Spontaneous and Ionizing Radiation-induced Chromosomal Abnormalities in p53-deficient Mice

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ABSTRACT

Chromosomal abnormalities have been assessed in p53-deficient mice. The in vivo frequency of spontaneous stable aberrations in bone marrow cells was elevated by approximately 20-fold in p53 nulls and 13-fold in p53 heterozygotes compared to wild-type. No excessive induction of stable aberrations by γ-irradiation was observed, but p53 deficiency resulted in excess radiation-induced hyperploidy (>10-fold wild-type frequency). No influence of p53 genotype on sister chromatid exchange or G2 chromatid damage was observed in mitogen-stimulated spleen cell cultures; however, a p53 effect on postirradiation mitotic entry was seen. Abnormalities in chromosome segregation and mitotic delay following irradiation in p53-deficient mice suggest a G2-M checkpoint role for p53 and are broadly consistent with data on tumorigenesis in these animals.

INTRODUCTION

Mutation of the p53 gene is among the most frequent genetic alterations noted in human tumors (1, 2). This frequent alteration suggests that the function of p53 protein is important in the maintenance of normal cellular phenotype. This proposition is supported by studies of the hereditary cancer-prone disorder Li-Fraumeni syndrome, a disease linked to germline mutations in p53 (3, 4) where patients are at increased risk for the early development of various tumors. A dramatic demonstration of the role of p53 in carcinogenesis comes from studies with transgenic “knock-out” mice. Mice homozygous for a null p53 allele develop tumors at very high rates early in life (5–8). The latent period for spontaneous tumors in heterozygous p53 knock-out (+/−) animals lies between that of nulls and wild-types (6–8). In addition, the tumor latent period in p53 heterozygotes can be very significantly reduced by treatment with the chemical carcinogen, dimethylnitrosamine (6), or by ionizing radiation exposure (7). As the effect of radiation on p53 nulls is a function of age, it has been suggested that complete loss of p53 function is rate limiting in carcinogenesis (7). Irradiation of young p53 nulls reduces the already very short tumor latency period in these animals (7), indicating that alteration of additional genomic targets is required for tumorigenesis.

The biological role of p53 is complex and not yet fully understood. Cells lacking functional p53 are defective in entry into apoptosis following some, but not all, stimuli, and radiation-induced apoptosis is p53-dependent (9–12). In cellular in vitro systems, p53 plays a role in G1 cell cycle arrest caused by DNA damage and other cellular stresses; functional loss of p53 reduces or abolishes G1 arrest following a number of treatments including ionizing radiation (13–16).

The related observations led to the suggestion that p53 occupies an important position in the signaling pathway between DNA damage and cellular consequences such as death by apoptosis, cell cycle arrest, and repair of damage (17). In broad agreement with this argument, p53 has been found to play a role in genetic stability as judged by permissiveness for drug-induced DNA amplification (15, 16) and control of chromosome number (15, 18, 19).

Cytogenetic analysis is one of the few methods available for screening, albeit on a gross scale, alterations to the entire genome of individual cells. We have used a number of cyogenetic techniques to examine genome stability in p53-deficient mice in vivo. Through the use of direct bone marrow preparations, the levels of spontaneous and radiation-induced stable chromosomal aberrations (translocations, deletions, chromosome gains, and losses) in hemopoietic stem and progenitor cells of adults were assessed. Hemopoietic tissue is of particular relevance since acute leukaemia is common in Li-Fraumeni patients (3) and p53-deficient mice frequently develop lymphoid tumors spontaneously and following irradiation (6, 7). The experiments reported here relied upon G-band analysis of stable aberrations; the scoring of large numbers of cells was facilitated by the use of an automated karyotype analysis system (20). The use of mitogen-stimulated spleen cell cultures allowed examination of shorter-term radiation effects such as chromatic breakage and sister chromatid exchange induction in p53-deficient genotypes. These approaches allow the following questions to be addressed: (a) does p53 status influence the in vivo burden of damaged cells in the hemopoietic system of mice spontaneously or following ionizing irradiation? (b) is the frequency of sister chromatid exchanges influenced by p53 status? (c) are early postirradiation cytogenetic responses affected by p53 status? and (d) can cytogenetic features of hemopoietic cells from p53-deficient mice be correlated with their abnormal spontaneous and radiation-induced cancer incidence?

MATERIALS AND METHODS

Wild-type (p53 +/+), p53 heterozygote (+/−), and p53 null (−/−) mice, originally obtained from L. Donehower and A. Bradley (Baylor College of Medicine, Houston, TX), were maintained in a mixed 129-NIH/Ola genetic background as described (7). The genotype of animals was determined by Southern blot analysis of tail tip DNA samples (21). For the analysis of radiation-induced chromosomal changes in bone marrow, adult mice were treated with a 3 Gy whole-body dose of cobalt-60 gamma radiation at 0.85 Gy min−1. Bone marrow samples were taken 2 weeks or 4 weeks postirradiation; all animals were between 12 and 16 weeks of age at the time of sampling. Direct femoral bone marrow metaphase preparations were produced by published methods (22). Briefly, animals were sacrificed by cervical dislocation, femora were removed, and marrow from each bone was flushed into 10 ml hypotonic solution (0.56% KCl) containing 100 μl 10× trypsin-EDTA solution (GIBCO) and 0.02 μg/ml Colcemid (Ciba). Following incubation at 37°C for 25–30 min, cells were fixed in three changes of 3:1 methanol/acetic acid. Slides were prepared and G-banded as described (20). Metaphases were analyzed using the automated karyotyping system of Piper and Breckon (20), and full G-banded karyotypes were produced for each cell examined.

In vitro irradiation studies were performed on spleen cell cultures. Spleens were removed from animals and disaggregated in RPMI 1640 (GIBCO) containing 10% fetal calf serum, using scissors, followed by sequential passage through 19 and 23 gauge needles. Following storage on ice for a maximum of 24 h, 5-ml cultures were set up in round-bottomed plastic universals, each with 1–5 × 10⁷ cells total in RPMI 1640 containing 20% fetal calf serum and 2
### RESULTS

Spontaneously Occurring Chromosomal Abnormalities in Bone Marrow. Direct bone marrow chromosome preparations allow the examination of metaphases from animals without extended culture periods. Metaphase cells are collected within minutes of removal from the animal and so reflect, as closely as possible, the chromosome constitution of cells in vivo. Full G-banded karyotypes of 40–50 cells, selected at random from three individuals each of wild-type, p53 constitution of cells in vivo. Full G-banded karyotypes of 40–50 cells, examined of metaphases from animals without extended culture scored as chromatid gaps; when lateral chromatid displacement was present, experiments, chromatid discontinuities of greater than a chromatid width were stained with 5% Giemsa in pH 6.8 phosphate buffer for G2 damage experiments. Cultures were X-irradiated at either 280 kVp at 0.1 Gy/minute or 250 kVp at 1 Gy/minute. Total culture times were 24 or 48 h, with the final two h in the presence of 0.004 μg/ml Colcemid. Cultures for analysis of SCE were irradiated at 24 h and harvested at 48 h. Chromosome preparations were made using a 15-min hypotonic treatment (0.56% KCl; room temperature) and three changes of fixative (3:1 methanol:acetic acid). Slides were prepared and stained with 5% Giemsa in pH 6.8 phosphate buffer for G2 damage experiments or, for SCE analysis, by established methods (23). In G2 damage experiments, chromatid discontinuities of greater than a chromatid width were scored as chromatid gaps; when lateral chromatid displacement was present, the aberration was classified as a chromatid break.

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The abbreviation used is: SCE, sister chromatid exchange.
Fig. 1. Representative G-banded karyotypes from bone marrow cells. A, normal mouse karyotype from an unirradiated wild-type animal; note the variation in the number of chromosomes in the centromeric region, which represents natural polymorphism between the 129 and NIH/Ola strains. B, karyotype from a 3-Gy irradiated, 29-day sample time p53 heterozygote showing a small inversion in the left hand chromosome 6 and monosomy of chromosome 10. C, karyotype from a 3-Gy irradiated, 14-day sample time p53 heterozygote; note the translocation between chromosomes 6 and 17, terminal deletion of material from chromosome 7, and monosomy of chromosome 15. D, karyotype from a 3-Gy irradiated, 29-day sample time p53 null animal; note the additional copies of chromosomes 1, 6, 8, 9, 12, 17, and Y, as well as monosomy of chromosome 2.

Gy-nulls had a somewhat enlarged spleen; the remaining sample groups were of three or four individuals.

For each genotype, the stable aberration frequencies observed at 14–15 days were not significantly different from those at 29–30 days after irradiation; data for the two time points were, therefore, combined for statistical analysis. Comparing average breakpoints/cell, 3-Gy irradiation showed a significant effect on aberration induction in only wild-types ($P < 0.001$, $\chi^2$ test). The failure to observe a significant radiation effect in heterozygotes and nulls may be due in part to the elevated spontaneous frequencies of stable aberrations (Table 1) in that it is more difficult to demonstrate a statistically significant effect in the presence of high background, although none of the aberration frequencies scored in this study is saturating.$^5$ As with the data from unirradiated animals, no evidence of clonality was observed. As with the results from unirradiated animals, the aberration distribution amongst cells was overdispersed (average variance:mean ratio, 1.8); again, this was taken into account for statistical testing. A range of aberration types was observed in irradiated wild-type animals with no one form predominating. Similarly, a range of aberration types was noted in nulls and heterozygotes, although these genotypes seemed more prone to terminal (telomeric) translocation induction. A related observation here is the occurrence of telomeric association events in irradiated nulls and heterozygotes (Table 1, far righthand column). Telomeric associations represent end-to-end linkage of two entire chromosomes, whereas terminal translocations appear as the fusion of a chromosome fragment broken at an interstitial site with the end of another chromosome. Telomeric association has been noted as a frequent event in high-passage-number Li Fraumeni syndrome fibroblast cultures (18).

The unstable aberrations, chromatid gaps, breaks, and exchanges were observed in irradiated p53-deficient animals but not wild-type. Radiation did not significantly elevate the frequency of chromatid gaps, breaks, or exchanges in any of the genotypes (Table 1). Double minutes, very small fragments of chromosome, were present in irradiated heterozygotes and more clearly in nulls but not in wild-type animals.

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$^5$ S. D. Bouffler, unpublished data.
Radiation effects on bone marrow cell ploidy were assessed by chromosome counting in 50 randomly selected cells from the G-banded preparations. Comparing irradiated and unirradiated cells (Table 2), it is evident that radiation treatment decreased the number of euploid cells in all genotypes. Statistically significant induction of hypodiploid cells was seen in wild-type animals (P < 0.001, χ² test). Again, the elevated level of hypodiploidy seen in untreated p53-deficient cells, abnormalities in S phase or entry of chromosomes into mitosis. The contribution of mitotic delay can be assessed by scoring the mitotic index after irradiation. Table 2 gives quantitative estimates of the radiation effect on mitotic cell frequency; the control (unirradiated) mitotic frequencies in the three genotypes are not statistically distinguishable. These data demonstrate a dose-dependent decline in mitotic frequency in wild-type, whereas in heterozygotes and nulls, no such decline was seen. In fact, linear regression analysis of the data suggests that it is statistically valid to suggest a dose-dependent increase in mitotic yield in nulls. Data from heterozygotes did not produce a good fit.

**DISCUSSION**

The chromosome analysis presented here of hemopoietic stem cells and progenitors from bone marrow of normal and p53-deficient mice has shown that p53 plays an in vivo role in maintaining some aspects of genome stability, even in the absence of DNA-damaging treat-
chromosomally abnormal cells, this explanation seems unlikely, and single functional p53 allele have been noted by others (15, 19). Similar strong phenotypic effects in cells believed to be carrying a genotype of each individual cell scored, we cannot exclude the possibility that a proportion of the chromatid damage be converted to chromosomal mechanism. In the bone marrow system used in these studies, factors such as clonal growth advantage and cell death are superimposed upon the processing of initial damage to genetic alterations. Although these factors might obscure some damage-processing defects, they are, in our view, unlikely to mask a major role of p53. Since such in vivo factors are likely to affect the early phases of the neoplastic process, the statistically insignificant differences in the observed frequency of induced stable aberrations per se are unlikely to account for the differences between p53 genotypes in sensitivity to radiation carcinogenesis (7). Despite the lack of an excessive overall chromosomal radiation effect in p53-deficient animals, some types of induced aberration appeared more commonly. Telomeric association events were noted only in irradiated nulls or heterozygotes. Similar events have been observed in unirradiated Li Fraumeni syndrome fibroblast cultures (18). Terminal translocation events were also more frequent in heterozygous and null cells (15, 16), but might also represent small interstitial deletion events. An excess of other unstable aberration types was not observed or function and may relate to the activation of telomerase noted in immortal cells and cancer cells (36). Double minute chromosomes are another abnormality seen only in irradiated p53-deficient animals; again, the excess seen in nulls is statistically significantly different from wild type (P < 0.05, x² test). This high frequency of telomere-associated aberrations might be indicating a role for p53 in the maintenance of normal chromosome end structure or function and may relate to the activation of telomerase noted in immortal cells and cancer cells (36). Double minute chromosomes are another abnormality seen only in irradiated p53-deficient animals; again, the excess seen in nulls is statistically significantly different from wild type (P < 0.05, x² test). These double minutes may be a reflection of gene amplification, which is readily achieved in p53 null cells (15, 16), but might also represent small interstitial deletion events. An excess of other unstable aberration types was not observed after irradiation. Given the long period after irradiation in the experiment, this is expected, because the cells scored have most probably undergone several cycles since irradiation; therefore, radiation-induced unstable aberrations will have been eliminated. These observations do, however, argue against the induction by radiation of persistent genomic instability in p53-deficient animals. Cellular ploidy appeared to be strongly influenced by radiation exposure. Chromosome losses were evident in wild-type cells, whereas cells of p53-deficient animals suffered both gains and losses. For this end point, there does seem to be a major difference in the damage response of different p53 genotypes. Thus, p53 appears to play a role in the control of ploidy, both spontaneously and in response to ionizing radiation damage. Because gains and losses of chromosomes were seen, the true level of whole chromosome changes is probably being underestimated, since some apparently 2n cells might be expected to have unbalanced karyotypes. The inter-genotype variation seen in these data seems to reflect most closely the carcinogenesis results of Kemp et al. (7). The significant radiation induc-
tion of hyperploidy in heterozygotes might account for the characteristic high frequency loss of the normal p53 gene and duplication of mutant allele noted in radiation-induced tumors in these animals (7).

Whether this mechanism is involved in the full range of tumor types that develop in the various mutant p53 backgrounds (5–8, 37) requires further investigation. Gains and losses of chromosomes presumably occur at a late stage of the cell cycle, and the data could, therefore, be indicating a role for p53 in the control of chromosome segregation in G2 or M of the cell cycle.

To look more closely at the influence of p53 status on radiation damage processing in the absence of in vivo selective pressures, a number of short-term cytogenetic assays using spleen cells were carried out. Concanavalin A, used in these experiments, is a T-cell-specific mitogen and was chosen because a high proportion of spontaneous and radiation-induced tumors in p53-deficient mice are T-cell lymphomas (5–8). Spleenic T-cells of neither p53 nulls nor heterozygotes had baseline SCE frequencies significantly different from wild-type. Low doses of X-rays (0.25 and 0.5 Gy) induced similar numbers of SCEs in all genotypes. These experiments indicate that p53 has no major role in the S phase recombinational events leading to SCE formation.

DNA damage processing at a later cell cycle stage was examined using a G2 irradiation chromatid damage assay. Ionizing radiation was very effective at causing this form of chromosome damage in all genotypes. The maximal levels of damage observed were comparable in all genotypes, suggesting that the conversion of radiation damage to chromatid lesions is independent of p53. However, peak levels of chromosomal damage were observed earlier in p53 heterozygotes and nulls than in wild-types following 1 Gy; therefore, differences in kinetics might be of importance. Parshad et al. (38) observed a higher frequency of G2 chromatid lesions in lymphocytes of affected individuals in a Li-Fraumeni syndrome pedigree at 0.5–1.5 h after irradiation. There was not, however, a simple relationship between high G2 damage responders, cancer proneness, and p53 mutations in the pedigree examined (38). There are, nonetheless, data available that suggest that G2 chromatid damage may be associated with cancer predisposition (39, 40).

The G2 damage data reported here could be interpreted as damaged cells of p53-deficient genotype being more likely to proceed rapidly through to mitosis than similarly damaged wild-type cells. Two of the possible explanations for this could be that wild-type cells are more likely to die prior to entry into mitosis, or wild-type cells are more able to remain in a viable but G2 blocked state than p53-deficient cells. An attempt to gain insight into this second possibility was made by assessing the numbers of mitotic cells in control and irradiated cultures. In wild-type cultures, a radiation dose-dependent decrease in postirradiation mitotic index was observed. The data for p53 heterozygotes were more variable, whereas the postirradiation mitotic yield in nulls was higher than in wild type and increased with dose. These data are contrary to the currently prevailing notion of p53 status having little or no effect on G2 cell cycle blockage (13, 14, 41, 42). Support for this latter hypothesis comes from flow cytometric determinations of cell cycle distributions after irradiation. The work of Kastan et al. (13) shows that human cells with mutant p53 accumulate in the G2-M phases at 13–16 h after irradiation. These cells were also found to continue to enter S phase from G2 after irradiation, with the overall radiation effect being an emptying of the G1 compartment and cells in G2 increasing. The data as presented did not distinguish between G2 and M cells, nor whether they are viable or dead. The radiation doses used in those experiments were fairly high (~2 Gy), where one would expect lethality to be at or above 50–60%, even for cells with mutant p53 (43). Data for radiation-induced cell cycle blockage in embryo fibroblasts from p53-deficient mice are also available (41). In these assays, again using flow cytometric techniques, little evidence of G2 blockage and G1 emptying postirradiation can be seen in the published data for any of the three p53 genotypes, although it is clear that p53-deficient cells are more likely to enter S phase following irradiation. A clear demonstration of effective G2-M blockage in p53 null T cells 1 day following 1 Gy gamma irradiation has been reported recently (42); cell cycle stages were determined by flow cytometry. The apparent disagreement between the G2-M blockage data from flow cytometric studies and those reported in this paper may be accounted for by the different sampling times used and by uncertainties on the viability of cells. Mitotic indices in the present study were scored up to 5 h after irradiation, whereas the flow cytometry data were largely taken from 13–24 h after irradiation (13, 41). Recently, data has been published that suggests that the expression of very high levels of wild-type p53 in rat embryo fibroblasts can cause arrest of cells in the G2-M phases of the cell cycle (44). Furthermore, Cross et al. (45) have demonstrated that p53 null mouse embryonic fibroblasts fail to arrest following prolonged exposure (22 h) to the spindle inhibitors, Colcemid or nocodazole; this failure to arrest leads to hyperploidy.

To conclude, in the hemopoietic system of mice, the principal effects of p53 deficiency appear to be a constitutive abnormality giving rise to elevated frequencies of stable aberrations and a late cell cycle defect, leading to increased spontaneous and radiation-induced aneuploidy together with faulty control of the entry of cells into mitosis. The intrinsic chromosomal instability that may be attributable to abnormalities in the chromosomal DNA replication machinery would be expected to contribute to the tumor susceptibility of p53-deficient mice and, by implication, to Li-Fraumeni patients. We suggest that, in addition to the established role for p53 in G1 cell cycle arrest (13, 14, 17, 41), p53 may play an important role in a late cell cycle stage (G2-M) damage-response pathway that regulates postirradiation entry into mitosis. In the absence of excessive radiation induction of stable aberrations in bone marrow cells and of SCEs in spleen cell cultures, the role for p53 in early phase cell cycle responses to damage (13, 14, 17, 41) does not appear to be ubiquitous or necessarily predominant. In the context of ionizing radiation, recent observations point towards a distinct tumorigenic mechanism involving loss of wild-type and duplication of mutant p53 alleles in tumors induced in p53 heterozygotes (7). The data on aneuploidy reported here suggest that this may be associated with whole chromosome 11 loss and gain; whereas chromosome 11 was not found here to be preferentially involved in gains or losses, numerical changes to chromosome 11 were observed. This will be the subject of further investigation.

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REFERENCES


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