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Advances in Brief

Concomitant *p53* Gene Mutation and Increased Radiosensitivity in Rat Lung Embryo Epithelial Cells during Neoplastic Development[1]

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Abstract

A rat lung cell population had been treated with benzo(*a*)pyrene, and a set of different epithelial cell lines was derived from it. These cell lines carried either a wild-type or mutant *p53* gene and represented grading states of neoplastic development. We demonstrate here that the cells lacking both wild-type *p53* alleles display a significant decrease in survival after γ-irradiation with doses of 2 to 12 Gy, compared with their counterparts carrying wild-type *p53* alleles. This is the first reported model in which cells bearing a mutation of the *p53* gene display enhanced sensitivity to ionizing radiation.

Introduction

In the past few years, an increasing accumulation of evidence has helped to provide new insights into the function of the *p53* gene in resistance to genotoxic injuries. According to data which are still controversial, the *p53* gene is one of the key genes that govern radioresistance by fine tuning of the balance between efficient DNA repair and programmed cell death (1). It is now well established that, after ionizing radiation, cells exhibiting the wild-type *p53* genotype display an inhibition of the replicative DNA synthesis characterized by arrest in G_1 (2, 3). In contrast, γ-irradiation led to arrest in G_2 whatever the *p53* gene status (4, 5). These events may be critical for preventing the fixation of genetic lesions leading to death or neoplastic transformation. The radiation-induced G_1 arrest is an active physiological response, since it has been shown to be sensitive to cycloheximide (5).

The p53 protein appears to be involved in the control of cell differentiation and proliferation, apoptosis, and DNA repair. It belongs to the signaling pathway by which cells might regulate the G_1-S transition following genotoxic insult; in this way, p53 might play a major growth-controlling role, especially in stressed cells (6, 7). Wild-type p53 protein levels rise dramatically after exposure to ionizing radiation and various DNA-damaging agents, especially in hematopoietic cells (5). This rise results from as yet undefined changes in the posttranscriptional modifications undergone by the p53 protein such as phosphorylation, binding to other proteins, or oligomerization. At subsequent end points of DNA-damage, a prolonged half-life was observed as well as increased DNA binding activity of p53 protein and enhanced transcriptional transactivation activity driven by this protein (8). This DNA damage-induced stabilization of the p53 protein is thought to switch off replication in some cell lines such as fibroblasts until DNA damage is repaired. But if such repair fails, p53 may trigger cell suicide by apoptosis in other cell lines, including hematopoietic cell lines and the human colon tumor-derived EB cell line (reviewed in Ref. 1). This fine tuning of the balance between DNA repair and apoptosis may be mediated by the DNA binding properties of the p53 protein and by its transactivation of gene transcription (reviewed in Ref. 9).

In contrast, cells carrying mutant *p53* alleles or no *p53* alleles were only partially blocked and continued to progress through the cell cycle after DNA damage (10). Consequently, these cells might be expected to have increased sensitivity to radiation. However, the data reported so far suggest that, after ionizing radiation, cells with altered or absent p53 protein exhibit increased survival. These data concerned cells arising, for instance, from erythroid or myeloid cell lines in which mutant *p53* status or *p53* deletion might abrogate the tendency towards apoptosis (11). However, it must be kept in mind that after γ-irradiation, cell lines maintained their G_2 arrest in all cases, irrespective of their *p53* status (5). Since most human cancers are of epithelial origin, we focused our attention on a rodent epithelial cell model allowing step-by-step analysis of carcinogenesis (12). This model enabled us to isolate cells in culture at various stages of neoplastic progression. This approach prompted us to study the resistance to ionizing radiation of cells before they underwent homozygous inactivation of their normal *p53* function, in this case in the parental BP cell line and in BPwt clones, and after this inactivation, in HE clones. The BP-T cell line, derived from a tumor formed upon transplantation of the parental BP population, was also studied. This line carried the same homozygous *p53* mutation as the HE clones (12). For definitions of cell lines and clones, see "Materials and Methods." This experimental model was very suitable for screening the radiosensitivity of cells concomitantly with their endogenous *p53* status (wild-type *versus* mutant form) and stage of neoplastic progression.

Materials and Methods

Cells. All cell lines were described in a recent investigation (12). They were derived from an epithelial cell population arising from embryonic rat lung explants treated with the potent carcinogen B(*a*)P.[3] This population is referred to here as BP cells. At the early passages following B(*a*)P treatment, almost all these cells (99% at passage 15 and 93% at passage 20) exhibited the wild-type *p53* genotype (12). In syngeneic rats, these cells were immortal but not tumorigenic. Several clones exhibiting the wild-type *p53* genotype were isolated from the BP population at passage 19 and were called BPwt clones. At passage 23, *p53* high expressor clones were isolated from foci emerging in BP culture and were called HE clones; all these cloned cells, whose growth was enhanced, displayed a mutant *p53* genotype. At passage 28, BP cells were injected into syngeneic rat, and the BP-T line was established from the resulting tumor. Of these tumor cells, 100% had the mutant *p53* genotype. The numbers of BP-T cells passages were counted for tumor isolation. All of our cell lines which carried a mutant *p53* gene *i.e.*, the HE clones and BP-T population, exhibited the same single AAG→AGG transition at codon 130. One point of critical importance in our experiments is that all of the cells tested were derived from the same syngeneic parental population. These cell lines

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[3] The abbreviation used is: B(a)P, benzo(*a*)pyrene.

were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 units/ml of penicillin, and 100 units/ml of streptomycin. Cells were incubated at 37°C in humidified incubator with 5% CO_2.

Ionizing Procedure. All cell lines were seeded at the same density in all experiments at 1000 cells per 25-cm^2 culture flask and incubated with 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. One day later, cells were exposed to radiation doses of 0, 2, 4, 6, 8, 10, or 12 Gy. Irradiation was carried out at room temperature (20°C) using a ^{60}Co source at a dose-rate of 1 Gy/min. Mediums were changed every 2 days. After about 7 days in culture, cells were fixed and stained with 0.2% methylene blue. Only colonies of more than 50 cells, as evidenced with a low magnification microscope, were counted. Survival curves were established as the percentage of clones appearing after γ-irradiation compared to mock-irradiated cells. Each cell line was assayed several times, as indicated in the text; in each independent experiment, the mean for three flasks was calculated with its SD, and the mean of the individual survival curves was determined.

Results and Discussion

Many authors have recently developed different models for assessing the role of *p53* gene alterations in tumorigenesis. In our approach, we chose to work with a parental cell line derived from embryonic rat lung explants treated with the potent chemical carcinogen B(*a*)P. After removal of the carcinogen, the cells were dispersed and expanded into the BP cell line. For up to 5 passages, these immortalized cells were not tumorigenic and only acquired a tumor-forming potential at later passages. One object of this work was to study four different epithelial cell lines (BP, BPwt, HE, and BP-T) originating from the same syngeneic parental population but representing different stages of neoplastic progression. These cell lines were carefully analyzed for their transformed characteristics, especially with regard to their *p53* gene status. It is noteworthy that all of the cell lines which carried a mutant *p53* gene *i.e.,* the HE clones and the BP-T population derived from the tumor formed on injection of BP cells into syngeneic rats, exhibited the same AAG→AGG mutation at codon 130 on both alleles (12). The percentage of cells bearing this mutation in the BP population increased with the number of passages of cells in culture and was directly correlated with the emergence of an increasing number of foci and increasing cell tumorigenicity. Clones were isolated from several foci and analyzed for *p53* status, tumorigenicity, and DNA content. BPwt clones displayed a normal genotype for *p53* status ($p53^{wt/wt}$), whereas HE clones exhibited a homozygous mutation ($p53^{mutant}$). The latter clones expressed high levels of p53 protein as evidenced by immunoprecipitation assay of [^{35}S]methionine-labeled p53 protein; futhermore, in these clones, the DNA content shifted towards a bimodal pattern of triploid and tetraploid chromosome numbers with complete loss of diploid cells. This result was expected, because several reports had shown that the loss of the wild-type genotype correlated with genetic instability, compared with the stability of counterpart populations that did not lose their wild-type *p53* alleles (6). A dramatic enhancement of the tumorigenic potential was observed in syngeneic rats with the HE clones. A large degree of similarity was observed between the BP-T cells and HE clones. In particular, BP-T cells carried the AAG→AGG transition at codon 130 of the *p53* gene. We also noted that the HE and BP-T cells retained various features of their epithelial origin, such as a marked cobblestone shape and the expression of cytokeratins (12).

In accordance with the hypothesis that p53 protein might act as a guardian of genomic integrity by controlling DNA repair processes, we carried out independent irradiation experiments to compare the radiosensitivity of these different cell lines. We began by focusing our attention on the parental BP cell line and the HE1 clone. As shown in Fig. 1, we observed that for radiation doses as low as 4 Gy, the survival of HE1 cells dropped significantly more than that of their

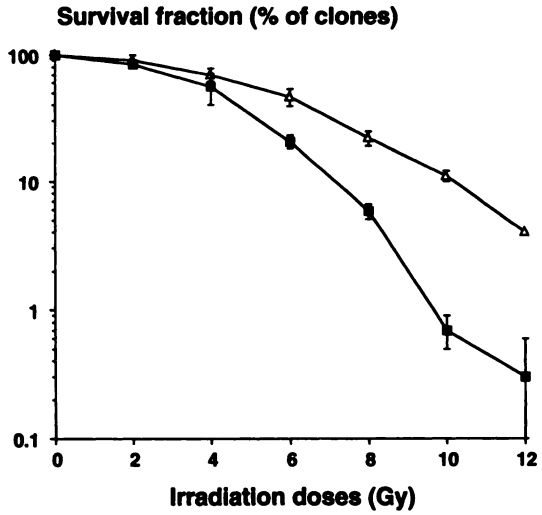

Fig. 1. Effect of γ-irradiation on the survival of B(*a*)P-treated rat epithelial lung cells carrying wild-type *p53 versus* the survival of cells carrying mutant *p53*. The parental BP cell line (passage 15 with 99% of wild-type *p53* alleles) and the HE1 clone (passage 65; 100% of mutant *p53* alleles) were seeded at 1000 cells per 25-cm^2 culture flask. Twenty-four h later, cells were irradiated with doses ranging from 0 to 12 Gy from a ^{60}Co source (with a dose-rate of 1 Gy/min). About 1 week later, clones were fixed and stained. *Points,* means of three culture flasks; *bars,* ± SD. △, progenitor BP (p15) cell line; ■, HE1 clone (p65).

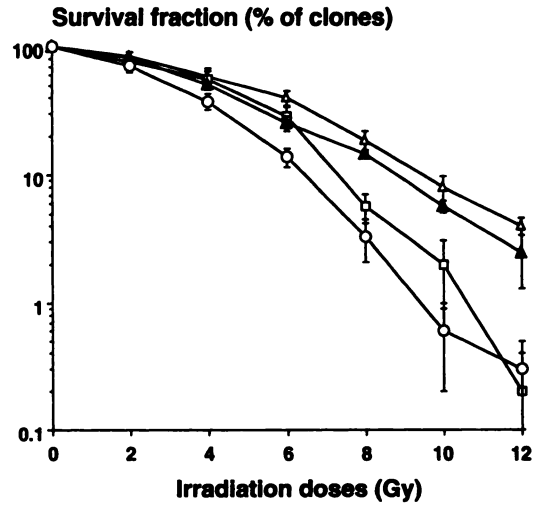

Fig. 2. Survival of BP cell lines. Summed results of several independent experiments. The following cell lines were irradiated as described in "Materials and Methods": the parental BP cell population at passages 15, 16, 18, and 20, which corresponded to between 99 and 93% of wild-type *p53* alleles; the BPwt1 clone at passages 28 and 30, 100% of wild-type *p53* alleles; the HE1 clone at passages 65, 65, 66, and 68, 100% of mutant *p53* alleles; and the BP-T population at passages 19 and 21 after tumor isolation, 100% of mutant *p53* alleles. For each experiment, three culture flasks were cocultivated and the means were determined; *bars,* SD. The different assays were carried out at different times with different frozen pools of cells and different culture periods. For this compilation curve, the mean value for each cell line ± SD was calculated. △, BP (4X); ▲, BPwt1 (2X); □, HE1 (4X); ○, BP-T (2X).

counterparts exhibiting the wild-type *p53* genotype (*i.e.,* BP population at a passage in which more than 93% of the cells displayed a $p53^{wt/wt}$ phenotype). We then extended this experiment to other cell lines exhibiting either a wild-type or mutant *p53* genotype, such as the BPwt 1 clone and the tumor-derived BP-T cell line. Thus, the BPwt 1 and BP-T populations were irradiated in two completely independent experiments (Fig. 2). Next, the HE1 clone and parental BP population were similarly irradiated in four independent assays. In each of the above experiments, three culture flasks per dose were counted. When all of the results were summed, we observed that, at doses exceeding

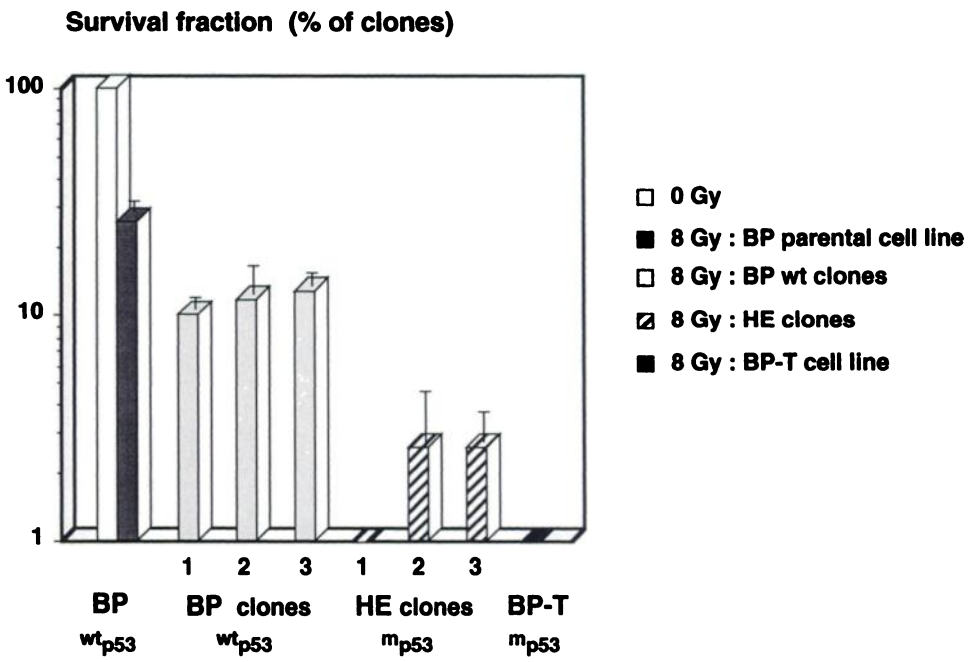

Fig. 3. Survival of BP cell lines and comparison of different clones. In another separate experiment, the following cell lines were irradiated as described in "Materials and Methods": three BPwt clones at passages 24, 25, and 25, respectively (wild-type *p53*), the early passage parental BP cell line at passage 19 (wild-type *p53*), three HE clones at passages 33, 43, and 40 (mutant *p53*), and the BP-T population at passage 22 after tumor isolation (mutant *p53*). For each experiment, three culture flasks were cocultivated and the mean value for the three culture flasks was calculated; *bars*, SD. All values were normalized to the relative cell survival of the corresponding mock-irradiated controls (0 Gy, BP cell line, wt *p53*), which represented 100% of cell survival.

6 Gy, sensitivity to γ rays was significantly more enhanced in the HE1 and BP-T cells, which carried a mutant *p53* gene, than in the BPwt1 and BP cells, which, at the early passages 19 and 21, exhibited the wild-type *p53* genotype (Fig. 2). Furthermore, it was striking to observe a significant increase in the radiosensitivity to γ-irradiation of the tumor-derived cell line BP-T for doses as low as 2 Gy. These results were highly reproducible in all experiments performed under the same conditions. In the next experiment, a panel of different BP clones carrying either the wild-type *p53* genotype (BPwt 2, BPwt 3, and BPwt 4) or a mutant *p53* gene (HE2 and HE3) were irradiated at the same time as the BP (p19), HE1, and BP-T (P22) cell lines. As expected, we observed a high incidence of irradiation-induced cell death in the cells which did not have the wild-type *p53* genotype (Fig. 3). This was particularly striking when we compared the clonogenic survival of BP (p19) cells after irradiation with 8 Gy with the survival of BP-T (P22) cells (25.6 ± 2.7% *versus* 0.6 ± 0.6%). We could not rule out the possibility that genomic instability might affect radiosensitivity, especially in the HE and BP-T cell lines carrying mutated *p53* alleles. In fact, however, after testing the HE1 cells at different passages ranging from 43 to 68, we observed no significant variations in radiosensitivity as a function of the number of passages (Figs. 1–3).

A considerable number of recent findings suggest that p53 protein-triggered apoptosis might be a critical event in the response to radiation or to DNA-damaging agents, especially in cells of hematopoietic lineage (11, 13–16). This response was usually correlated with very high expression or stabilization of the p53 protein. Thus, Slichenmeyer *et al.* (13), who recently studied the radiosensitivity of fibroblasts arising from mice in which zero, one, or two *p53* alleles had been disrupted, observed a striking difference between two independent clones, each of them carrying two disrupted *p53* alleles; with one clone, these authors noted a significant decrease in clonogenic survival at radiation doses of 1 to 8 Gy (13). However, no mention of this observation was made in the body of their paper, and these authors concentrated their attention on the second clone tested, which was more resistant. This difference in survival revealed a heterogeneous response by these fibroblasts to ionizing radiation, which may have been due to the putative genomic instability of these cell lines. In a similar approach using p53-deficient mice, Harvey *et al.* (14) observed that mice lacking one or two wild-type *p53* alleles are highly susceptible to tumor formation, either spontaneously or after daily treatment with low doses of dimethylnitrosamine for a long period. In contrast, mice carrying two wild-type *p53* alleles were very resistant to tumor development. It is noteworthy that almost all of the homozygous $p53^{-/-}$ mice tested by these authors developed malignant lymphomas, whereas most of the heterozygous $p53^{+/-}$ mice developed soft tissue sarcomas and osteosarcomas. This result might be due to the two alternative responses of the cells to DNA damage, *i.e.*, either DNA repair or apoptosis, the choice of which pathway to follow being dependent on cell type specificity.

In a recent paper, Brachman *et al.* (16) found no correlation between *p53* status (wild-type *versus* mutant) and radioresistance in a variety of head and neck cancer cell lines irradiated with a single dose of 2 Gy. When we used this dose here, we observed no significant difference between the various cell lines tested, except for the tumor-derived cell line, but we did find a significant decrease in cell survival at doses higher than 6 Gy in cells carrying a mutant *p53* gene. This suggests that, in our system of rat lung epithelial cells representing various stages of neoplastic progression, the p53 protein plays a key role in resistance to radiation. However, we cannot rule out the possibility that other events in neoplastic progression, closely associated with the loss of p53 function, could be involved in the decrease in radioresistance.

The data reported here show that, in this experimental system, the loss of both wild-type *p53* alleles is accompanied by increased sensitivity to γ-irradiation. At first sight, this observation seems to be at variance with previous reports that mouse hematopoietic cells, which have structurally or functionally lost both normal *p53* alleles, were relatively radioresistant. On the other hand, in a study of sister lines of normal embryonic fibroblasts from mice in which zero, one, or two *p53* alleles had been disrupted, it was demonstrated that *p53* status did not directly affect the sensitivity of these cells to the lethal effects of ionizing radiation. Although we cannot rule out the possibility that a phenotypic or genotypic change accompanying the loss of both wild-type *p53* alleles might help to enhance radiosensitivity, the simplest assumption is that the modulatory effect on radioresistance due to the loss of both wild-type *p53* alleles is associated with other important parameters such as the organ, species, model system, and environmental factors. This point is illustrated in a study by Takahashi and Suzuki (17) who demonstrated that insulin-like growth factor I abol-

ished the inhibition of growth induced by serum starvation of the human MCF-7 breast cancer cell line. In addition, this cell line was also responsive to TGF-β1, which inhibited the phosphorylation of the p53 protein and stimulated its cell cycle suppressive activities (18). This raises the question of whether other environmental agents or cell-cell interaction, which both trigger either the protein kinase C cascade or the tyrosine kinase pathway, are also involved in the p53 response to radiation and affect the radiosensitivity of target cells. Furthermore, it is not unreasonable to assume that, in tumor cells containing a *p53* mutation, the nature and site of the mutation are also involved in the cell response to irradiation, since different mutations are known to alter different properties of the p53 protein, such as its DNA binding or transactivating activities (19, 20).

In conclusion, our results, taken together with the reported data by others, suggest that the p53 protein plays a complex role in the mechanism modulating radioresistance. It is now important to elucidate how wild-type p53 monitors the balance between induced DNA repair and apoptosis and to define the effects of species/organ/cell specificity and environmental factors in the response of the cell to irradiation.

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