Radiation-induced Mutations at the Autosomal Thymidine Kinase Locus Are Not Elevated in p53-null Cells

Yao-Yu Eric Chuang, Qi Chen, and Howard L. Liber

Department of Radiation Oncology, Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract

To explore further the possibility that some forms of mutated p53 may increase mutagenesis in a positive manner, a double p53 knockout cell line was created, using a promoterless gene targeting approach. The identity of these p53-null cells was confirmed by Southern blot and Western blot analyses. Radiation-induced toxicity and mutagenicity was then compared among p53-null cells, TK6 cells with wild-type p53, and WTK1 cells with a p53 point mutation in codon 237. At the autosomal, heterozygous thymidine kinase locus, p53-null cells had equivalent background mutation frequencies and were approximately equally mutable as TK6, whereas WTK1 was much more sensitive to spontaneously arising and X-ray-induced mutation. Thus, these results indicate that the lack of wild-type p53 did not lead to increased mutagenesis.

Introduction

Genome instability is a characteristic of many human cancers. It has been well documented that alterations in the tumor suppressor p53 are related to genomic instability at the chromosomal level, using end points such as karyotypic instability and gene amplification. The p53 protein has been implicated in multiple cellular responses related to DNA damage, including apoptosis, cell cycle control, as well as DNA replication and repair and transcription. Alterations in any of these processes could be related to increased genomic instability.

Our recent work has focused on the effect of mutated p53 on spontaneously arising and radiation-induced gene locus mutations. The human B lymphoblast cell lines WTK1 and TK6, both of which are heterozygous for the autosomal thymidine kinase locus (1, 2), were derived from the same progenitor, WIL2. We and others have shown that WTK1 (3, 4), and its direct parent WIL2-NS (5, 6), overexpress a mutant form of p53 (methionine to isoleucine substitution at codon 237) and no wild-type p53 protein, whereas TK6 is wild-type for p53. These lines respond quite differently to ionizing radiation. Compared with TK6, WTK1 is less sensitive to radiation-induced cytotoxicity and more sensitive to the induction of mutations at both the TK and HPRT loci. After exposure to 1.5 Gy of X-rays or without treatment, the difference in MF between the two cell lines at tk is about 15-fold (7), and the mutational spectrum was shifted toward large-scale alterations (deletions and interchromosomal recombination) in WTK1 (8). To prove that particular p53 mutations may be associated with both a mutator and hypermutable phenotype, we transfected the known dominant-negative alanine-143 and also the ile237 p53 cDNAs into TK6 (which are p53 null) and thereby obtained isogenic cells that varied only in the status of the p53 gene. We demonstrated that the alteration of p53 status directly led to increases in spontaneous and X-ray-induced mutation frequencies, similar in magnitude to those observed in WTK1 (9).

Studies from other laboratories on the effect of p53 on induced mutagenesis have also demonstrated the importance of this gene product on the mutagenic process. It was found that X-rays induced significantly more mutations at the hpri locus in preB cells from p53 knockout mice compared with the wild type; the increases in MF were associated mainly with gross gene rearrangements (10). Experiments with TK6 cells demonstrated that abrogation of p53 function by HPV16 E6 resulted in enhanced radiation mutagenesis (11). Mekeel et al. (12) showed that intraplasmid recombination frequencies were greatly elevated in p53 mutant cells, including cells with mutated p53 and cells that were null.

Perhaps the simplest explanation for increases in spontaneous and induced gene locus mutation associated with mutated p53 alleles is that the loss of function of the protein is responsible for increased genomic instability, which eventually leads to the transformed state. Certainly there is evidence to support this, including the observations noted above that cells from p53 knockout mice exhibit increased levels of induced mutations, and that E6 inactivation leads to increased mutagenesis. Interestingly, however, when these methods were used to alter p53 status, the investigators did not observe nearly as large an increase in MF as we saw when p53 was altered by a point mutation at ala143 or ile237. Therefore, it is tempting to postulate that in some instances, mutations in p53 could result in a "gain-of-function," i.e., that the continuous presence of a high level of certain mutant forms could act in a positive fashion to increase genetic instability. A number of studies support this hypothesis, with tumorigenesis as the end point (13–16). In addition, the sensitivity to ionizing radiation as well as some anticancer drugs in a p53-null human osteosarcoma cell line transfected with mutant p53 genes has been shown to vary with the position of the mutation of the p53 gene introduced (17, 18). Finally, a gain-of-function p53 mutation that could disrupt spindle checkpoint control was reported (19).

To explore further the possibility that some forms of mutated p53 may increase mutagenesis in a positive fashion, we created a double p53 knockout cell line by using a promoterless gene targeting approach. We then compared radiation-induced toxicity and mutagenicity among p53-null cells and cells with wild-type p53 (TK6) or with mutated p53 (WTK1). The results showed that at the TK locus, p53-null cells had equivalent background MFs and were approximately equally mutable as TK6, whereas WTK1 was much more sensitive to spontaneously arising and radiation-induced mutation. Thus, these results indicate that the lack of wild-type p53 does not lead to increased mutagenesis.

Materials and Methods

p53 Targeting Vectors. The selection conditions used to knockout the two p53 alleles were the neomycin phosphotransferase (neo) and the histidinol dehydrogenase (hisD) genes. The targeting vectors containing these, p53-neo...
and p53-his, were kindly provided by Dr. John Sedivy at Brown University (Providence, RI). Except for containing different selective markers, these targeting vectors are identical. The critical portion of the p53-neo targeting vector is shown in Fig. 1. As can be seen, the vector contains most of the first intron (~10 kb) of p53, the 3' end of exon 2 up to the normal ATG site, and then the neo gene with a polyadenylation signal. The vector continues with normal genomic p53 sequence from the second intron through to the middle of the fourth intron, where it terminates. Replacement of the second exon with the neo gene results in the addition of ~1 kb to this region of p53. It is important to note that Dr. Sedivy designed these vectors so that after proper targeting, the original exon 2 of p53 is no longer intact. Because the normal ATG start site for endogenous p53 is in exon 2, after correct targeting, only translation of the neo (or hisD) gene is possible. The poly(A) region ensures that translation will be terminated and that no portion of the p53 protein will be made.

Cell Culture and Transfection. Human lymphoblast TK6 cells were maintained as exponentially growing cultures in RPMI 1640 supplemented with 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in stationary culture in loosely capped tissue culture flasks at 37°C in 5% CO2 at densities of 1–10 × 10^5 cells/well.

Transfections were performed as described previously (9). Briefly, 10–30 μg of plasmids were linearized with either EcoRI/SalI or PacI/SalI and introduced into 5 × 10^5 cells by electroporation in a total volume of 0.8 ml, shocked with 250 V at 960 μF with Bio-Rad Gene Pluser. Three days after electroporation, cells were plated at 10,000 cells/well in 96-well microtiter plates in medium containing selective agents (1,000 μg/ml G418 or 1.2 mM hygromycin B, histidinol; histidinol-containing medium was prepared by mixing RPMI 1640 with standard levels of histidine. Resistant clones were picked after 16–17 days and expanded in normal medium without selective agents. 

Southern Blot Analysis. DNA (10 μg) was digested with HindIII restriction enzymes according to the methods recommended by the supplier. Restriction-digested DNA samples were electrophoresed on 0.8% agarose gels overnight at 25–35 V. The molecular weight standard was a HindIII-digested lambda DNA. Genomic DNA of wild-type cells was used as a positive control. The gels were stained in ethidium bromide and photographed. Then, DNAs were transferred from agarose gels to nylon membrane using TurboBlotter transfer system (Schleicher & Schuell, Keene, NH). A flanking 3' probe for the p53 locus was provided by Dr. John Sedivy (Brown University). The 3' probe was labeled with [32P]dCTP using a random primer labeling system (Life Technologies, Inc., Gaithersburg, MD). Southern hybridizations were performed by standard methods. Autoradiograms were exposed for 1–7 days.

Western Blot Analysis. Cells were harvested 3 h after irradiation, and protein samples were extracted as described (11). Briefly, cells were pelleted and washed with cold PBS twice, and then cells were lysed on ice for 20 min in a lysis solution [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% NP40]. The protein concentration of each sample was quantified, and equal amounts of protein (50 μg) from each sample were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred onto a nitrocellulose membrane. Filters were probed with different antibodies. The signals were detected by the enhanced chemiluminescence system (New England Nuclear).

Determination of Toxicity and Mutagenicity of Ionizing Radiation. Immediately after treatment, lymphoblast cells were seeded into 96-well microtiter plates at densities of 1–10 cells/well, depending on dose; after 12 days, colonies were counted, and the Poisson distribution was used to calculate the plating efficiency. The surviving fraction was determined by dividing the plating efficiency of a treated culture by the plating efficiency of the untreated control (20).

After treatment, cultures were grown in nonselective medium for 3 days to allow phenotypic expression prior to plating for determination of mutant
I\textit{ONIZING RADIATION MUTAGENESIS IN p53-null CELLS}

Fig. 4. X-ray-induced mutations at the TK locus in NH32, TK6, and WTK1 cells. NH32 and TK6 cells were treated with 0, 100, 200, and 300 cGy of X-irradiation. WTK1 cells were treated with 0 and 200 cGy of X-irradiation. Each point is the mean of three independent experiments; bars, SE. In NH32, the TK$^-$ mutant frequencies were $3.2 \pm 0.6 \times 10^{-5}$, $26.7 \pm 8.0 \times 10^{-6}$, $48.7 \pm 3.1 \times 10^{-6}$, and $45.0 \pm 3.6 \times 10^{-6}$, respectively. In TK6, the TK$^-$ mutant frequencies were $2.2 \pm 0.4 \times 10^{-5}$, $28.6 \pm 8.2 \times 10^{-6}$, $23.9 \pm 1.0 \times 10^{-6}$, and $38.5 \pm 2.7 \times 10^{-6}$, respectively. In WTK1, the TK$^-$ mutant frequencies were $125.9 \pm 30.7 \times 10^{-6}$ and $2638.0 \pm 582.4 \times 10^{-6}$.

fraction. Cells then were plated in microtiter plates in the presence of TFT (2.0 $\mu$g/ml). Cells from each culture also were plated at 1 cell/well in the absence of TFT to determine plating efficiency. All plates were incubated for 11 days prior to scoring colonies. Mutation plates were refed with fresh TFT medium and incubated for an additional 7 days to observe the appearance of any late-appearing mutants. The mutant fractions were calculated with the Poisson distribution (20).

\textbf{Results and Discussion}

\textbf{Construction of p53-null Cells.} To determine whether p53 acts on the mutational process in a positive or negative fashion, we constructed p53-null cells from TK6 human lymphoblast cells, using a promoterless gene targeting approach. The approach relies on a fusion vector between the target gene, p53, and the selectable markers neo or his. Because the marker is not linked to a promoter, it is not expressed after transfection unless it integrates near an endogenous promoter. The p53 vector genomic sequences, linked 5' and 3' of neo, target the construct to the correct location, where the p53 promoter is then able to drive expression of neo (Fig. 1). This approach can be used to knock out both alleles of p53, sequentially. In this study, two "knockout" vectors for p53 were used, including p53-neo and p53-his.

In the first step, the p53-neo targeting vector was introduced into TK6 cells. A total of 5 x 10$^7$ cells were transfected, and 200 G418R colonies were obtained. Correctly targeted events were detected by Southern blot analysis using a flanking probe (Fig. 1), which contained p53 sequence immediately 3' to the genomic p53 sequences in the targeting vector. Due to the fact that the presence of the neo gene in the targeting vector adds about 1 kb to the region, after digestion of genomic DNA with HindIII, Southern blot analysis using this 3' probe should detect a 3.6-kb fragment from correctly targeted events, compared with a 2.65-kb fragment derived from the wild-type p53 allele (Fig. 2). Fig. 2 shows that one transfec\textsuperscript{t}ant (NE72, Lane 3) was correctly targeted by the p53-neo targeting vector, and thus that one allele of wild-type p53 gene in TK6 cells had been knocked out.

For the second step, the heterozygous knockout (NE72) was used as a target for the histidinol dehydrogenase-containing (p53-hisD) targeting vector to create a p53-null (knockout of two alleles) cell line. A total of 5 x 10$^7$ cells were transfected, and 200 histidinol$^R$ colonies were obtained. Among these, 16 transfec\textsuperscript{t}ants were confirmed by Southern blot analysis to be correctly targeted to the remaining p53 wild-type allele, thus producing a putative p53 null. As seen in Fig. 2, NH32 is such a double knockout for p53, because it exhibits only a 3.6-kb band. To confirm that these cell lines were indeed double knockouts, Western blot analyses were done with the p53 antibody Ab6 (Oncogene) to confirm the lack of p53 protein, constitutively or after irradiation (Fig. 3). It also was demonstrated that p21$^{WAF1/CIP1}$ was not induced after irradiation (Fig. 3). On the basis of all of these data, we concluded that we had successfully constructed p53-null cells from TK6.

\textbf{Quantitative Studies with p53-null Cells.} Previous studies showed that X-ray-induced $tk$ mutation frequencies are very different in closely related human lymphoblast cell lines that vary in the status of the p53 gene; it was demonstrated that $tk$ mutations induced by 1.5 Gy X-rays in WTK1 (mutant p53 with ile237) were about 15 times higher than in TK6 (wild-type p53; Ref. 7). To further investigate whether p53 acts on the mutational process in a positive or negative fashion, in this study, X-ray-induced toxicity and mutagenicity were compared among cells with wild-type p53 (TK6), p53-null cells (NH32), and cells with mutated p53 (WTK1). WTK1 was most resistant to X-ray-induced toxicity, with a $D_{50}$ of 150 cGy; TK6 was most sensitive ($D_{50}$, 81 cGy), and NH32 was only slightly more resistant ($D_{50}$, 88 cGy). Similar to our previous study, the induced mutation frequencies at the TK locus after 2 Gy of irradiation were more than 50 times higher in WTK1 than TK6 (Fig. 4). Interestingly, the p53-null NH32 exhibited MFs similar to those of TK6 (wild-type p53), rather than those of WTK1 (mutant p53). Similar results were obtained from another p53 double knockout transfec\textsuperscript{t}ant, NH33 (data not shown).

Therefore, these results indicate that the lack of p53 protein did not result in significantly elevated spontaneous or radiation-induced mutation frequencies. They suggest that wild-type p53 may not affect mutational processes in these human lymphoblast cells and instead that the presence of the mutant p53 protein might be responsible for elevated mutation frequencies and survival. With the availability of p53-null cells, we will be able to determine whether there is a positive effect of p53 mutant protein itself on mutational processes. In future experiments, we will add back different p53 mutant variants to the p53-null cells and elucidate the specificity of different p53 mutations on mutational processes.

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\textbf{References}

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