Original Paper

Journal of Biomedical Science

J Biomed Sci 1999;6:285–292

Received: December 18, 1998
Accepted: March 15, 1999

The Tumor Suppressor p53 Can Reduce Stable Transfection in the Presence of Irradiation

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Key Words

Tumor suppressor p53 · Radiation · Plasmid integration · Transfection, stable · Recombination · Genetic instability

Abstract

The tumor suppressor p53 is believed to play an essential role in maintaining genome stability. Although it is currently unknown how p53 is involved in this important biological safeguard, several previous publications indicate that p53 can help to maintain genome integrity through the recombination-mediated DNA repair process. The integration of linearized plasmid DNA into the host chromosome utilizes the same repair process, and the frequency can be measured by clonogenic assays in which cells that were stably transfected by plasmid integration can be scored by their colony-forming abilities. To gain insight into whether p53 has a direct role in plasmid integration into the host chromosome, we determined the frequency of stable transfection with CHO cells expressing either wild-type or mutant p53 in the presence and absence of irradiation. We found that low-dose irradiation (~50 to 100 cGy) increased stable transfection frequencies in CHO cells regardless of their p53 status. However, the increase of transfection frequency was significantly lower in CHO cells expressing wild-type p53. Our data thus suggest that wild-type p53 can suppress plasmid DNA integration into the host genome. This p53 function may play a direct and significant role in maintaining genome stability.

Introduction

Mammalian cells rapidly respond to genotoxic agents such as ionizing radiation by inducing a multitude of signal transduction pathways to repair the damage [15]. This cellular response may result in enhanced survival for the damaged cells. Ironically, however, transformation potentials may also be elevated by the increased survival [4]. This elevated transformation potential is, at least in part, due to inappropriate repair after irradiation, which may eventually lead to the development of genetic abnormalities such as gene amplification [9, 24, 30, 31, 36]. Although the underlying mechanism for this is poorly understood, irradiation may provide an environment conducive to an increase in illegitimate recombinational repairs by generating single- and double-strand breaks [2, 9, 23, 27].

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The tumor suppressor p53 can recognize damaged DNA, bind to single-stranded DNA ends, and catalyze DNA renaturation and strand transfer reactions, in addition to its well-known late G1 checkpoint function and induction of apoptotic pathways [1, 5, 8, 17]. Recently, it has also been shown that p53 possesses exonuclease activity [28]. These observations together suggest that p53 is directly involved in aiding fidelity during the repair process. Consistent with this hypothesis, Xia and Liber [39] and others [3, 25, 37] have reported that p53 can reduce mutation rates. In line with this observation, it was found that inactivation of p53 by viral oncogenes or by a dominant negative mutant significantly increased homologous recombination [3, 25]. Furthermore, p53, in association with RAD51/RecA, can directly participate in the recombination process by binding to and cleaving the Holliday junctions [16, 34].

Since radiation-mediated DNA damage can result in increases of wild-type p53 protein levels [11, 12, 21], the function of p53 in damage repair may be more pronounced in irradiated cells. Using plasmids and different DNA-damaging agents, Mallya and Sikpi [22] found that the rejoining capacity of double-strand breaks differed both qualitatively and quantitatively in irradiated p53 wild-type and p53 mutant hosts. Based on their observations, these authors suggested that p53 can modulate excision repair fidelity and the double-strand rejoining process in irradiated cells. Their hypothesis predicts that wild-type p53 can reduce plasmid integration into the host chromosome after irradiation. We tested this hypothesis by examining transfection frequencies in hamster cells expressing either wild-type or mutant p53. We found that low dose irradiation significantly increased transfection frequencies in the cells with mutant p53. Irradiation also increased transfection frequencies in the cells expressing wild-type p53; however, the increase was much smaller than in cells with mutant p53. Since the plasmids used in this study have little sequence homology with host chromosomal DNA, p53 may be able to suppress certain types of illegitimate recombination that do not require extensive homology.

Materials and Methods

Cell Culture and Generation of the CHO K1 Cell Line Expressing Wild-Type p53

The Chinese hamster ovary cell line, CHO K1, and the Chinese hamster embryo fibroblast cell line, CHEF 18-1, were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (HyClone) in 5% CO_2 and 95% air. To generate a CHO K1 cell line expressing wild-type p53, a 1.8-kb *Bam*HI/*Eco*RI cDNA fragment containing the entire coding region of wild-type Chinese hamster p53 [14] was cloned into pCDNA3 (Invitrogen), which contains a neomycin-resistant gene under control of the SV40 promoter. The resultant plasmid, pCDNA-Chp53, was then linearized at the single *Sca*I site and transfected into CHO K1 cells by electroporation (see below) to generate a CHO K1 cell line expressing wild-type p53 (CHO-p53).

Fluorescence-Activated Cell Sorting Protocol

To prepare cells for fluorescence-activated cell sorting (FACS) analysis, cells were washed once with cold phosphate-buffered saline (PBS; 137 m*M* NaCl, 5.4 m*M* KCl, 1.1 m*M* KH_2PO_4, 1.1 m*M* Na_2HPO_4, pH 7.2) and were then trypsinized and washed once again in cold PBS. The pellet was resuspended in 1.5 ml cold PBS, and centrifuged for 5 s at full speed in an Eppendorf microcentrifuge. The cell pellet was resuspended in DNA staining solution [0.1% (wt/vol) sodium citrate, 0.3% (vol/vol) Nonidet-40, 100 μg/ml RNAse A, 100 μg/ml propidium iodide] which was used immediately or stored at 4°C up to 7 days.

Irradiation and Electroporation Protocols

Radiation treatments were performed with a GammaCell 40 (energy source, Cs-137; Atomic Energy Canada) at 100 cGy/min. Cells were irradiated either in 1.5 ml tubes or 4-well cluster dishes (Nunc) which were placed on ice to inhibit repair during the irradiation procedure. Electroporation was performed with PG200 Progenitor II (Hoefer) as follows. Cells were trypsinized and washed with MEM containing 10% fetal bovine serum. The cell pellet was washed again in serum-free MEM, and resuspended in cold serum-free medium at a density of 1×10^7 cells/ml. Three micrograms of linearized plasmid were added to the cell preparation (5×10^6 cells in 0.5 ml) and incubated for 5 min on ice before electroporation. The plasmids used were either pCDNA3 or pRep10 (Invitrogen) and linearized at the unique *Sca*I or *Sac*I sites, respectively. pRep10 contains a hygromycin B-resistant gene driven by the HSV thymidine kinase promoter and a truncated EBNA-1 gene. Digesting pRep10 with *Sac*I completely inactivates the remaining EBNA-1 gene, since *Sac*I cleaves within the EBNA-1 gene. All the electroporations were done at the capacitance of 980 μF and 600 V/cm, which resulted in approximately 50% survival rates for both CHO and CHEF 18-1 cells. After electroporation, cells were further incubated for 10 min on ice and then plated on 10-cm dishes containing complete MEM.

Transfection and Cell Survival Assays

Stably transfected cells were selected by growing them for 7–10 days in complete MEM containing 500 μg/ml G418 (for pCDNA3 transfection) or 280 μg/ml hygromycin (for pRep10 transfection) starting 2 days after electroporation. These drug concentrations and the period of treatment were the minimum required to kill 100% of nontransfected CHO and CHEF 18-1 cells. The colonies were then stained with carbol-fuchsin (Sigma) as follows. Briefly, cells were washed once with cold PBS before fixing in 10% formaldehyde for 10 min. Cells were then stained with 0.25% carbol-fuchsin for 30 min. The dish containing cell colonies was rinsed with tap water and air-dried. Only those colonies containing 50 or more cells per colony were counted. To determine survival rates, cells were grown in nonselective medium for 5–7 days after irradiation or electroporation, stained, and colonies counted.

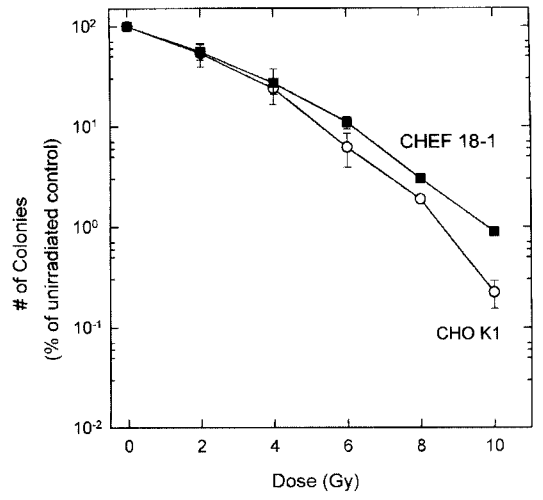

Fig. 1. Radiation survival curves for CHO K1 and CHEF 18-1 cells. Asynchronous cells were irradiated at various doses and surviving cells were scored by colony-forming ability as described in Materials and Methods. The data represent at least three different experiments.

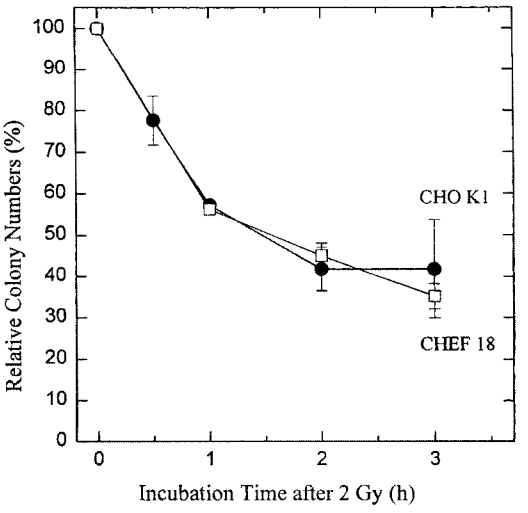

Fig. 2. Incubation of irradiated cells in normal culture conditions prior to transfection reduced transfection-frequency. CHO K1 and CHEF 18-1 cells were irradiated at 2.0 Gy and incubated for various times in a cell culture incubator. The cells were then transfected with linearized pCDNA3 by electroporation. Subsequently, the transfection efficiency for each cell line was determined by scoring colony numbers as described in Materials and Methods. The data represent three independent experiments.

Table 1. Number of G418-resistant colonies at various irradiation doses (number of colonies/5 × 10^6 cells)

	Dose, cGy					
	0	10	20	50	100	200
CHO K1	130±2	328±35	469±70	591±67	589±10	665±16
CHEF 18-1	35±3	101±10	126±17	167±29	176±27	157±6

Results

Transfection Frequencies of Chinese Hamster CHO K1 and CHEF 18-1 Cells

We have previously found that p53 in CHO K1 cells contained a missense mutation at codon 211 (Thr^{211} to Lys^{211}), which could abrogate the ability of cells to arrest in G1 [14]. Presumably, the p53 missense mutation in CHO cells also allows the cells to amplify drug-resistant genes such as dihydrofolate reductase [26] and adenylate deaminase [7]. The CHEF 18-1 cell line, on the other hand, was found to retain wild-type p53 [14], which may explain the lack of gene amplification in this cell line [32]. Interestingly, CHEF 18-1 cells were stably transfected at much lower frequencies than CHO cells when these two cell lines were subjected to transfection with linearized pCDNA3 plasmids (table 1). Since the difference in transfection frequency could be the result of the different survival rates after irradiation, we determined long-term survival rates of the two cell lines after radiation challenges. As shown in figure 1, the survival rates for both CHO and CHEF 18-1 cells as determined by clonogenic assays were

essentially the same at radiation doses up to 2.0 Gy. Therefore, higher transfection frequencies shown by CHO with or without radiation (table 1) were not likely due to the different radiation sensitivity of the two cell lines.

Incubation after Irradiation Reduced the Transfection Frequencies for Both CHO K1 and CHEF 18-1 Cells

Since radiation increased transfection frequencies in a dose-dependent manner in both CHO K1 and CHEF 18-1 (e.g., at 2.0 Gy, 5.1- and 4.5-fold increased, respectively), we hypothesized that radiation-induced damage repair may affect transfection frequencies. To test this hypothesis, we incubated the irradiated cells (2.0 Gy) for various periods at 37°C prior to transfection. Samples were then mixed with plasmid DNA and transfected at 0.5, 1.0, 2.0, and 3.0 h postirradiation. As shown in figure 2, the stable transfection frequencies (presumably by integration of plasmid DNA into the host chromosome) rapidly decreased when cells were incubated at 37°C and reached 55% of the control levels after 1-hour incubation following irradiation. This time-dependent decrease in transfection frequency is similar to the time required for DNA repair, as it was previously shown that most DNA damage was repaired within 1–2 h following irradiation [19, 29]. This data establishes the correlation between radiation-induced damage/repair and the increase/decrease of transfection frequencies.

Generation of a CHO Cell Line Expressing Wild-Type p53

Since the p53 status is, among others, one obvious difference between the two Chinese hamster cell lines (CHO K1 and CHEF 18-1), we postulated that wild-type p53 might be responsible, at least in part, for the lower transfection frequencies in CHEF 18-1 cells. To accurately determine the role of p53 in stable transfection in Chinese hamster cells, therefore, we generated a CHO cell line expressing wild-type p53 by transfecting CHO K1 cells with wild-type p53 cDNA (pCDNA3-Chp53 [14]). To identify the cells expressing functional p53, we examined the radiation-mediated late G1 checkpoint in several G418-resistant colonies by FACS analysis. As shown in figure 3, the CHO-p53 cell line operated a G1 checkpoint in response to irradiation (panels M and P), while parental CHO cells (panels O and R) and cells containing vector alone (CHO-pCDNA3; panels N and Q) did not. Since the operation of the G1 checkpoint is known to be the hallmark of wild-type p53, we concluded that the CHO-p53 cells contained and expressed Chinese hamster wild-type p53.

Since p53 is also an important modulator of apoptosis [6, 21], wild-type p53 in CHO cells may activate the p53-mediated apoptotic pathway in response to irradiation. If this occurs, the results of subsequent transfection experiments in the presence of irradiation would be difficult to interpret. Therefore, we examined the radiation-mediated cell killing effects in CHO-p53 cells and the parental CHO cells. As shown in figure 4, the survival rates of cells containing wild-type p53 (p53WT) and mutant p53 (CHO or vector) showed little difference up to 2.0 Gy, although the CHO-p53 cell line showed lower survival rates at higher radiation doses. We found that a subpopulation of CHO-p53 cells died by apoptosis at doses higher than 4.0 Gy. However, we have not observed apoptotic cell death at doses lower than 2.0 Gy in either CHO or CHO-p53 cells.

Wild-Type p53 Significantly Reduced Radiation-Induced Transfection in CHO Cells

To gain a better understanding about whether p53 is involved in the regulation of transfection in CHO cells, we compared the long-term transfection frequencies by clonogenic assay between parental CHO cells and CHO-p53 cells with or without radiation challenges. Since CHO-p53 cells were already neomycin-resistant, linearized pRep10 (Invitrogen), which contained a hygromycin-resistant gene, was used to transfect CHO and the CHO-p53 cells. Because a subpopulation of CHO-p53 cells underwent apoptosis at higher radiation doses but not at low doses, we examined transfection enhancement ratios at doses only up to 100 cGy. Surviving cell colonies were selected in MEM containing 280 μg/ml hygromycin as described in Materials and Methods. As shown in figure 5, the transfection frequencies for CHO-p53 and parental CHO cells were similar in the absence of irradiation (0 Gy in fig. 5). Lower levels of radiation (i.e., 50–100 cGy) increased the transfection frequencies for both the Chinese hamster cell lines; however, the increase in colony-forming frequency was much lower in the cells expressing wild-type p53. For example, the transfection frequencies increased ~2.9-fold and ~7.3-fold at 100 cGy for the CHO-p53 cell line and the parental CHO cell line, respectively. Thus, the CHO-p53 cell line showed at least 2.5-fold lower long-term transfection frequencies compared to the parental CHO cell line at 100 cGy (fig. 5).

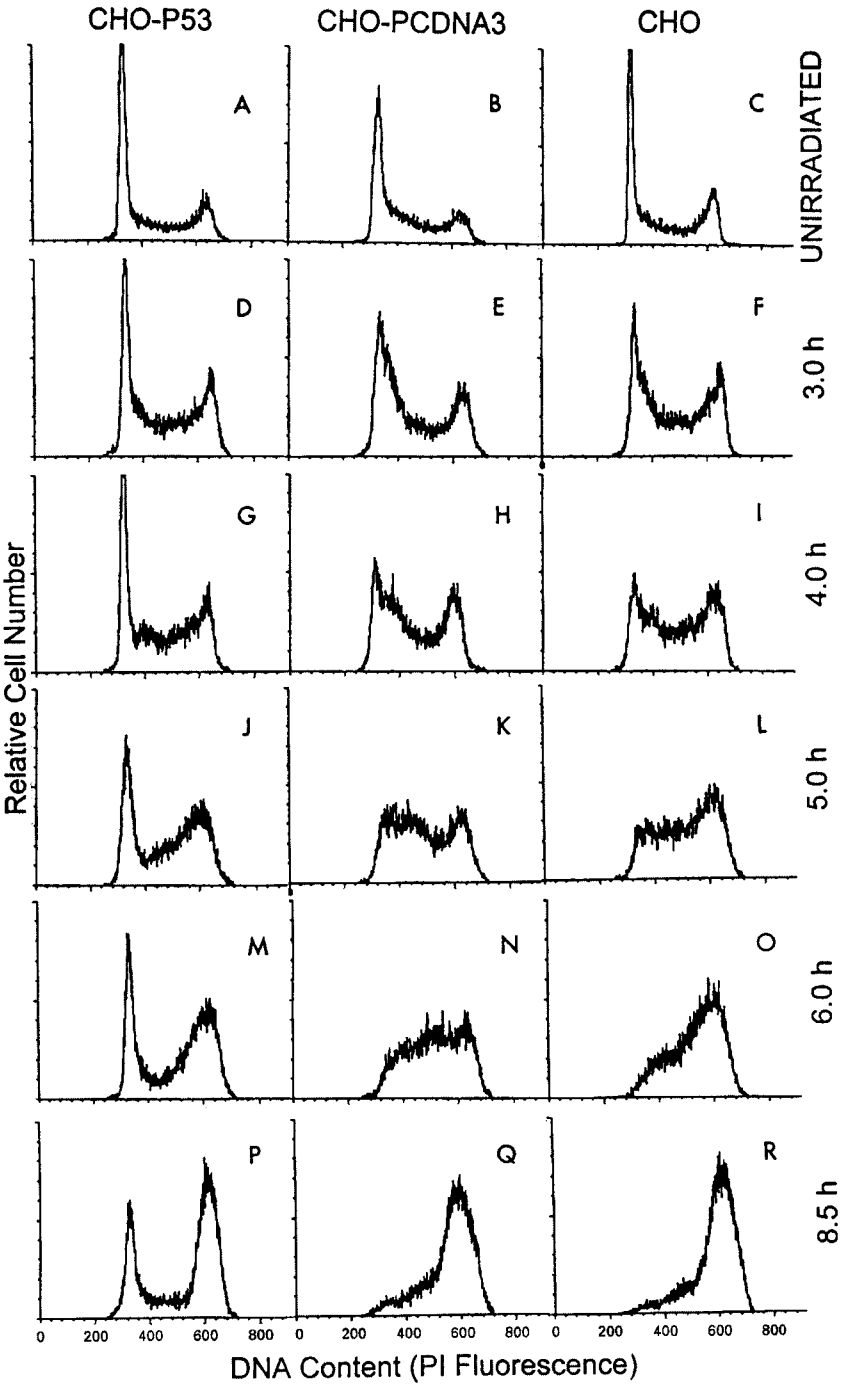

Fig. 3. Expression of wild-type p53 restored the G1 checkpoint in CHO cells. Cell cycle progression of CHO-p53 cells (CHO cells expressing wild-type p53), CHO-pCDNA3 cells (CHO cells containing vector alone), and parental CHO cells was investigated after asynchronously growing cells were irradiated with 10 Gy. Each cell line is indicated at the top of the panels. Panels A, B, and C are unirradiated controls of cell lines, CHO-p53, CHO-pCDNA3, and CHO, respectively. The times indicated at the right of the panels are the times sampled after irradiation. The G1 arrest of CHO-p53 is obvious by 6 h after irradiation (compare panels M, N, and O).

Discussion

We report here that radiation-mediated increases of the long-term transfection frequencies are much lower in CHO cells expressing wild-type p53 than mutant p53. Considering the fact that all three cell lines (i.e., parental CHO, and cells with wild-type p53 or vector alone) showed essentially the same survival rate up to 2.0 Gy (fig. 4), the lower long-term transfection frequencies observed with pCDNA-p53 cells at radiation doses up to

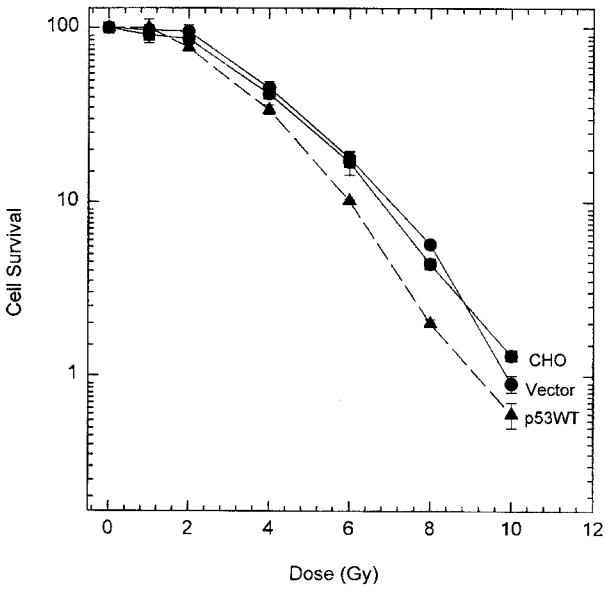

Fig. 4. Radiation survival curves for p53-positive and p53-negative CHO cells. Experiments were done as per figure 1 legend and Materials and Methods. Note that cell survival rates are essentially the same among the three cell lines at least up to 2.0 Gy, although p53-positive CHO cells are more radiosensitive at higher doses. CHO, vector, and p53WT denote parental CHO, CHO cells containing pCDNA3 vector, and CHO cells expressing wild-type p53, respectively.

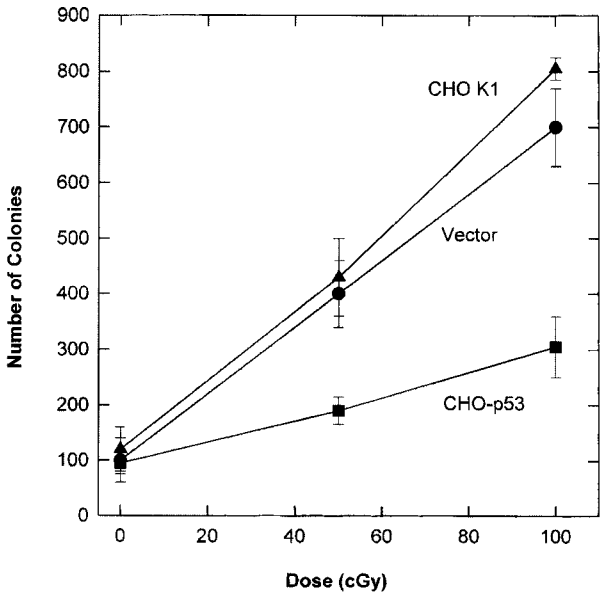

Fig. 5. Radiation-mediated transfection enhancement of CHO parental cells (CHO) and CHO-expressing wild-type p53. Transfection by electroporation was carried out immediately after irradiation at the indicated doses. Scoring colony numbers was done as described in Materials and Methods. Vector denotes the CHO K1 cell line containing vector sequence only.

1.0 Gy were not caused by differences in radiosensitivity of the three cell lines. Since CHO and CHO-p53 cells are genetically identical except for their p53 status, wild-type p53 can apparently suppress the long-term transfection frequencies in the presence of irradiation in Chinese hamster cells. The radiation-mediated transfection increase, which is consistent with the previous observation [33], is likely facilitated by DNA damage and/or the repair process since transfection frequencies were substantially reduced by incubating irradiated cells for 30–60 min before transfection (fig. 2). The long-term transfection frequencies should accurately reflect the frequencies of plasmid integration into the host chromosome, since the measurement was done by clonogenic assays in which linearized plasmids were used. No colony could be formed without plasmid integration under these conditions (data not shown).

Why is there a significant decrease in transfection frequencies with irradiated CHO-p53 cells but not with unirradiated samples (fig. 5)? This may be because there is a relatively higher amount of wild-type than mutant p53 protein in irradiated CHO-p53 cells. It is well known that radiation can rapidly increase the protein levels of wild-type p53 [11, 12, 18, 21] but not mutant p53 [10–12]. Since CHO-p53 cells express both wild type (exogenous) and mutant p53 (endogenous), the wild-type p53 functions may be masked by mutant proteins in the absence of irradiation. However, the relative amount of wild-type p53 protein can be substantially higher than mutant p53 protein in irradiated CHO-p53 cells. Therefore, even if some of the wild-type p53 proteins are inactivated by mutant p53 in irradiated CHO-p53 cells (i.e., dominant negative effect), there could be enough wild-type p53 molecules to facilitate normal p53 function. This notion is supported by the fact that CHO-p53 could operate a late G1 checkpoint (fig. 3). Also consistent with this, CHEF 18-1 cells, which express only wild-type p53, showed much lower stable transfection frequencies than CHO cells with or without irradiation (table 1). However, we could not confirm this by Western analysis, since no antibody to detect Chinese hamster p53 is presently available.

How does wild-type p53 reduce integration of plasmid DNA into the host chromosome? p53 may achieve this by directly participating in checking fidelity during the damage repair process [1, 3, 5, 17, 25, 28, 34, 39]. Since p53 is involved in joining strand breaks [22], it is conceivable that this tumor suppressor protein can inhibit incorrect end joining. In fact, it was found by others that p53 could suppress certain recombination processes [3, 16, 25, 34, 37]. This p53 function could be important in maintaining genome integrity in the presence of genotoxic agents such as irradiation. It is perhaps noteworthy that gene amplification, which seldom occurs in the cells with wild-type p53, is initiated by double-strand breaks and incorrect rejoining [20, 35, 40]. Taken together, our observations raise the possibility that p53 may inhibit gene amplification by suppressing undesirable end joining. (Also, note that this p53 function can result in more frequent induction of apoptotic signals due to high numbers of unrepaired residual strand breaks, which can also contribute to genome stability by eliminating cells with high oncogenic potential.) A similar situation may also exist in the process of viral DNA integration into the mammalian host genome. For example, integration of a human papilloma virus (HPV) into the host chromosome coincides with increased oncogenic potential in cervical carcinomas, and the integration process can be facilitated by inactivation of p53 by HPV oncoprotein [13, 38]. Suppressing foreign DNA integration by p53 may be a critical mechanism to maintain genome integrity and thus reduce oncogenic potential.

Acknowledgment

This work was, in part, supported by the Startup Fund from Cancer Care Ontario.

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