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Werner's Syndrome Protein Is Required for Correct Recovery after Replication Arrest and DNA Damage Induced in S-Phase of Cell Cycle

Pietro Pichierri,*† Annapaola Franchitto,*† Pasquale Mosesso, and Fabrizio Palitti‡

Laboratorio di Citogenetica Molecolare e Mutagenesi, DABAC, Università degli Studi della Tuscia, 01100 Viterbo, Italy

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Werner's syndrome (WS) is a rare autosomal recessive disorder that arises as a consequence of mutations in a gene coding for a protein that is a member of RecQ family of DNA helicases, WRN. The cellular function of WRN is still unclear, but on the basis of the cellular phenotypes of WS and of RecQ yeast mutants, its possible role in controlling recombination and/or in maintenance of genomic integrity during S-phase has been envisaged. With the use of two drugs, camptothecin and hydroxyurea, which produce replication-associated DNA damage and/or inhibit replication fork progression, we find that WS cells have a slower rate of repair associated with DNA damage induced in the S-phase and a reduced induction of RAD51 foci. As a consequence, WS cells undergo apoptotic cell death more than normal cells, even if they arrest and resume DNA synthesis at an apparently normal rate. Furthermore, we report that WS cells show a higher background level of DNA strand breaks and an elevated spontaneous induction of RAD51 foci. Our findings support the hypothesis that WRN could be involved in the correct resolution of recombinational intermediates that arise from replication arrest due to either DNA damage or replication fork collapse.

INTRODUCTION

Werner's syndrome (WS) is a rare autosomal recessive disorder characterized by premature aging (Salk *et al.*, 1985) and early onset of various neoplasms, including different types of carcinomas and sarcomas (Goto *et al.*, 1981; Hrabko *et al.*, 1982; Sato *et al.*, 1988). This disorder arises as a consequence of mutations in a gene coding for a protein that is a member of RecQ family of DNA helicases, WRN. One of the hallmarks of WS patients is the genomic instability as evidenced by the spontaneous chromosome anomalies and large deletions in many genes (Salk *et al.*, 1985; Fukuchi *et al.*, 1989). It has been demonstrated that WRN exhibits DNA unwinding activity (Gray *et al.*, 1997; Suzuki *et al.*, 1997) and exonuclease activity residing in the N-terminal region (Huang *et al.*, 1998). The cellular function of WRN is still unclear. It has been proposed that helicases are required for various DNA metabolic activities, including progression of replication fork, segregation of newly replicated chromosomes, disruption of the nucleosome structure, DNA supercoiling, transcription, recombination, and repair (Duguet, 1997). In addition, it has been suggested that RecQ family of DNA helicases has an important role in the maintenance of genomic integrity during DNA replication (Karow *et al.*, 2000). Studies from yeast show that DNA replication does not proceed normally in absence of RecQ helicase function (Stewart *et al.*, 1997) and in *Xenopus laevis* the ortholog of WRN is absolutely required for proper formation of replication foci (Yan *et al.*, 1998). Functional interaction between proteins involved in DNA replication, such as replication protein A (RPA) and DNA polymerase δ, and WRN also have been reported (Shen *et al.*, 1998; Brosh *et al.*, 1999; Kamath-Loeb *et al.*, 2000). Furthermore, in yeast, the RecQ-like proteins seem involved in suppression of hyperrecombination, S-phase checkpoint control, and correct DNA segregation (Gangloff *et al.*, 1994, Watt *et al.*, 1995; Stewart *et al.*, 1997; Davey *et al.*, 1998; Yamagata *et al.*, 1998; Frei and Gasser, 2000). Noteworthy, yeast mutants in such RecQ-like helicases are extremely sensitive to agents that cause replication arrest, such as hydroxyurea because of uncontrolled illegitimate recombinational events (Stewart *et al.*, 1997; Yamagata *et al.*, 1998). The observed sensitivity of WS cells to 4-nitroquinoline-1-oxide (Gebhart *et al.*, 1988; Ogburn *et al.*,

* Present address: CNRS, UPR2169 "Genetic Instability and Cancer", Institut de Recherches sur le Cancer, André Lwoff, 7, Rue Guy Moquet 94801 Villejuif Cedex, France.
† These authors contributed equally to this study.
‡ Corresponding author. E-mail address: palitti@unitus.it.

1997) has prompted a search for sensitivity toward drugs that interfere with DNA replication and transcription. More recently, the finding that WS cells show sensitivity to camptothecin, an agent that produces replication-dependent DNA damage, reinforces the hypothesis that WRN functions mainly during the DNA replication processes (Lebel and Leder, 1998; Poot *et al.*, 1999; Pichierri *et al.*, 2000). For these reasons, it has been proposed that the primary defect of this disorder is "unprotected synthesis," that is, the failure of cells to protect the integrity of the genoma during the DNA replication process (Chakraverty and Hickson, 1999). More recently, models were proposed to explain WRN function in replication restart and/or recombinational repair after replication fork stalling (Chakraverty and Hickson, 1999; Shen and Loeb, 2000), also cooperating with RAD51, a key enzyme in homologous recombination (HR) (reviewed by Haber, 2000), and an activity of WRN in the translocation of Holliday junctions has been proposed (Constantinou *et al.*, 2000).

In this work, the hypothesized involvement of WRN in the restart of DNA synthesis after replication fork collapse has been further investigated, taking into consideration the proper activation of the RAD51-dependent recombinational pathway of DNA synthesis restarting. To induce perturbation of DNA replication or replication-dependent DNA damage we treated WS cells with camptothecin (CPT) or hydroxyurea (HU), two drugs that interfere with DNA replication. double strand breaks (CPT) stabilizes topoisomerases I–DNA cleavable complex and induces replication arrest and replication-dependent DSBs (Hsiang *et al.*, 1989; D'Arpa *et al.*, 1990). HU blocks replication fork progression by reducing the nucleotide pool, triggering replication fork collapse and cell death (Skog *et al.*, 1992).

MATERIALS AND METHODS

Cell Culture

Normal human primary fibroblasts (VH16, GM0842) and WS primary fibroblasts (AG00780G) were cultured in Earle's modified essential medium, supplemented with 15% fetal bovine serum, 2 mM L-glutamine, vitamins, and 1× concentration of essential and nonessential amino acids. Primary fibroblasts were used at passage 7 to 12. GM0842 and WS fibroblasts were obtained from Coriell Cell Repositories (Camden, NJ); VH16 cells were a gift from Prof. A.T. Natarajan (State University of Leiden, Leiden, The Netherlands). The EBV-transformed lymphoblasts used were two normal (SNW646 and AHH1) and two WS (KO375 and DJG). SNW646, KO375, and DJG lymphoblast cell lines were originally obtained from Dr. George Martin (University of Washington, Seattle, WA) and were a gift from Dr. V.A. Bohr (National Institute on Aging, National Institutes of Health, Baltimore, MD). The lymphoblast cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. All the cell lines were incubated at 37°C in a 5% CO_2 atmosphere (100% humidity nominal). The growth kinetics of the wild-type and WS lymphoblasts used in these studies is similar, whereas WS primary fibroblasts showed the typical slow-growing phenotype. Under our experimental conditions, WS lymphoblasts showed a higher spontaneous frequency of apoptosis (WS cells ranging from 5 to 7% whereas normal cells from 1 to 3%), whereas in WS primary fibroblasts the spontaneous frequency of apoptosis was lower (~2–3% respect to 0% of normal cells).

Considering the extremely poor growth of the primary WS cells, it was necessary to largely use lymphoblastoid cell lines for our experiments. Furthermore, several authors for many other previous studies on WS have used these cell lines.

Chemicals

CPT and HU were obtained from Sigma (St. Louis, MO). CPT was dissolved in dimethyl sulfoxide, and a stock solution (2.5 mM) was prepared and stored at −20°C. HU was prepared in sterile RPMI medium as 500 mM solution; aliquots were stored at −20°C.

For treatment of cultures, CPT was added at indicated concentrations such that the final dimethyl sulfoxide concentration did not exceed 1%. When not appositely specified, all other chemicals were purchased from Sigma.

CPT and HU Treatments

Lymphoblast cells and fibroblasts were exposed to different doses of CPT (0.1, 1, 15, and 45 μM) for 1 h, washed twice, and allowed to recover in complete medium for 14 h, before processing for assessment of apoptotic induction.

HU treatment was performed treating lymphoblasts with 2 mM HU for 2 h and cells were allowed to recover in drug-free medium for 4, 8, and 14 h. At the end of the treatments, cells were processed to evaluate the apoptotic response. To analyze apoptotic response in S-phase cells, normal and WS lymphoblastoid cell lines were pulse-labeled for 30 min with 30 μg/ml 5-bromo-2′-deoxyuridine (BrdUrd) and then exposed to equitoxic doses of CPT (1 and 45 μM) for 1 h. After treatment, cells were washed and harvested after different recovery times (from 2 up to 14 h). Samples were processed for immunodetection of BrdUrd incorporation essentially as already described (Franchitto *et al.*, 1998). For each time point the percentage of apoptotic cells and the incidence in the population of apoptosis that incorporated BrdUrd (labeled apoptosis, i.e., apoptosis from cells in the S-phase at the treatment) were determined. The percentage of labeled apoptosis was calculated by counting at least 200 apoptotic nuclei.

Evaluation of Apoptotic Response

After treatments, cells were harvested and fixed with methanol/acetic acid 7:1 for 5 min at room temperature and seeded onto clean slides. Apoptotic morphology was determined after staining cells with bis-benzimide H33342 (Sigma) at 0.1 μg/ml in phosphate-buffered saline (PBS) for 20 min at room temperature. At least 1000 cells were scored for each experimental point with the use of a fluorescence microscope (Zeiss, Oberkochen, Germany); only cells with highly condensed chromatin or fragmented nuclei were considered as apoptotic.

Apoptosis was also visualized with the use of the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay with the use of the *In Situ* Cell Death Detection kit (Roche Molecular Biochemicals, Milan, Italy) according to the procedure indicated by the manufacturer for analysis on slides. Results obtained with the two different methods were similar.

Analysis of CPT-induced DNA Damage by Comet Assay

CPT-induced DNA double-strand breaks as well as their repair kinetics were evaluated by Comet assay (single cell gel electrophoresis) in denaturing conditions as described in Olive *et al.* (1991). Comets were evaluated with the use of the public domain software NIH Image combined with an additional comet macro created by Helma and Uhl (2000). Residual DNA damage was evaluated as percentage of the initial tail moment. We preferred to use denaturing conditions instead of nondenaturing ones to simultaneously determine the induced DNA damage, as well as the spontaneous background levels of DNA single-strand breaks simultaneously both in normal or WS cells.

The use of the denaturing assay does not alter the significance of the results because cytotoxicity of CPT is entirely due to the formation of long-living DSBs and does not derive from the short-living single strand breaks (SSBs). A minimum of 200 cells was analyzed for each experimental point. Because an elevated number of apoptotic cells could create confusion in the assessment of DNA damage by means of the Comet assay, resulting in artificial higher mean tail moments, we recorded comets with apoptotic morphology separately (smaller comet head and extremely larger comet tail) and did not use them for the analysis of the tail moment.

Analysis of Induction of RAD51 Foci

Lymphoblasts cells were either exposed to 1 and 45 μM CPT for 1 h and sampled after 2, 4, 6, 8, 10, and 14 h of recovery, or to 2 mM HU for 2 h and sampled after 2, 4, 6, 8, 10, and 14 h of recovery. Slides were prepared by smearing cellular suspension onto poly-L-lysine-coated slides. Cells were fixed in 4% paraformaldehyde-buffered solution and immediately processed for immunochemical detection of RAD51 as already described (Maser *et al.*, 1997), with the use of rabbit polyclonal anti-RAD51 antibody (Oncogene Research, Cambridge, MD) and Alexa546-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). For each experimental point 200 nuclei were examined and RAD51 foci scored by eye at a magnification of 100× with the use of a Zeiss epifluorescence microscope. Only nuclei showing more than five bright foci were considered as positive.

Cell Cycle Analysis

To evaluate the perturbations in cell cycle progression induced by different doses of CPT, normal and WS lymphoblasts were treated for 1 h with CPT and then harvested at different time points. Thirty minutes before CPT treatment cultures were pulsed and chased with 45 μM BrdUrd to analyze cellular progression from the S-phase. Cells were processed for flow cytometry as follows: for each time point 1×10^6 cells were collected and after two washes in PBS fixed in 50% cold methanol. After fixation, cells were exposed to acid denaturation (3 M HCl), neutralization (1 M sodium tetraborate), and blocking solution (10% normal goat serum/PBS). After that, samples were incubated in series with a primary anti-BrdUrd antibody (1:100 in blocking solution) and then with a secondary fluorescein isothiocyanate-conjugated antibody (1:50 in blocking solution). Samples were resuspended in 20 μg/ml propidium iodide before analysis.

For each time point the percentage of labeled S-phase cells (S*); labeled G2-phase cells (G2*); unlabeled G1, S, and G2 phase cells (G1, S, and G2, respectively), as well as subG1 labeled cells (apo*), were evaluated with the use of the gates described in Figure 6.

Recovery of DNA Synthesis after CPT or HU Treatment

To verify whether WS cells properly arrest DNA synthesis after CPT or HU treatments, cells were exposed to 1 μM CPT for 1 h or to 2 mM HU for 2 h and recovered in drug-free medium for different time points. BrdUrd (30 μg/ml) was added 1 h before harvesting and BrdUrd incorporation was evaluated as previously described (Franchitto *et al.*, 1998). At least 500 interphase cells were scored to evaluate the percentage of labeled nuclei. Only nuclei displaying a more or less uniform BrdUrd labeling in the entire volume were considered as actively replicating. Percentage of cells undergoing DNA synthesis at each time point was calculated as fraction of the treated cells versus untreated controls.

All the reported data are presented as mean ± SE and derived from at least three repeated experiments. Statistical analysis was performed with the use of the Chi-square test.

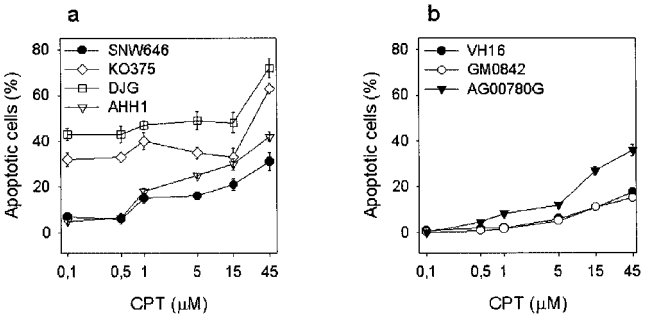

Figure 1. WS cells present a higher induction of apoptosis after CPT treatment. Analysis of induction of the apoptotic cell death by pulse treatment with different doses of CPT in lymphoblast (a) and fibroblast (b) normal and WS cell lines. Normal (SNW646, AHH1, VH16, GM0842) and WS (KO375, DJG, AG00780G) cell lines were exposed to 0.1, 1, 15, and 45 μM CPT for 1 h and apoptosis evaluated 14 h later. In the figure the results from the morphological analysis by bis-benzimide staining are presented but similar results were obtained by TUNEL assay. Points represent mean ± SE from at least three experiments.

RESULTS

WS Cells Are Hypersensitive to CPT Treatments

Hypersensitivity of WS cells to CPT-induced apoptosis has already been reported (Poot *et al.*, 1999). To investigate the recovery processes after CPT-induced DNA damage, we have exposed cells to 1-h pulse treatment with CPT and analyzed sensitivity in terms of apoptotic response after 14 h of recovery. Both lymphoblasts and fibroblasts from WS were more sensitive to CPT-induced apoptosis; WS cell lines showed about a 4- to 10-fold higher sensitivity to CPT than the normal cells, depending on the sampling time, and the DJG cell line appeared particularly sensitive (Figure 1). These data confirm the hypersensitivity of WS cells to CPT also after a pulse treatment.

The analysis of the time course of the CPT-induced apoptosis (Figure 2) showed that normal cells underwent apoptosis only 10 h after treatment, whereas in WS cells a significant induction of apoptotic cell death over the spontaneous level was already observed after 2 h of recovery. The apoptotic response increased with time and reached the highest value after 14 h of recovery in all the cell lines. However, it is important to note that in WS cells the earlier response did not appear dose-dependent. The analysis of the incidence of labeled apoptosis (apoptosis from S-phase cells; Figure 3) in the total apoptotic population demonstrated that in wild-type cells the apoptotic population was almost entirely composed of cells treated in the S-phase, whereas in WS cells composition of the apoptotic population changed with the posttreatment time. In fact, in WS cells the earlier apoptotic cells appeared unlabeled (i.e., cells not treated in the S-phase of the cell cycle). The percentage of unlabeled apoptotic cells was highest between 2 and 4 h of recovery and decreased thereafter, when, conversely, labeled apoptosis became predominant, reaching 100%. These data indicate that hypersensitivity of WS cells after a pulse treatment is due to increased apoptosis not only of cells damaged in S-phase but also to apoptosis of cells that are damaged outside of S-phase.

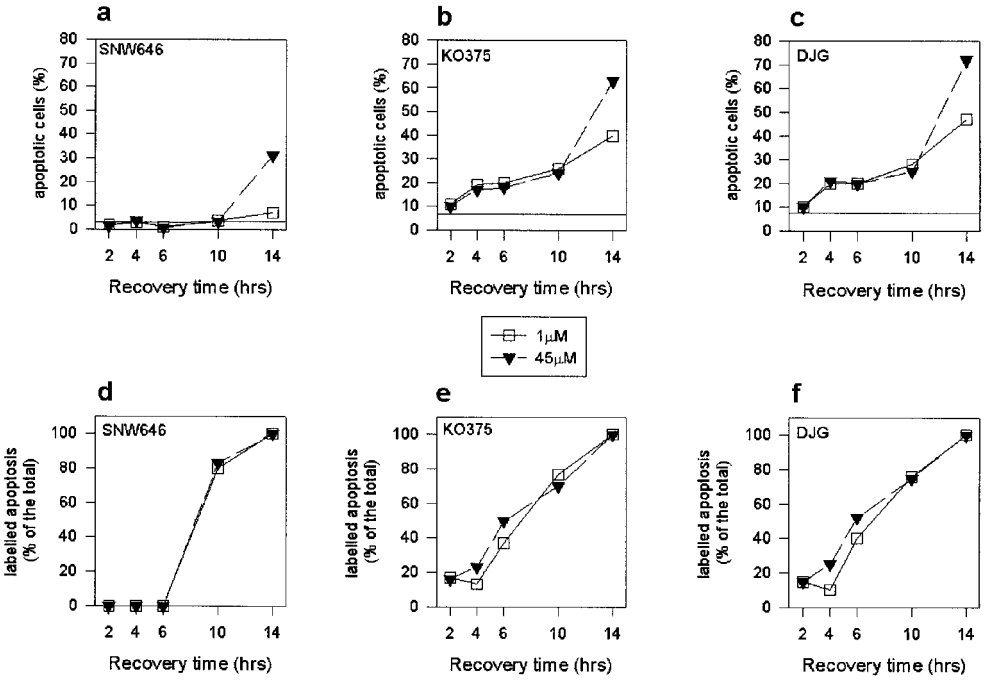

Figure 2. In WS cells CPT induces an early apoptotic response in non-S-phase cells and a later response in S-phase cells. Time course of the apoptotic induction after 1-h CPT treatment in normal (SNW646) and WS (KO375, DJG) cells (a–c); analysis of the percentage of labeled apoptotic nuclei (i.e., cells treated in the S-phase) after 1-h CPT treatment and different recovery times (d–f). Cells were pulse-labeled with BrdUrd for 30 minutes to label S-phase cells and then exposed to CPT for 1 h. Apoptotic cell death was evaluated at the indicated recovery times as described in MATERIALS AND METHODS. Because, in wild-type cells no apoptosis was detected at the earlier times after CPT (Figure 2a), the data reported in Figure 2d at 2, 4, and 6 h represent the absence of any apoptotic response rather than the absence of labeled apoptotic cells. Points represent mean from at least three experiments. Error bars were not indicated for means of clarity; SE were <10%. The horizontal reference line (a–c) represents the mean value of the spontaneous level of apoptosis in the three cell lines.

WS Cells Are Hypersensitive to HU Treatments

To further investigate apoptotic response of WS cells to agents interfering with DNA replication we treated cells with HU. In WS cells HU treatment induced a significant apoptotic response, whereas wild-type cells were affected very little or not at all (Figure 4). The apoptotic induction started after 4 h of posttreatment and gradually increased, reaching the highest value after 14 h (Figure 4a). All the apoptotic cells derived by HU treatment showed BrdUrd incorporation, proving that they were induced to undergo apoptosis while in S-phase (Figure 4b).

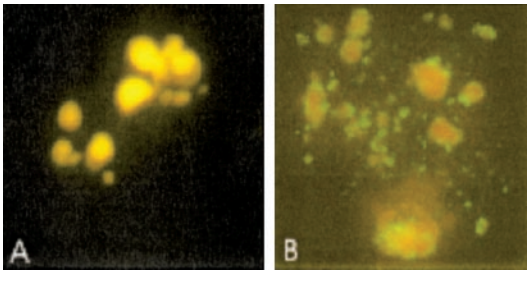

Figure 3. Example of immunocytochemical detection of BrdUrd incorporation in apoptotic nuclei. Cells were handled as described in MATERIALS AND METHODS. (A) BrdUrd-negative apoptosis (unlabeled), representing a cell that was not treated during the S-phase; note that the nucleus is only positive for the DNA. (B) BrdUrd-positive apoptosis (labeled), representing a cell treated during the S-phase.

WS Cells Arrest and Resume DNA Synthesis at a Normal Rate after CPT or HU Treatment

To verify whether the increased hypersensitivity of WS cells to agents that interfere with DNA replication is accompanied by altered kinetic of DNA synthesis arrest and resumption, cells were exposed to CPT or HU and replication rate assessed by analyzing the number of BrdUrd-incorporating cells. CPT and HU treatments resulted in arrest of DNA replication in both normal and WS cells. The rate of replication arrest was similar between wild-type and WS cells and a comparable reduction of BrdUrd-incorporating cells was observed after CPT or HU pulse exposure (Figure 5). Also, the resumption of the induced replication arrest appeared similar between the normal and the WS cells. Consistent results were obtained from the cytofluorometric analysis of the distribution of labeled cells after 1-h pulse treatment with 1 μM CPT or 2 mM HU and different recovery times (our unpublished observation).

To further investigate the progression of cells out of S-phase after CPT-induced DNA replication arrest and evaluate the possibility that the non-S-phase apoptotic response observed in WS is attributable to cells in G2-phase during the time of treatment, S-phase cells were labeled by a short BrdUrd pulse before treatment and analyzed with the use of bivariate cytofluorometry. After treatment labeled S-phase cells (S*) accumulated in both normal and WS cells. Cells remained in the S* compartment up to ~6 h then they started to accumulate in the subsequent G2-phase (G2*). Noteworthy, also bivariate cytofluorometric analysis showed that in WS cells later apoptosis only derived from cells arrested in S-phase after treatment (apo*). In fact, from

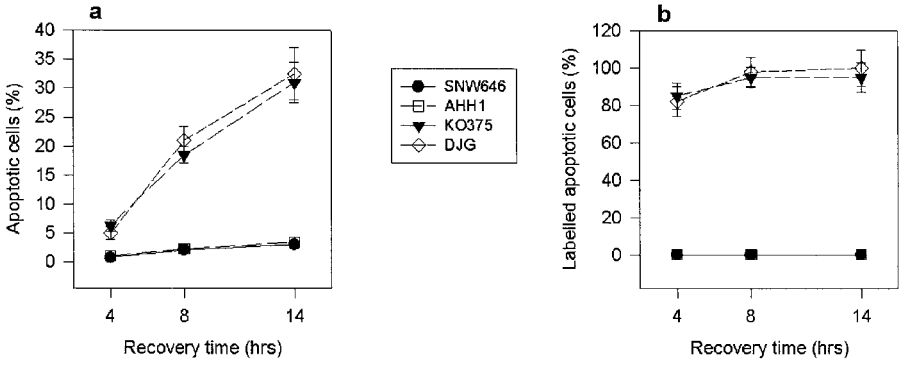

Figure 4. Replication blockage by HU leads to the induction of apoptotic cell death in WS. Cells were exposed to 2 mM of HU for 2 h followed by different recovery periods (a). The apoptotic induction was evaluated at the indicated time by bis-benzimide staining of cells smeared onto microscopic slides as described in MATERIALS AND METHODS. Similar results were obtained by TUNEL assay; analysis of the percentage of labeled apoptotic nuclei (i.e., cells treated in the S-phase) after 2-h HU treatment and different recovery times (b). Cells were pulse-labeled with BrdUrd for 30 minutes to label S-phase cells and then exposed to HU for 2 h. Apoptotic cell death was evaluated at the indicated recovery times as described in MATERIALS AND METHODS. Points represent mean ± SE from at least three experiments.

6 to 10 h of recovery percentage of S* cells was drastically reduced in WS cells when, conversely, the percentage of labeled subG1 cells was increased (apo*). At the same harvesting times normal cells not only showed little or not apoptotic induction but also did not present any reduction in the percentage of the S* population. It is also interesting to note that in WS cells at 2 and 4 h of recovery the proportion of unlabeled G2-phase cells (G2) is reduced in agreement with the possible occurrence of apoptotic cell death in such cells, actually proving that unlabeled apoptosis evidenced by immunocytochemical methods (Figure 2) belonged to cells treated in their G2-phase. In contrast, our results did not show differences in the progression of unlabeled G1 cells (G1) in the unlabeled S-phase compartment (S), as well as, at the later recovery time, both normal and WS cells similarly resume progression of S* cells into the G2* compartment.

WS Cells Repair with Lower Efficiency CPT-induced DNA Damage

We then evaluated the possibility that hypersensitivity of WS cells to CPT is attributable to a higher level of induced DNA damage and/or an altered repair. Cells were exposed to 1 μM CPT and analyzed after different recovery periods for the presence of DNA strand breaks through alkaline Comet assay (Figure 7). Even though CPT treatment resulted in a similar yield of induced DNA damage between normal and WS cell lines (our unpublished observation), repair of the damage appeared slower in WS cells compared with wild-type (Figure 7a).

Spontaneous levels of DNA damage were significantly higher with respect to wild-type cell lines (Figure 7b); interestingly, we observed in untreated WS cells a higher percentage of damaged cells (~10–18% of cells with comets) and not only a more pronounced damage (greater comet tails).

WS Cells Show a Higher Spontaneous Number of RAD51-positive Cells and an Altered Focus-forming Response to CPT or HU Treatments

Replication fork stall triggers recombination as a mode to reinitiate DNA synthesis and yeast defective in the RecQ class helicase show an aberrant recombination rate after DNA replication arrest. RAD51 is a key enzyme in the recombinational repair pathway that takes place between homologous chromosomes (Haber, 2000). Such a pathway is thought to be highly active during the S-phase (Takata *et al.*, 1998; Sonoda *et al.*, 1999), leading also to the formation of sister chromatid exchanges (Sonoda *et al.*, 1999). Furthermore, several models have been proposed to hypothesize a concerted action of WRN and RAD51 (Chakraverty and Hickson, 1999; Shen and Loeb, 2000). We observed that WS cells showed a higher background frequency of RAD51-positive nuclei with respect to normal cell lines (Figure 8b),

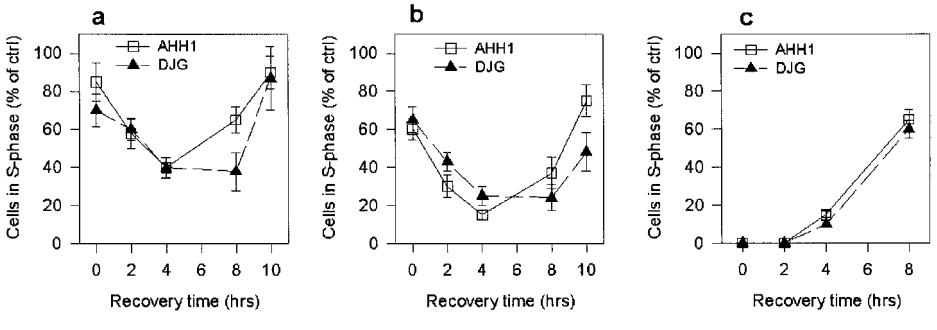

Figure 5. WS cells arrest and resume DNA synthesis as normal cells after CPT or HU pulse treatment. Normal (SNW646) and WS (DJG) cells were exposed to 1 μM (a) or 45 μM (b) CPT for 1 h, or to 2 mM HU for 2 h (c) and recovered at the indicated times. Percentage of S-phase cells was determined labeling DNA synthesis by adding BrdUrd in the last hour before harvesting. BrdUrd incorporation was assessed as described in MATERIALS AND METHODS. Similar results were obtained also with the other normal (AHH1) and WS (KO375) cells. Points represent mean ± SE from at least three experiments.

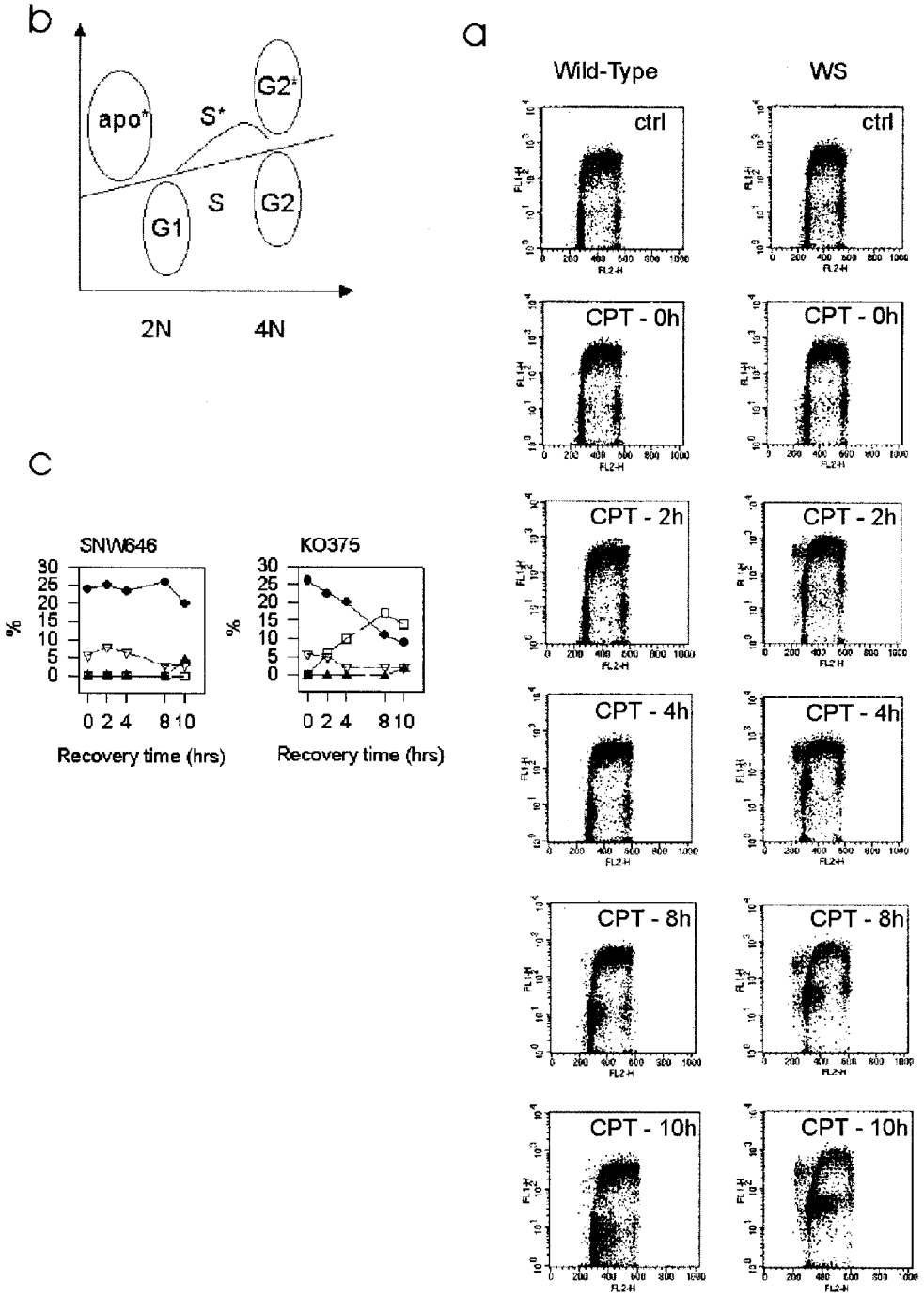

Figure 6. Cytofluorometric bivariate analysis of the CPT-induced cell cycle perturbations. Normal, wild-type, (SNW646), and WS (KO375) cells were pulse labeled with BrdUrd for 30 min to monitor S-phase cells, washed, and then exposed to 45 μM CPT for 1 h. At the indicated recovery times cells were collected and processed for the analysis as described in MATERIALS AND METHODS. Cell cycle progression of wild-type and WS cells after CPT treatment; the horizontal and the vertical axis represent the DNA content and the BrdUrd content (i.e., cells at the S-phase during the treatment), respectively (a). Cell populations were analyzed by gating as described in b: S*, S-phase cells positive for the BrdUrd incorporation; G2*, G2 cells from the S-phase compartment positive for the BrdUrd incorporation; apo*, apoptosis (subG1 population) from S-phase cells positive for the BrdUrd incorporation; S, S-phase cells negative for the BrdUrd incorporation (i.e., G1 or G2 cells entering S-phase). (c) Percentage of cells from different stages of the cell cycle after 1-h pulse treatment with 45 μM CPT. ●, S*; □, apo*; ▲, G2*; ▽, G2. Points represent mean from at least three experiments. Error bars were not indicated for means of clarity; SE were always <10%.

even if, on average, WS cells displayed a similar number of RAD51 foci/nucleus compared with wild-type cells (our unpublished observation). Because no difference was observed in cell cycle distribution between untreated wild-type and WS cells (Figure 6), we can exclude that the higher percentage of RAD51-positive nuclei is due to a greater fraction of late S/G2 cells. We tested whether RAD51 foci formation was altered in cells lacking WRN when treated with either CPT or HU. We found that normal cells showed a time-dependent focus-forming activity after both treatments. When treated with CPT, positive nuclei appeared in normal cells starting at 6 h of recovery and reached the top at 8 h (Figure 8, c and d). A similar trend of RAD51 foci formation was observed at 1 or 45 μM CPT in normal cells, with a higher percentage of RAD51-positive nuclei detected at the higher dose level (Figure 8d). Exposure to HU led to foci formation already at 4-h posttreatment (Figure 8e). On the contrary, in WS cells the

RAD51 response after both treatments was significantly reduced (Figure 8, c–e). Furthermore, if the spontaneous induction of RAD51 foci is subtracted, the RAD51 focus-forming activity is abolished in WS cells at later harvesting times (Figure 8, c–e).

DISCUSSION

Hypersensitivity of WS Cells to CPT and HU

A hypersensitivity of WS cell to CPT has already been reported with the use of several end points (Lebel and Leder, 1998; Poot *et al.*, 1999; Pichierri *et al.*, 2000) and a higher induction of apoptosis has been previously obtained by Poot *et al.* (1999). We confirm the higher induction of apoptosis by CPT in WS cells also after a short pulse treatment. In addition, we find that apoptotic cell death is triggered not only in S-phase cells but also in the G2-phase. It has been reported that aphidicolin prevents CPT-mediated cytotoxicity (D'Arpa *et al.*, 1990). Interestingly, the G2-phase–specific apoptosis seen in WS cells is not prevented by aphidicolin (our unpublished observation), but can be reduced almost entirely by preventive inhibition of RNA polymerase II-driven transcription (Pichierri *et al.*, 2000). In addition, WS cells are highly sensitive to HU exposure, resulting in a strong apoptotic response restricted to cells in the S-phase. The effect of HU in WS cells is interesting because the sensitivity to HU is one of the phenotypical hallmarks of the yeast mutants in the orthologs of WRN, sgs1 and Rqh1. In the RecQ yeast mutants the sensitivity to replication arrest, as induced by HU, results in hyperrecombination and in the formation of the "cut" mitotic phenotype (Stewart *et al.*, 1997; Yamagata *et al.*, 1998). Such a phenotype, resulting from unequal distribution of the genetic material between the two daughter cells, is also associated with mutations in the Rad genes, which are responsible of the checkpoint response (Enoch and Nurse, 1990). However, yeast defective in the RecQ helicases Rqh1 and Sgs1 do not show an altered checkpoint response after replication arrest (Stewart *et al.*, 1997; Davey *et al.*, 1998; Frei and Gasser, 2000). In fact, it has been demonstrated that the reversible S-phase arrest (i.e., arrest followed by retain of cell viability) also requires protective functions that are distinct from cell cycle checkpoint controls (Stewart *et al.*, 1997).

We find that WS cells arrest and resume DNA synthesis after CPT and HU exposure at an apparent similar rate respect to the wild-type cells that is consistent with a role of WRN in human cells similar to that exerted by Sgs1 or Rqh1. Thus, sensitivity to agents that interfere with DNA replication could not be attributed to a direct role of WRN in the checkpoint response. However, WS cells treated in S-phase do not reach mitosis as the RecQ yeast mutants do, but die when still in S-phase or immediately after exit from the S-phase, without apparent accumulation in the subsequent G2-phase (Figure 6).

Lack of Functional WRN Protein Results in Higher Spontaneous Yield of DNA Strand Breaks and Elevated Frequency of RAD51-positive Nuclei

The observed sensitivity of Rqh1 and Sgs1 mutants to arrest of replication fork progression proved that repression of illegitimate recombination is essential for fruitful recovery from S-phase arrest (Stewart *et al.*, 1997; Davey *et al.*, 1998; Frei and Gasser, 2000). An anti-hyperrecombinational role of WRN has been proposed in several models (Chakraverty and Hickson, 1999; Shen and Loeb, 2000) and it is consistent with the partial suppression of the sgs1 mutant phenotype by transfection with WRN (Yamagata *et al.*, 1998). The models proposed to describe the function of WRN in the control of recombination events envisage cooperation with RAD51 (Chakraverty and Hickson, 1999). We find that the absence of functional WRN gives a higher spontaneous focus-forming activity of RAD51. This higher spontaneous frequency of RAD51-positive nuclei could account for an elevated background level of DNA lesions, which trigger HR, as seen for *Xeroderma pigmentosum* group A cells (Radershall *et al.*, 1999). Actually, we observe that WS cells present a significantly larger amount than normal controls of DNA strand breaks in untreated cultures (Figure 7). This observation is consistent with the possibility that in the absence of active WRN the spontaneous damage at the replication fork cannot be resolved properly, leading to recruitment of HR molecular apparatus. In addition, persistence of DNA strand breaks in WS cells could be consistent with the observed higher rate of DNA rearrangements (Gebhart *et al.*, 1988; Fukuchi *et al.*, 1989). Furthermore, a possible consequence of this higher yield of DNA strand breaks is that the effect of the reported interruption of RNA synthesis by CPT, which results in an SSB in the transcribed strand (Barrows *et al.*, 1998; Mosesso *et al.*, 2000), combined with one gap in the other strand, could lead to cytotoxic DSB in G2 cells, thus resulting in cell death.

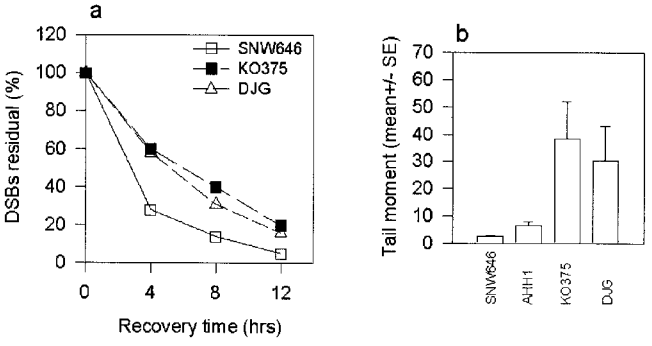

Figure 7. WS cells present by Comet assay an elevated background level of DNA strand breaks and a slower repair of the CPT-induced DNA damage. Normal (SNW646, AHH1) and WS (KO375, DJG) cells were exposed to CPT for 1 h and recovered in drug-free medium for different times. At the indicated time, samples were collected and analyzed by the Comet assay as described in MATERIALS AND METHODS to obtain the time course of extinction of the CPT-induced DNA breakage (a). Similar results as reported for the SNW646 cell line were obtained with the other normal cell line (AHH1). (b) Evaluation of the spontaneous yield of DNA strand breaks in normal (SNW646, AHH1) and WS (KO375, DJG) cells, as evaluated by the tail moment analysis through Comet assay. Points represent mean ± SE from at least three experiments.

Engagement of RAD51-dependent Recombination after CPT or HU Treatment Results in Apoptotic Cell Death in Absence of Functional WRN

Both CPT and HU cause replication fork stall and collapse and recently it is has become clear that HR can play an

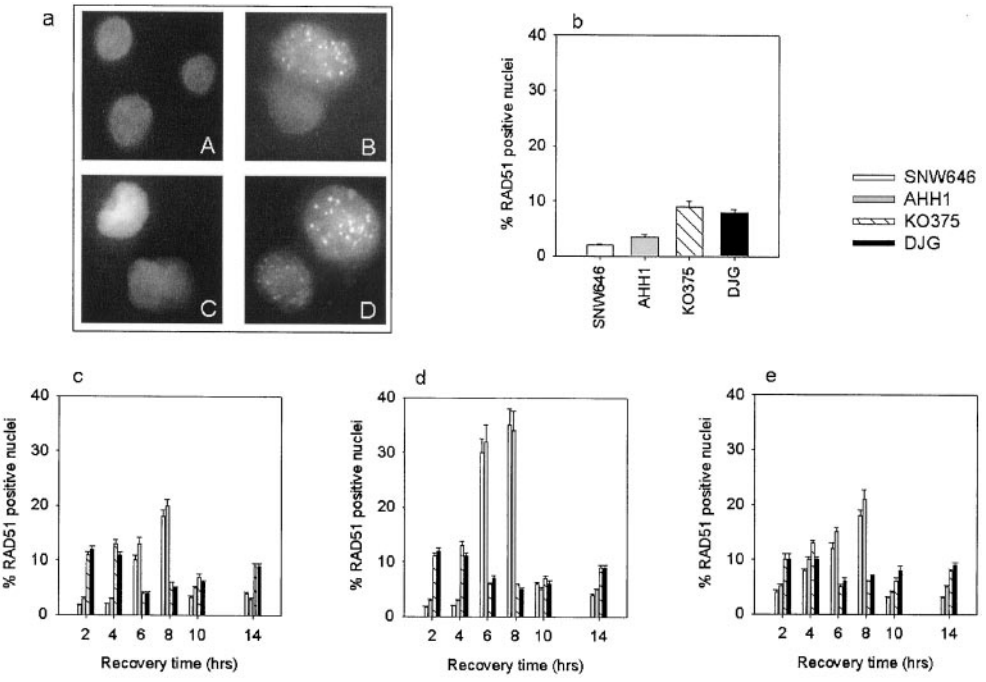

Figure 8. WS cells have an higher level of RAD51 foci but a lack of focus-forming activity after either CPT or HU treatment. Normal (SNW646, AHH1) and WS (KO375, DJG) cells were pulse exposed to CPT or HU and recovered in drug-free medium for different times. At the indicated time, samples were collected and analyzed to detect the presence of RAD51 foci as described in MATERIALS AND METHODS. (a) Representative RAD51 patterns in wild-type (A and B) and WS cells (C and D). The left column shows nuclei without RAD51 spots; the right column presents nuclei containing RAD51 focal activity. Similar focal distribution was observed either in untreated cells or after CPT and HU treatments. (b) Spontaneous level of RAD51-positive nuclei in normal and WS cells; time course of the induction of RAD51 foci after either CPT (c and d) or HU (e) treatment. Points represent mean ± SE from at least three experiments.

important role in the repair of stalled or broken replication fork to permit the reinitiation of DNA synthesis (Haber, 1999; Rothstein *et al.*, 2000). Recently, it has been reported that WRN forms nuclear foci after treatment with HU, colocalizing with RPA upon replication arrest (Costantinou *et al.*, 2000). Accordingly, we analyzed whether in the absence of functional WRN protein the RAD51 foci form properly after CPT or HU treatment. It has already been reported that RAD51-positive cells increase after DNA damage in a time-dependent manner (Haaf *et al.*, 1995; Maser *et al.*, 1997). So, we expected a similar time-dependent increase in RAD51 focus-forming activity after CPT or HU treatments. In addition, we expected that in WS cells the observed elevated spontaneous induction of RAD51 foci (Figure 8b) and those induced in response to treatment were added, resulting in a larger amount of RAD51-positive nuclei. Consistent with the role of HR in bypassing the DNA damage induced by CPT or HU we find that in normal cells RAD51 focus-forming activity is increased in response to treatments in a time-dependent manner and precedes reinitiation of DNA synthesis. Surprisingly, in WS cells we observe a decrease of RAD51-positive nuclei after treatment. However, it is noteworthy that the disappearance of RAD51 foci in WS cells strictly correlates with the increase of the apoptotic cell death in S-phase cells. Furthermore, the percentage of these apoptotic events roughly corresponds to the frequency of RAD51-positive nuclei observed in normal cells. It is also important to note that at the posttreatment sampling times corresponding to the absence of apoptosis derived from S-phase cells, RAD51 foci are still observed, suggesting that, in WS cells, apoptosis is a consequence of the engagement of HR. Supporting our hypothesis that the absence of a functional WRN could result in apoptosis due to hyperrecombination, we were not able to observe a higher induction of sister chromatid exchanges after CPT treatments in WS cells (our unpublished observation), despite the effectiveness of this drug to induce these events (Degrassi *et al.*, 1989). The observation that yeast sgs1 or srs2 mutants die for the accumulation of hyperrecombination events (Gangloff *et al.*, 2000) could be extended in human cells according to our findings. Moreover, because it has been recently reported that WRN relocalizes to sites of replication arrest after HU treatment, colocalizing with RPA (Costantinou *et al.*, 2000), and on the basis of the documented interaction of RAD51 with RPA (Golub *et al.*, 1998), our observation could also reinforce the hypothesized interaction of WRN and RAD51 in resolving replication conflicts.

CONCLUSION

A lot of evidence, regarding mainly studies on bacterial and yeast model systems, suggests that RecQ family of helicases may resolve aberrant DNA structures that arise from DNA metabolic processes such as replication, recombination, and repair (Harmon and Kowalczykowski, 1998; Chakraverty and Hickson, 1999; Karow *et al.*, 2000). In particular, bacterial and yeast RecQ helicases seem to be involved in the suppression of illegitimate recombination after collapse and disassembly of the replication fork, caused either by replication arrest or DNA damage (reviewed by Kuzminov, 1995; Chakraverty and Hickson, 1999; Michel, 2000).

Taken together, our data support the hypothesis of a role of WRN in the control of hyperrecombination after perturbation of the replication fork progression. WRN seems to be essential to avoid illegitimate recombination events after formation of RAD51 foci, ensuring genomic stability and cell viability. In the absence of WRN, cells are able to form

RAD51 foci, but the following aberrant recombination events result in the activation of the apoptotic program.

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