/-}$ DT40 cells are very similar in their phenotype to human BS cells. $BLM^{-/-}$ DT40 cells showed a hypersensitivity to several genotoxic agents, such as 4-NQO, MMS, CPT, etoposide, to the same extent as UV irradiation. CPT is known to stabilize topoisomerase I resulting in formation of dsDNA breaks during the S phase (Tsao *et al*., 1993). Etoposide stabilizes topoisomerase II

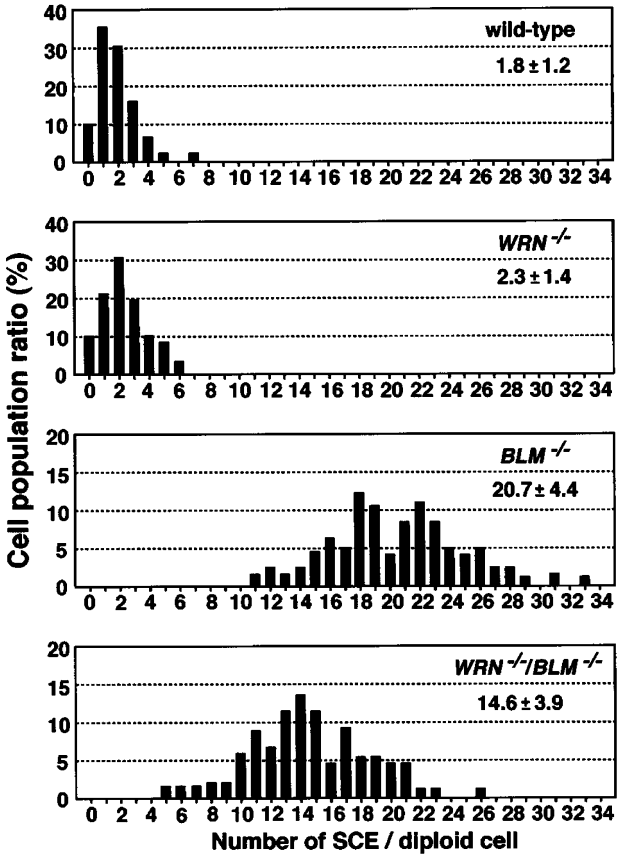

Figure 5 Distribution of population of wild-type, $BLM^{-/-}$, $WRN^{-/-}$ and $BLM^{-/-}/WRN^{-/-}$ cells with indicated frequency of SCEs. Cells were labeled with BrdU during two cell cycles. Spontaneous SCE in the macrosomes of 200 metaphase cells were counted. Histograms show the frequency of cells with the indicated number of SCEs in each cell

resulting in formation of a cleavable DNA complex (Zhang *et al*., 1992). The other chemical genotoxins cause specific types of DNA damages. All of them contribute to the formation of certain types of mutagenic adducts in DNA (Galiegue-Zouitina *et al*., 1984; Smith and Grisham, 1983). UV irradiation also forms DNA adducts such as cyclobutane pyrimidine dimers (Tornaletti and Pfeifer, 1996). Usually, DNA adducts are repaired by the nucleotide-excision repair (NER) pathway (Wood, 1989). However, large numbers of DNA adducts may be overlooked by the NER pathway and may subsequently produce strand discontinuities during replication, leading to chromatid-type gaps and breaks (Sonoda *et al*., 1999; Yamaguchi-Iwai *et al*., 1999). Such strand discontinuities can be repaired by HR with the sister chromatid after replication, as is the case for recombinational repair in yeast cells (Kadyk and Hartwell, 1993). BLM has an ability to recognize DNA adducts or DNA structures associated with stalled replication forks, such as Holliday junctions, and to disrupt these DNA secondary structures leading to recombination repair (Imamura *et al*., 2001). In the presence of BLM, HR after replication might be prevented by rapid disruption of aberrant DNA secondary structures.

Transformed B-lymphoblastoid cells from WS patients are hypersensitive to 4-NQO and CPT (Gebhart *et al*., 1988; Ogburn *et al*., 1997; Poot *et al*., 1999). WS cells are also hypersensitive to etoposide (Elli *et al*., 1996). However, transformed B-lymphoblastoid cells from WS patients do not exhibit a hypersensitivity to other DNA-damaging agents, such as UV irradiation, most other alkylating agents, bleomycin, H_2O_2 or X-ray irradiation (Fujiwara *et al*., 1977; Gebhart *et al*., 1988; Higashikawa and Fujiwara, 1978; Okada *et al*., 1998). In the present study, $WRN^{-/-}$ DT40 cells showed marked hypersensitivity to MMS, etoposide, 4-NQO and CPT, but not to UV irradiation. These phenotypes characterized in $WRN^{-/-}$ DT40 cells are similar to the phenotypes in transformed B-lymphoblastoid cells from WS patients. WRN has been shown to bind to and/or functionally interact with RPA (Brosh *et al*., 1999; Constantinou *et al*., 2000), proliferating cell nuclear antigen (PCNA), DNA topoisomerase I (Lebel *et al*., 1999), Ku 86/70 (Cooper *et al*., 2000; Orren *et al*., 2001), and DNA polymerase δ (Kamath-Loeb *et al*., 2000). Each of these interacting proteins is involved in DNA manipulations including those that resolve alternative DNA structures or repair DNA damages. The synergistic increases in sensitivities of $WRN^{-/-}/BLM^{-/-}$ DT40 cells to genotoxic agents suggest the complementary involvement of WRN and BLM in repair of damaged DNAs. Moreover, the hypersensitivity of $BLM^{-/-}$ cells to UV irradiation is enhanced by the concomitant disruption of *WRN* despite the fact that $WRN^{-/-}$ cells show no increase in sensitivity to UV irradiation. These findings suggest that WRN may have a role on DNA transitions in a manner different from BLM. This explanation might be supported by characteristics unique to WRN where WRN exonuclease preferentially hydrolyzes alternative DNA that contains bubbles, extra-helical loops, 3-way or 4-way junctions (Machwe *et al*., 2000; Shen and Loeb, 2000). In addition, WRN as well as BLM can resolve aberrant DNA structures such as G-quadruplex and G-triplex DNAs (Brosh *et al*., 2001; Sun *et al*., 1998).

The synergistic increase in sensitivity of $WRN^{-/-}/BLM^{-/-}$ DT40 cells to UV irradiation is also supported by the data from karyotypic analyses. As previously reported (Imamura *et al*., 2001), the fact that UV irradiation in the G_1 to early S phase causes chromosomal aberrations such as chromatid-type gaps and breaks in $BLM^{-/-}$ cells suggests BLM may have a role in resolving DNA secondary structures occurring at stalled forks in the early S phase. In the present study, chromatid breaks were frequently observed in $WRN^{-/-}$ cells, as well as in wild-type cells, when irradiated with UV light during the S to G_2 phases. In $WRN^{-/-}/BLM^{-/-}$ cells, UV irradiation in the late S to G_2 phase synergistically enhanced the increases in the number of chromatid-type gaps and breaks without any remarkable change in the number of chromosome-type breaks and gaps. These results

imply that WRN may, in the late S to G_2 phase, contribute to repair of dsDNA breaks which occur due to the lack of rapid resolution of aberrant DNA secondary structures by BLM. Therefore, the sensitivity of DT40 cells to UV irradiation and to other DNA damaging agents might be increased synergistically when both *BLM* and *WRN* were disrupted. In addition, the marked increase in frequency of spontaneous chromosomal aberrations leading to cell death may explain the decreased rate of proliferation of $WRN^{-/-}/BLM^{-/-}$ cells without any change in cell cycle profile.

Possible involvement of WRN in homologous recombination

In BS cells, the increases in frequencies of SCEs and interchange between homologous chromosomes are observed (Chaganti *et al*., 1974; German, 1993). Like the yeast *sgs1* disruptants that show an increased frequency of spontaneous recombination (Watt *et al*., 1996; Onoda *et al*., 2001), $BLM^{-/-}$ DT40 cells have a hyper-recombination phenotype resulting in increased frequencies in both SCEs and targeted genome integration (Imamura *et al*., 2001). Sonoda *et al*. (1999) demonstrated that HR between sister chromatids is the primary mechanism for SCE in DT40 cells. The level of SCE in $BLM^{-/-}$ DT40 cells is considerably reduced in the absence of *Rad54* (Wang *et al*., 2000). These findings suggest that a large number of the SCEs in $BLM^{-/-}$ cells occur via HR.

$WRN^{-/-}$ DT40 cells also showed an increase in incidence of spontaneous recombination indicated by increased frequency of targeted genome integration. However, disruption of *WRN* partially diminished the SCE frequency that increased in $BLM^{-/-}$ DT40 cells despite the fact that the SCE frequency did not change in $WRN^{-/-}$ DT40 cells compared with wild-type cells, suggesting that WRN might be partially involved in accelerating the post-replicational HR repair occurring in $BLM^{-/-}$ DT40 cells. The above speculation is compatible with the recent report that the close proximity of a pair of sister chromatids allows efficient HR-mediated repair of dsDNA breaks during late S to G_2 phases (Sonoda *et al*., 2001). A possible role of WRN on HR repair contrasts with the function of BLM that may prevent HR by disrupting recombinogenic molecules arising at sites of stalled replication forks which lead to dsDNA breaks (Karow *et al*., 2000). Suppression of spontaneous HR by WRN, that is indicated by the increase in targeted genome integration upon the disruption of WRN, may be explained by an ability of WRN to unwind a DNA triple helix, as well as BLM, which is a joint molecule formed by aberrant recombination between exogenous DNA and its homologous genomic DNA (Brosh *et al*., 2001; Constantinou *et al*., 2000). Because human WRN and BLM can suppress the increased homologous and illegitimate recombination in the yeast *sgs1* mutant (Yamagata *et al*., 1998), both WRN and BLM have been considered as suppressors of HR. We believe this is the first report of a possible function of WRN to accelerate HR in response to DNA damage in vertebrate cells.

In summary, our results with chicken DT40 cells suggest (1) BLM accelerates branch migration of Holliday junctions encountering stalled replication forks and may occasionally overlook some stalled forks in which dsDNA breaks occurred and (2) WRN may accelerate post-replicational recombination repair of dsDNA breaks occurring at stalled forks. Thus, WRN and BLM may contribute to maintain genomic stability in a complementary fashion, resulting in the prevention of tumorigenesis.

Materials and methods

Cloning the chicken homolog of WRN gene

Degenerate primers corresponding to the amino acid residues in motifs I (TGGGKSLC) and V (ATIAFGMG) of human WRN were used to clone a cDNA fragment coding for a portion of the helicase domain of chicken WRN (GdWRN). The polymerase chain reaction (PCR) product was subcloned into a pGEM-T vector (Promega, Madison, WI, USA). The cloned cDNA fragment was used to screen a cDNA library of avian leukosis virus-induced B cell line DT40 (Buerstedde *et al*., 1990) to obtain full-length chicken *WRN* cDNA clones (GenBank/DDBJ/EMBL, Accession No. AB035866), which were verified by DNA sequencing.

Plasmid constructs

The 10-kb genomic chicken *WRN* locus was amplified from DT40 genomic DNA by long-range PCR using primers designed for the chicken *WRN* cDNA sequence. The positions of the exons and introns were located by sequencing. The chicken *WRN* disruption constructs *WRN*-his and *WRN*-bsr were made by replacing approximately 0.8 kb of *Bam*HI/*Bam*HI-linked genomic sequence with histidinol- or blasticidin-selection marker cassettes His^R or Bsr^R under the control of the *β-actin* promoter (Bezzubova *et al*., 1997). The *WRN* disruption constructs were made linear before transfection to DT40 cells by electroporation. The chicken *BLM* targeting constructs were made as described by Imamura *et al*. (2001) using His^R, Bsr^R, puromycin- and neomycin-selection markers.

Gene targeting and cell culture

DT40 cells were maintained in RPMI#1640 medium supplemented with penicillin, streptomycin, 10% fetal bovine serum and 1% chicken serum (Sigma, St. Louis, MO, USA) at 37°C. For DNA transfection, 10^7 cells were suspended in 0.5 ml of phosphate-buffered saline (PBS) containing 30 μg of linearized plasmid and were electroporated using a GENE Pulser apparatus (BioRad, Hercules, CA, USA) at 550 V and 25 μF. Following electroporation, the cells were transferred to 20 ml of fresh medium and were incubated for 24 h. The cells were then resuspended in 90 ml of medium containing the appropriate drugs and divided into four 96-well microtiter plates. After 7–10 days, drug-resistant colonies were transferred to 24-well plates. Gene disruption was confirmed by Southern and Northern blot analysis in addition to reverse transcriptase (RT)–PCR.

Northern blot analysis

10^7 cells were washed once with PBS and the total RNA was extracted using TRIzol Reagent (Gibco-BRL, Grand Island, NY, USA). RNA (20 μg/lane) was separated in a 1.2% formaldehyde gel, was transferred to a nylon membrane, and was hybridized with a ^{32}P-labeled chicken *WRN* or *BLM* cDNA fragment.

Measurement of sensitivity of cells to genotoxic agents

To determine the sensitivity to genotoxic agents, 50 to 5×10^5 cells were plated in triplicate onto 6-well clusters with 5 ml/well of 1.5% (w/v) methylcellulose (Aldrich, Milwaukee, WI, USA) plates containing D-MEM/F-12 (Gibco-BRL) with 15% fetal calf serum and 1.5% chicken serum in the presence or absence of various concentrations of genotoxic agents. Colonies resistant to the genotoxic compound were counted 7–10 days after inoculation. To measure the UV light sensitivity, cells were irradiated with various doses of UV light ($\lambda = 254$ nm) before plating. The percentage survival was calculated relative to the numbers of colonies from untreated cells.

Karyotype analysis

The karyotype was analysed as described by Sonoda *et al*. (1998). Briefly, cells were treated for 3 h with a medium containing 0.1 μg/ml colcemid (Gibco-BRL) before harvesting. The harvested cells were incubated in 1 ml of 0.9% sodium citrate for 15 min at room temperature and were fixed with 5 ml of a freshly prepared 3:1 mixture of methanol and acetic acid. The cell suspension was dropped onto a glass slide, which had been wetted with 50% ethanol, and was immediately flame-dried. The cells on the slides were stained with 3% Giemsa solution at pH 6.4 for 10 min.

Measurements of targeted integration frequencies

To analyse the targeted integrations at the *HPRT* locus, the disruption DNA cassette containing the hygromycin-resistance gene within the locus (Fukagawa *et al*., 1999) was transfected into cells and Southern blot analysis was used for selecting clones resistant to hygromycin.

Measurement of SCE levels

Cells were cultured together with 10 μM BrdU for 18–28 h (two cell cycles) at 37°C and were incubated with 0.1 μg/ml colcemid for the last 3 h. The harvested cells were treated with 75 mM KCl for 15–30 min at room temperature and were subsequently fixed with a freshly prepared 3:1 mixture of methanol and acetic acid for at least 30 min. The cells were fixed onto glass slides wetted with 50% ethanol, and were dried at 40–42°C. The dried slides were incubated with 10 μg/ml of Hoechst 33258 in PBS (pH 6.8) for 20 min, followed by rinsing with MacIlvaine solution (Sonoda *et al*., 1998). The slides were irradiated with black light ($\lambda = 352$ nm) for 60 min, and were incubated in 2 × SSC solution at 62°C for 1 h before staining with 3% Giemsa solution (pH 6.8) and were then subjected to microscopic observation.

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