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Advances in Brief

Altered p53 Status Correlates with Differences in Sensitivity to Radiation-induced Mutation and Apoptosis in Two Closely Related Human Lymphoblast Lines[1]

Fen Xia, Xi Wang, Yi-Han Wang, Ngan-Ming Tsang, David W. Yandell, Karl T. Kelsey, and Howard L. Liber[2]

Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts 02115 [F. X., X. W., N-M. T., K. T. K., H. L. L.]; Department of Medicine, Renal Division, Boston University School of Medicine, Boston, Massachusetts 02118 [Y-H. W.]; and Department of Pathology, University of Vermont, Burlington, Vermont 05405 [D. W. Y.]

Abstract

Previous work identified TK6 and WTK1 as human lymphoblast cell lines from one donor that have different capacities to catalyze recombination and that vary significantly in their response to ionizing radiation. WTK1 cells are more resistant to the toxic effects of X-rays yet more sensitive to induced mutation. We demonstrate here that although both cell lines contain equal levels of p53 mRNA, baseline protein levels are 4 times higher in WTK1. Irradiation leads to higher levels of p53 protein in both lines but to a greater extent in TK6. TK6 contains a wild-type p53 sequence, while WTK1 has a homozygous mutation in codon 237 of exon 7. We also observed a significant difference in the kinetics but not the overall degree of apoptosis induced by X-rays in these cells; apoptotic death is delayed for 3 days in WTK1. We hypothesize that this p53 mutation is responsible for the difference in apoptosis as well as for the differences in mutability and mutational spectra reported previously.

Introduction

Genomic instability is a hallmark of many human cancers (1, 2). The product of the *p53* tumor suppressor gene has been implicated as a molecule of central importance in this process (3) because of its involvement in DNA damage-induced G_1 arrest (4), apoptosis (5), DNA repair (6), and gene amplification (7). In previous studies with two human lymphoblast cell lines derived from a single donor, we demonstrated that one line (WTK1) was more resistant to the toxic effects of X-rays than the other line (TK6). Interestingly, however, WTK1 had a 10-fold higher spontaneous mutation rate at the autosomal heterozygous thymidine kinase (*tk*) locus, and was about 20 times more sensitive to mutations induced by 1.5 Gy X-rays (8). In addition, there were significant differences in the mutational spectra observed. WTK1 had a higher proportion of large-scale genetic changes than TK6, and the sizes of these changes were more extensive (9). WTK1 also had 7 times more capacity for catalyzing interplasmid recombination (9). Finally, chromosome 17q, which contains the *tk* gene, was seemingly identical in the two cell lines, as measured by RFLP analysis; however, the short arm, which contains *p53*, was heterozygous for two RFLP markers in TK6 but homozygous in WTK1. On the basis of these previous results, we decided to investigate the p53 status and X-ray-induced apoptosis in these two cell lines.

Materials and Methods

Cell Culture and X-irradiation. Human lymphoblastoid cell lines were maintained as exponentially growing cultures in RPMI 1640 supplemented with 10% horse serum. The cultures were incubated at 37°C in 5% CO_2 and 100% humidity and maintained at densities of 1–10 × 10^5 cells/ml. For serum deprivation experiments, cells were resuspended in serum-free medium and then sampled over the next 3 days. Irradiations were performed with a Philips MG-102 X-ray generator operating at 9.6 mA with 1 mm aluminum added filtration. The dose rate was approximately 0.76 Gy/min.

Northern Blot. Total RNA was extracted 2 h after X-irradiation using acid guanidinium thiocyanate and phenol/chloroform extraction. For each sample, 30 μg of RNA was electrophoresed with 4-morpholinepropanesulfonic acid buffer on a denaturing gel (1% agarose 2.2 M formaldehyde). RNA was transferred onto nylon and hybridized with a radiolabeled human p53 cDNA[3] probe. The ethidium bromide-stained gel scanned at the 28S rRNA band served as a standard for loading. Densitometry was used to quantify the autoradiogram.

Western Blot. Cells were harvested 2 h after X-irradiation and nuclear proteins were isolated. The protein content for each sample was quantified and equal amounts of protein (40 μg) from each sample were loaded on SDS-polyacrylamide gels. After separation, the protein was transferred onto nitrocellulose and blocked with 5% nonfat milk for 1 h at room temperature. Filters were incubated with primary antibody p53 Ab2 (Oncogene Science) for 3 h at room temperature. Finally, the peroxidase-conjugated rabbit anti-mouse antibody was incubated with the blot and detected by the enhanced chemiluminescence method (Amersham). Densitometry was used to quantify the Western blot.

PCR-SSCP. Oligonucleotide primers for PCR amplification of exons 5–9 were those of Toguchida *et al.* (10). PCR products were diluted 10-fold with 0.1% SDS-10 mM EDTA, mixed with an equal volume of stop solution (United States Biochemicals) and heated at 94°C for 4 min. Samples were cooled on ice and loaded immediately on a 6% nondenaturing polyacrylamide gel with 10% glycerol. The gel was electrophoresed at 4°C for 16 h at 15 W.

DNA Sequencing. DNA sequencing was performed using direct PCR product-sequencing methods, with the same primers as for SSCP. PCR product was prepared and sequenced using Sequenase PCR Product Sequencing Kit (United States Biochemicals). A 6% denaturing polyacrylamide gel at room temperature for 4 h was utilized for electrophoresis.

Apoptosis Measured by ELISA. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse mAbs directed against DNA and histones, respectively. This allowed the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. The experiment was performed according to the protocol provided by Boehringer Mannheim. Briefly, 5 × 10^5 cells were lysed on ice at different times after X-irradiation. Cytoplasmic fractions were collected and diluted with lysis buffer, such that 400 cells from each sample were analyzed by the ELISA. Steps in the ELISA included coating of the microtiter plate-modules with antihistone, incubation with sample solution, incubation with anti-DNA-peroxidase, and finally the substrate reaction. Absorbance was measured at 405 nm against substrate solution as a blank. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm from X-ray-treated cells was normalized to the untreated control cells.

Apoptosis Measured by Morphological Analysis. Samples of 4 × 10^5 cells in 1 ml were taken from treated and control cultures at different times after irradiation. Cells were stained with 20 μl of Hoechst 33342 (0.1 μg/ml) at 37°C for 30 min. Cells were centrifuged and fixed with methanol:acetic acid

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[2] To whom requests for reprints should be addressed, at Harvard School of Public Health, Department of Cancer Biology, 665 Huntington Avenue, 1-209, Boston, MA 02115.

[3] The abbreviations used are: cDNA, complementary DNA; SSCP, single strand confirmation polymorphism; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

(3:1), and then examined with a fluorescence microscope. Apoptotic cells were distinguished by the presence of micronuclei or apoptotic bodies. At least 500 cells were counted for each determination.

Results

Status of p53 in WTK1 and TK6. Northern blot analysis of TK6 and WTK1 indicated that there were approximately equal levels of p53 mRNA present in the two cell lines, either with or without 1.5-Gy X-rays (Fig. 1). However, baseline levels of p53 protein, as measured by Western blot, were 3.9-fold higher in WTK1 than in TK6. Two h after X-irradiation, there was a clear increase in the level of p53 protein in the wild-type TK6, of 3.6-fold after 1.5 Gy, and 4.4-fold after 3 Gy (Fig. 1). In WTK1, there was about a 50% increase after either 1.5 or 3 Gy (Fig. 1).

SSCP analysis of each individual p53 exon indicated that all exons from TK6 exhibited the same behavior as wild-type. In WTK1, only exon 7 appeared to be abnormal. DNA sequence analysis of exons 2–10 in TK6 revealed only wild-type sequence. Exon 7 from WTK1 contained a single base pair substitution, a transition of ATG to ATA in codon 237, resulting in a methionine to isoleucine amino acid substitution (Fig. 2).

Apoptosis in WTK1 and TK6 after Treatment with 1.5-Gy X-rays. Apoptosis was quantified by ELISA, which measured the overall generation of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates, and a cell morphology assay, which allowed a determination of the percentage of cells undergoing apoptosis. As seen in Fig. 3, background levels of apoptosis were similar in both cell lines. X-ray-induced apoptosis was apparent at the first time point of 5 h in TK6 but was delayed in WTK1. In TK6, apoptosis peaked after 2 (as measured by staining) or 3 (by ELISA) days, while in WTK1, it peaked after 5 (staining) or 6 (ELISA) days. Despite the difference in kinetics between the two lines, there was no significant difference in the overall level of apoptosis (P = 0.27) when measured by either method. The peak of apoptosis appeared 1 day later when measured by ELISA than by staining; one possible explanation for this difference between methods is that they may detect slightly different stages of the apoptotic process.

In contrast, when apoptosis was induced by serum deprivation, there were no differences between cell lines in either the kinetics or overall levels (Fig. 4).

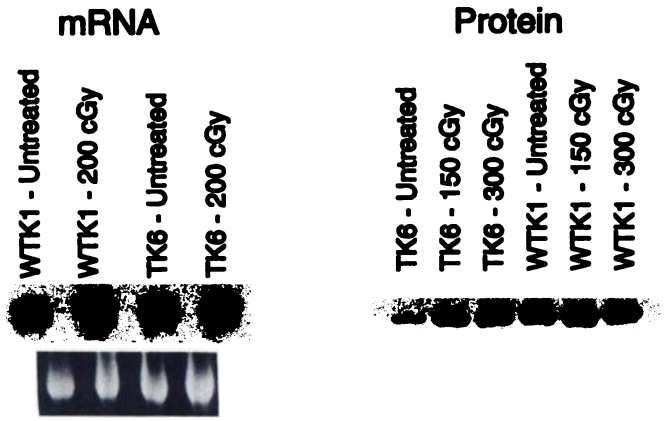

Fig. 1. p53 expression in WTK1 and TK6. Cells were X-irradiated with 1.5, 2 or 3 Gy, and total RNA and nuclear protein were extracted 2 h later. As described in "Materials and Methods," equal amounts of RNA or protein were loaded for each sample. p53 mRNA was detected by Northern blot using a radiolabeled human p53 cDNA probe. p53 protein was detected by enhanced chemiluminescence Western blot using antibody p53 Ab2. The results were quantified by densitometry.

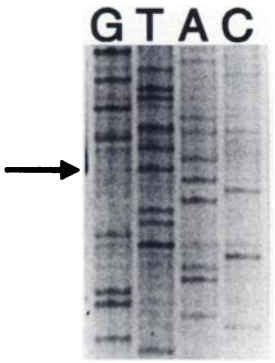

Fig. 2. Sequence analysis of exon 7 from *p53* of WTK1. Exon 7 was PCR amplified as described in "Materials and Methods." Antisense strand is shown. *Arrow,* C to T transition in codon 237 of the *p53* gene in WTK1 cells.

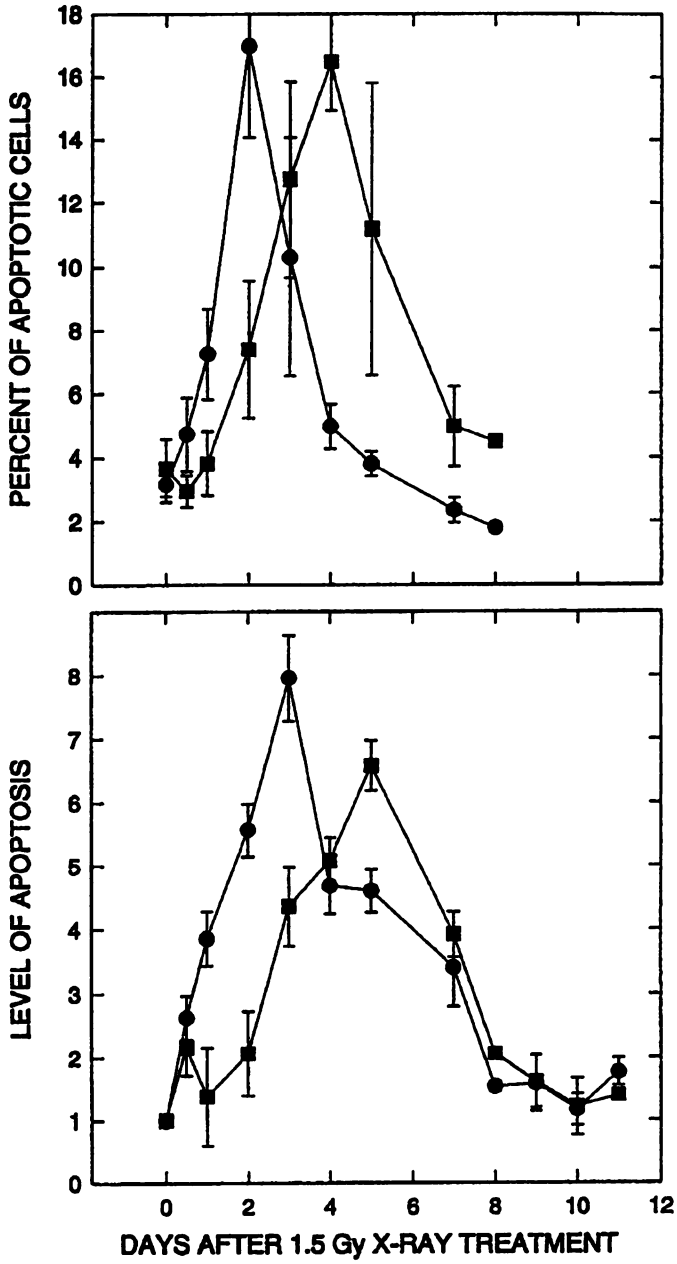

Fig. 3. Kinetics of apoptosis induced by X-rays. WTK1 (■) or TK6 (●) were treated with 1.5 Gy X-rays, and cells were sampled over the next 11 days. *Bottom,* the overall level of apoptosis was determined by ELISA. Y-axis, absorbance at 405 nm normalized to untreated cells; *points,* mean of seven replicates; *bars,* SEM. *Top,* the percentage of cells undergoing apoptosis was determined by staining with Hoescht and microscopic examination. At least 500 cells were scored for each point. *Points,* mean of five replicates; *bars,* SEM.

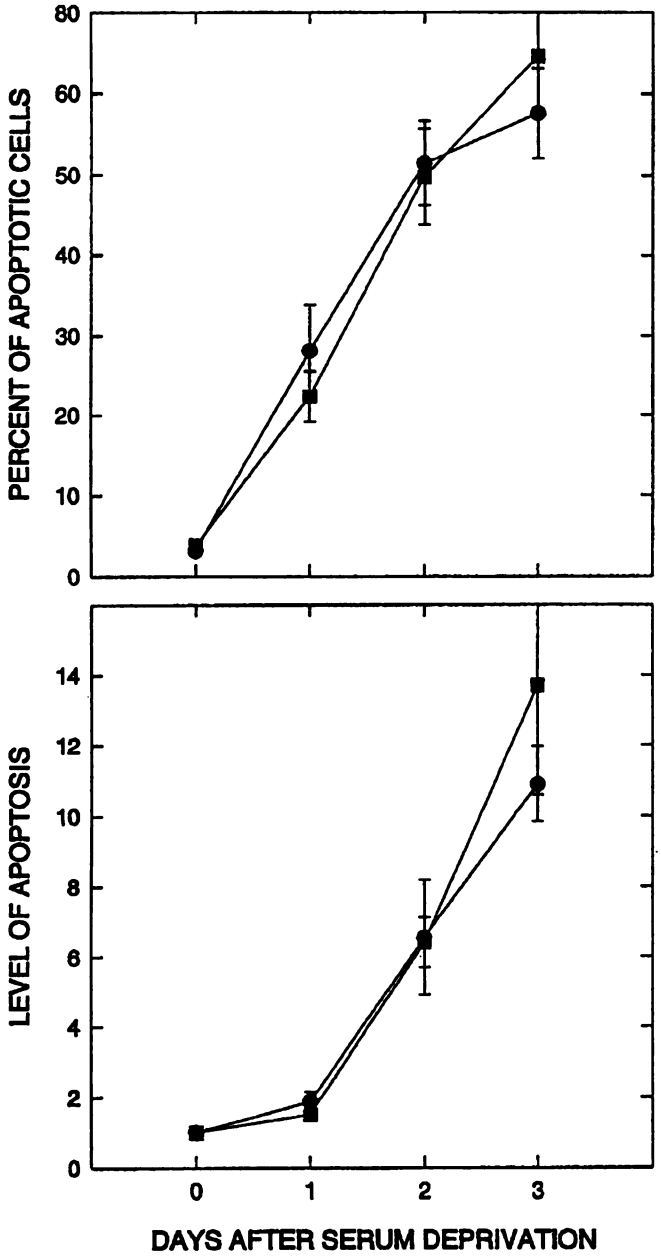

Fig. 4. Kinetics of apoptosis induced by serum deprivation. WTK1 (■) or TK6 (●) were resuspended in serum-free medium, and cells were sampled over the next 3 days. Apoptosis was measured as in Fig. 3. *Points*, mean of seven replicates; *bars*, SEM.

Discussion

WTK1 contains a single point mutation in exon 7 of the *p53* gene. SSCP analysis and direct genomic sequencing both revealed a single, simple pattern at this site, indicating that WTK1 is either homozygous or hemizygous for the allele. We believe that the mutation present in WTK1 is likely to have phenotypic consequences. It is located in a highly conserved region of exon 7 (11). This region is necessary for sequence-specific DNA binding and also contains the SV40 large T antigen-binding site (11). A recent report demonstrated that p53 protein with this mutation was unable to bind single stranded DNA as well as wild-type p53 protein (6). Finally, this same mutation has appeared in tumors (12). It is reasonable that the observed p53 alteration is responsible for increased spontaneous and X-ray-induced mutant frequencies seen in WTK1. Thus, p53 deficiency may be associated with both mutator and hypermutable phenotypes. However, it will be necessary to directly demonstrate that loss of wild-type p53 activity will convert the TK6 phenotype to the WTK1 phenotype, or *vice versa*. Experiments to do this using a known dominant-negative *p53* gene are in progress.

As seen in Fig. 1, there was no difference in the baseline levels of p53 mRNA in WTK1 and TK6. However, WTK1 contained about 4 times more p53 protein than TK6. This is consistent with studies showing that in many human cancers, overexpression of p53 protein is not due to increased p53 transcription, but results from increased p53 protein stability caused by mutations (including the codon 237 missense mutation) in the highly conserved region of the gene (13). Furthermore, in both cell lines, X-ray treatment led to higher levels of p53 protein but had no effect on mRNA. This too is consistent with observations demonstrating that nuclear accumulation of p53 protein following treatment with DNA-damaging agents also is due to increased protein stability and depends on translation but not enhanced transcription (14). Interestingly, the mutated p53 protein in WTK1 still was elevated after X-ray treatment, although to a lesser extent (50%) than in TK6 (3.6–4.4-fold). Therefore, it is possible that the mutation does not completely abolish all p53 protein function.

In previous work, we demonstrated that WTK1 was more resistant to the toxic effects of X-rays, with a D_o of 0.90 Gy, compared to 0.65 Gy for TK6 (8). We proposed recombinational repair as a possible mechanism to account for this finding (9). From the present work, an alternative hypothesis is suggested. Even though the two lines have the same overall level of X-ray-induced apoptosis, the difference in kinetics still could account for the difference in sensitivity to killing. In the earlier experiments, survival was determined by seeding cells in microtiter dishes at an average of 1 cell/well immediately after irradiation. TK6 cells cease to divide at once and begin to apoptose within the first few hours; thus, many wells that originally contained one cell would fail to produce a colony. However, WTK1 cells do divide at least once before apoptosis begins; thus, the average number of cells/well would have increased to at least 2. The probability of all cells in these wells dying by apoptosis would be lower; thus, the apparent surviving fraction would be higher.

Both delayed apoptosis and increased recombination might contribute to the mutator and hypermutable phenotype observed in WTK1. As in the case for survival, delayed apoptosis in WTK1 could increase X-ray-induced mutant frequencies. However, given that the effect on survival was at most a 50% increase, it is unlikely that aberrant apoptosis could have resulted in a 20-fold increase in mutation frequency. Therefore, we believe that increased recombination in WTK1, as demonstrated in our previous work (9), is mainly responsible for the hypermutable and mutator phenotype seen in WTK1. There are several examples of the involvement of recombination in the process of genomic instability. Aberrant V(D)J recombination contributes to genomic instability in lymphoid cells (15), and some specific sequence elements such as telomeric repeats (16) or inverted repeats (17) are involved in genomic rearrangements through recombination. Elevated spontaneous recombination has been observed in cells from patients with ataxia telangiectasia, a syndrome associated with chromosomal instability and increased cancer incidence (18).

Two possibilities could account for delayed apoptosis observed after X-ray treatment of WTK1. One explanation is that the missense mutation in p53 may have reduced p53 protein function without eliminating it entirely; thus, WTK1 may have retained some capability to trigger apoptosis. Furthermore, since it has been reported that p53 may not only regulate apoptosis but also may participate directly as part of the enzymatic machinery of apoptosis (19); the mutant protein may be able to perform one but not both of these functions. A second explanation is that delayed apoptosis in WTK1 after X-ray treatment may occur through a p53-independent pathway. The experiments with

serum deprivation corroborate the fact that apoptosis can be induced in these cells, and other reports in the literature have indicated that apoptosis resulting from growth factor deprivation is p53 independent (20). X-ray-induced damage may have triggered a p53-independent pathway directly, or a recombinational repair system operating at elevated levels in WTK1 may result in errors that serve as later signals for apoptosis.

Thus, WTK1 and TK6 are a pair of human cell lines from a single donor that vary in their p53 status. Since cells with the same phenotype as WTK1 are at very high risk of becoming malignant, these cells should serve as an important tool for future studies. Unresolved questions that we plan to examine include: (*a*) does p53 play a role in regulating recombinational processes; (*b*) what is the mechanism by which delayed apoptosis occurs; and (*c*) how does p53 status affect the spectrum of spontaneous mutations or those induced by environmental agents?

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