Attenuation of G_2 Cell Cycle Checkpoint Control in Human Tumor Cells Is Associated with Increased Frequencies of Unrejoined Chromosome Breaks But Not Increased Cytotoxicity Following Radiation Exposure[1]

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Abstract

The contribution of G_2 cell cycle checkpoint control to ionizing radiation responses was examined in ten human tumor cell lines. Most of the delay in cell cycle progression seen in the first cell cycle following radiation exposure was due to blocks in G_2 and there were large cell line-to-cell line variations in the length of the G_2 block. Longer delays were seen in cell lines that had mutations in *p53*. There was a highly significant inverse correlation between the length of G_2 delay and the frequency of unrejoined chromosome breaks seen as chromosome terminal deletions in mitosis, an observation that supports the hypothesis that the signal for G_2 delay in mammalian cells is an unrejoined chromosome break. There was also an inverse correlation between the length of G_2 delay and the level of chromosome aneuploidy in each cell line, suggesting that the G_2 and mitotic spindle checkpoints may be linked to each other. Attenuation in G_2 checkpoint control was not associated with alterations in either the frequency of induced chromosome rearrangements or cell survival following radiation exposure suggesting that chromosome rearrangements, the major radiation-induced lethal lesion in tumor cells, form before cells enters G_2. Thus, agents that act solely to overide G_2 arrest should produce little radiosensitization in human tumor cells.

Introduction

Cell cycle progression in mammalian cells is a tightly regulated process (1,2). Progression from one phase of the cell cycle to the next is dependent on the successful completion of the previous cell cycle event. Exposure to DNA damaging agents will also induce delays in progression. These cell cycle checkpoints are thought to act by preventing cyclin-dependent kinase phase transitions, and delaying cell cycle progression until the previous cell cycle event is completed or until DNA damage can be repaired (2-4). In yeast and mammalian cells, the presence of unrejoined chromosome breaks signal for G_2 delay, and most cells remain blocked in G_2 until the break is rejoined or otherwise modified (5-7). The observation that loss or attenuation in G_2 checkpoint control is often associated with ionizing radiation sensitivity in yeast and mammalian cells (5,8-12) suggests that attenuation in G_2 cell cycle checkpoint control confers sensitivity to the toxic effects of radiation exposure by allowing cells to progress into mitosis with unrejoined breaks.

The radiosensitization associated with G_2 checkpoint attenuation is puzzling, however, because more than 90% of induced DNA double-strand breaks are rejoined within 2 h of exposure in most mammalian cells (7,13), and cytotoxicity is not thought to result from unrejoined breaks, but instead is associated with chromosome ring and dicentric formation, which may develop long before G_2. Thus, one would expect that premature exit from G_2 should yield only a small increase in the number of lethal lesions. In the present study, we re-examined the relationship between attenuation of G_2 cell cycle checkpoint control and ionizing radiation sensitivity in human tumor cells, and determined the effect of attenuation on chromosome mutation induction as well as clonogenic survival. Our results suggest that the attenuation of G_2 checkpoint control does lead

to increased frequencies of mitotic cells with unrejoined breaks, but these unrejoined breaks apparently contribute little to overall radiation sensitivity. We also noted a correlation between the length of radiation-induced G_2 delay and chromosome stability suggesting a link between G_2 checkpoint control and the control of the mitotic spindle checkpoint.

Materials and Methods

Cell Lines and Tissue Culture. Ten human tumor cell lines were studied: eight were squamous cell carcinomas (SCC) and two were B-cell lymphoblastoid cell lines (Table 1). The SCC cell lines were established from tumor biopsy specimens. The methods of establishment and characterizations of the cell lines have been previously described (13). The SCC cell lines were maintained in media consisting of 72.5% Dulbecco's Modification of Eagle's Medium, 22.5% Ham's Nutrient Mixture F-12, 15% fetal bovine serum, 0.4 μg/ml hydrocortisone, 100 units/ml penicillin, and 100 μg/ml streptomycin. For all studies, cells of late (> 50) passages were used. The origins of the human B-lymphoblastoid cell lines TK6 and WI-L2-NS have been described in Amundson *et al.* (14). Both cell lines were derived from the same spleen cell isolate, WI-L2. By cytogenetic and restriction fragment length polymorphism analyses, TK6 and WI-L2-NS are nearly identical. They differ in *p53* status: the *p53* gene is mutated in WI-L2-NS (15). Lymphoblast cell lines were maintained under exponentially growing conditions in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were grown in suspension and cell densities kept between 2–10 $\times 10^5$ cells by subculturing every 3 to 4 days.

Cell Survival Analysis. For cell survival analysis, exponentially growing cultures were

trypsinized from stock cultures and between 500 and 40,000 cells were plated in 100-mm tissue culture dishes and allowed to enter exponential growth. Irradiation was carried out 18 h later with a 250 kV Maxitron operating at 26 mA at a dose rate of 1.07 Gy/min. All the cell lines had doubling times greater than 19 h (16), therefore delaying irradiation for 18 h should not result in any significant increase in multiplicity. This was checked for each cell line. Cells were irradiated at room temperature and then incubated at 37° C for 18–24 days, after which the cells were fixed and stained with crystal violet. Only colonies of more than 50 cells were scored as survivors. Results are the mean of 2–6 experiments. For the lymphoblastoid cell lines, asynchronous populations of cells were irradiated at room temperature with ^{60}Co gamma rays from a Gamma Beam 650 Irradiator (Atomic Energy of Canada) at a dose rate of about 2.5 Gy/min, and immediately after irradiation cells were plated for survival in 96-well microtiter plates at densities of 0.5 to 20 cells per well. Plates were incubated and scored for colony formation two weeks later. Survival levels were determined as previously reported (17). Results are the mean of three determinations. Multi-target radiation survival parameters, D_0 and the extrapolation number (n), were determined by least-squares regression analysis of all the data points.

Cytogenetic Analysis. For the cytogenetic analyses, cells were incubated for up to 48 h following radiation exposure, depending on the growth rate of the cell line, to maximize the scoring of cells that were irradiated in G_1 (17,18). Cells were arrested in metaphase with a 2-h treatment with 2×10^{-7} M colcemid. Cells were harvested, incubated in 0.075 M KCl for 20 min, and fixed in 3:1 methanol:acetic acid. Fixed cell suspensions were dropped onto slides, air-dried, and stained in a 5% Giemsa solution for later cytogenetic analysis. Cells were analyzed for chromosome-type aberrations, i.e., chromosome dicentrics, rings, interstitial deletions, and

terminal deletions, as previously described (7,18). Fifty cells per dose point were analyzed. Cells were exposed to 0-6 Gy x rays and dose response curves determined from two to three independent experiments.

Cell Cycle Analysis. Cell cycle progression after radiation exposure was determined as previously described (7,16,17). Briefly, exponentially growing cultures were exposed to 0-20 Gy gamma rays. At regular intervals following exposure, a sample of cells (10^6 cells) was fixed in 70% ethanol. Fixed samples were stained in 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI), and the fluorescence histogram was accumulated on a Partec PAS II cytometer (Basel, Switzerland). The distribution of cells throughout the cell cycle was calculated using software designed by Phoenix Flow Systems (San Diego, CA). Cell cycle progression was evaluated in at least two experiments per dose. The width of the curves at half-maximal levels of G_2 delay were used as a measure of the length of G_2 delay. As has been reported by us and others (7,10), G_2 delay is dose-dependent over the range we studied. The data were combined to determine a rate of induction of delay and to present the results as the length of delay/Gy of exposure.

Results and Discussion

Ten human tumor cell lines, eight SCC cell lines established from tumor biopsy specimens (13), and two B-cell lymphoblastoid cell lines (14, 15, 17) were studied. Exponentially growing cultures of each cell line were irradiated and cell survival, chromosome mutation frequency, and cell cycle progression determined. Some of these results have been previously reported (13, 16-18). As *p53* has been reported to play an important role in checkpoint control (2), the results were compared to previously reported observations concerning the status of the *p53* gene in these

cell lines (15,19). The results are summarized in Table 1 and figure 1.

The inherent radiation sensitivity was determined from survival curves. Both the D_0 and the extrapolation number (n) are presented in Table 1. The 10 cell lines differed primarily in D_0, which ranged from 0.9 Gy to 3.5 Gy. In the lymphoblastoid cells, the relative radioresistance of WI-L2-NS compared to TK6 probably is due to the mutation in *p53* and the resulting reduction in apoptosis (15,17). Of the eight SCC tumor cell lines studied here, three have normal *p53* expression, while the other five have mutations in *p53* (19). The *p53*- mutant and normal cells did not differ in inherent radiosensitivity. The mean ± s.e.m. D_0 of the *p53*-normal SCC cell lines is 1.95 ± 0.25 Gy; that of the *p53*-mutant cell lines is 2.11 ± 0.27 Gy.

Exponentially-growing cells were exposed to 0–20 Gy gamma rays and the effects on cell cycle progression determined by flow cytometry. Most of the delay in cell cycle progression was due to blocks in G_2. The width of the curves at half-maximal levels of G_2 delay were used as a measure of the length of G_2 delay. As has been reported by us and others (7,10), G_2 delay is dose-dependent over the range we studied. The data were combined to determine a rate of induction of delay and to present the results as the length of delay/Gy of exposure. The range of G_2 delay following radiation exposure was 0.8–3.0 h/Gy (Table 1). There was no relation between the length of the G_2 delay and overall cell doubling time, and no significant relationship between radiation-induced G_2 delay and inherent radiation sensitivity in these human tumor cell lines (Table 1).

The G_2 delay was longer in *p53*-normal cells when both SCC and lymphoblastoid cell lines were considered together, but the difference was not significant. The mean ± s.e.m. delay for the *p53*-normal group was 2.3 ± 0.3 h/Gy, while that for the *p53*-mutant cells was 1.6 ± 0.3 h/Gy.

When the SCC cell lines were considered separately, however, the *p53* mutants had a significantly shorter G_2 delay (p=0.03). The mean ± s.e.m. for the *p53*-normal SCC group was 2.3 ± 0.4 h/Gy while that for the *p53*-mutant cells was 1.4 ± 0.2 h/Gy. This may be an artifact due to the absence of a G_1 checkpoint that would lead to a faster entry of damaged cells into G_2. Alternatively, *p53* may play a role in the G_2 checkpoint response in non-lymphoid cells or at least influence the degree of attenuation in G_2 checkpoint control in transformed cells. The role of *p53* in G_2 checkpoint control remains unclear, but some have noted correlations between *p53* mutations and attenuation in G_2 checkpoint control (20).

Unrejoined chromosome breaks are the primary signal for G_2 delay (5-7). Defects in G_2 checkpoint control should lead to increased frequencies of cells with chromosome terminal deletions, the cytogenetic equivalent of unrejoined breaks. Terminal deletions are defined as large acentric chromosome fragments not associated with any obvious chromosome exchange-type aberration (dicentrics, acentric rings). There was a significant relationship ($r^2 = 0.54$, $p = 0.02$) between the length of G_2 delay and terminal deletion frequency in these non-isogenic cell lines (Table 1, Figure 1b). The cells with shorter delays had greater frequencies of terminal deletions as one would predict. All of the cell lines were equally sensitive to break induction, and all but one were proficient in break rejoining ability (13,17), so the differences in terminal deletion frequency were not due to alterations in chromosome break induction or repair. One of the cell lines, SCC-61, is deficient in DNA double-strand break rejoining (unpublished observation). It also falls off the regression line in figure 1b. The reduced capacity to rejoin breaks might lead to higher levels of unrejoined breaks and therefore change the general relationship between G_2 delay and terminal deletion frequency. If one considers only the other eight cell lines, the relationship

between G_2 delay and terminal deletion frequency is much tighter ($r^2 = 0.85$, $p = 0.0007$). Thus, attenuation of G_2 checkpoint control and premature exit from G_2 is the major factor defining chromosome terminal deletion frequency in repair-proficient cell lines.

As mentioned above, there was no significant relation between the length of G_2 delay and inherent radiosensitivity (D_0) and thus there is no significant relationship between terminal deletion sensitivity and radiation sensitivity (D_0). While terminal deletions are lethal lesions, other factors are more important in defining the radiation sensitivity of these cells. For the lymphoid cell lines, apoptosis is the major factor that defines radiation sensitivity (15,17). For the SCC cell lines, it is the formation of asymmetric chromosome rearrangements, i.e., dicentrics, rings, and interstitial deletions, that define radiation sensitivity (18). In the SCC cell lines, there was a significant linear relationship between cytotoxicity and the frequency of induced asymmetric chromosome rearrangements (Figure 1c). The mean lethal dose corresponded to approximately 1.5 induced asymmetric chromosome rearrangements per cell. Alterations in G_2 checkpoint control influenced only terminal deletion frequencies, not chromosome rearrangement frequencies.

In our cytogenetic analyses, we noted that all but one of the tumor cell lines were aneuploid (Table 1). Modal chromosome number ranged from 46 to 82 per cell. Furthermore, there was a suggestion of a relationship between chromosome number and G_2 delay (Figure 1d), which was significant for the *p53*-mutant cell lines ($r^2 = 0.69$, $p = 0.04$). Attenuation of G_2 checkpoint control was associated with increased levels of aneuploidy. This karyotype instability manifested itself primarily as increased numbers of whole sets of chromosomes (euploidy). Increases from diploid to triploid or tetraploid chromosome numbers can result from aberrant chromosome segregation in mitosis, a manifestation of loss or attenuation in the mitotic spindle

checkpoint (21). The correlation between G_2 checkpoint control and chromosome number suggests that the G_2 and mitotic spindle checkpoints may be linked. This linkage could explain why transformation of human fibroblasts with SV40 large T antigen leads to both an attenuation in G_2 checkpoint function (20) and karyotype instability (22).

In conclusion, while the G_2 checkpoint defines the frequency of unrejoined chromosome breaks in mitosis, it plays little direct role in radiation sensitivity because unrejoined chromosome breaks represent only a minor fraction of induced lethal lesions in normal repair-proficient cells. It has been suggested that the G_2 checkpoint would be an appropriate target for cancer therapeutic agents (11,12). Caffeine treatment, which abrogates G_2 checkpoint control, has been shown to be an effective radiosensitizer in some cases (8,11,12). Our studies suggest that attenuation of G_2 checkpoint control only influences chromosome terminal deletion frequency. As terminal deletions make up only a minor fraction of the total number of lethal lesions, it is unlikely that caffeine-mediated radiosensitization is due solely to its effects on G_2 delay. Caffeine has been reported to affect other processes as well including inhibiting repair and enhancing chromosome fragile site expression (23). Perhaps the caffeine radiosensitization is due to its effects on these other processes.

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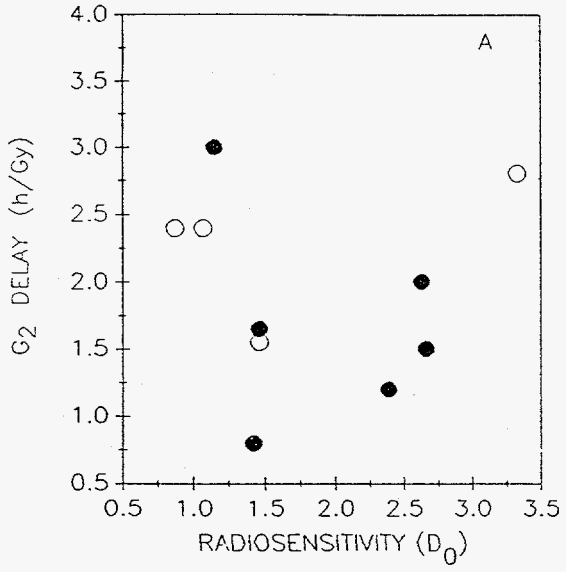
Table 1. Characteristics of Human Tumor Cell Lines[1]

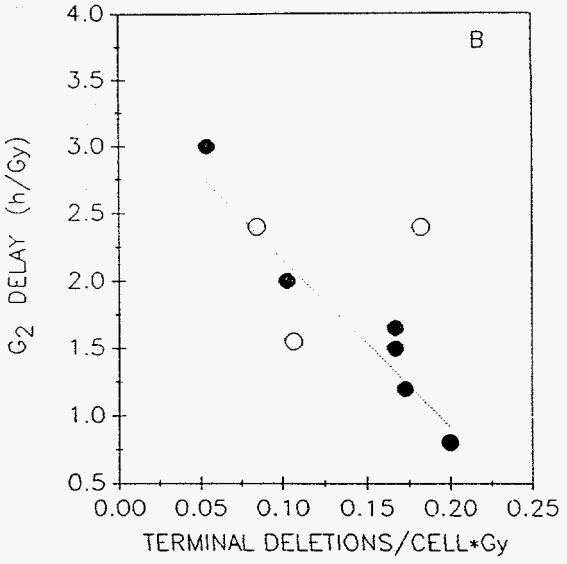
Cell Line	Type	*p53*	D_0 (Gy)	n	CHR/Cell	AGT (h)	G_2 Delay (h/Gy)	TD/cell•Gy
JSQ-3	SCC	M	2.63	1.7	57	19.2	2.0	0.103
SQ-20B	SCC	M	2.39	1.4	68	21.7	1.2	0.173
SCC-12B.2	SCC	M	2.66	2.1	72	27.2	1.5	0.167
SQ-9G	SCC	M	1.46	1.4	52	38.3	1.6	0.167
SCC-25	SCC	M	1.42	1.5	72	24.7	0.8	0.200
SQ-38	SCC	N	1.46	1.8	82	23.2	1.6	0.107
SCC-61	SCC	N	1.07	1.8	46	32.2	2.4	0.183
HNSC-143	SCC	N	3.33	1.2	70	ND	2.8	ND
WI-L2-NS	LYM	M	1.15	1.1	47	18.2	3.0	0.054
TK6	LYM	N	0.87	1.2	47	16.8	2.4	0.085

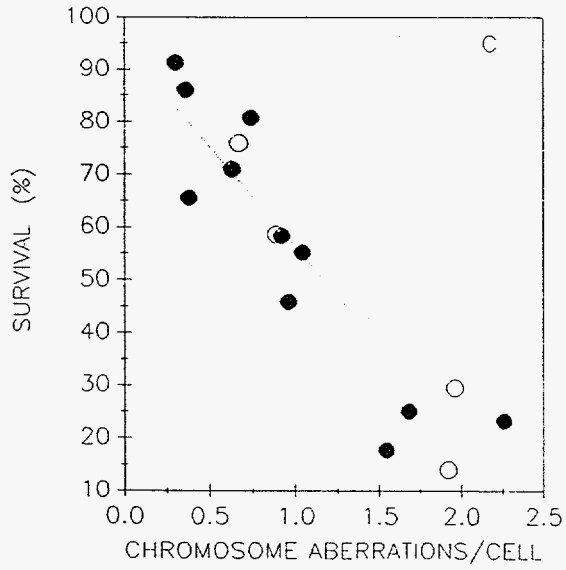
Abbreviations: SCC, squamous cell carcinoma cell lines; LYM, lymphoblastoid cell lines; M, *p53* mutation; N, *p53* normal; TD, terminal deletion; CHR, mean number of chromosomes/cell; AGT, average doubling times; ND, not determined.

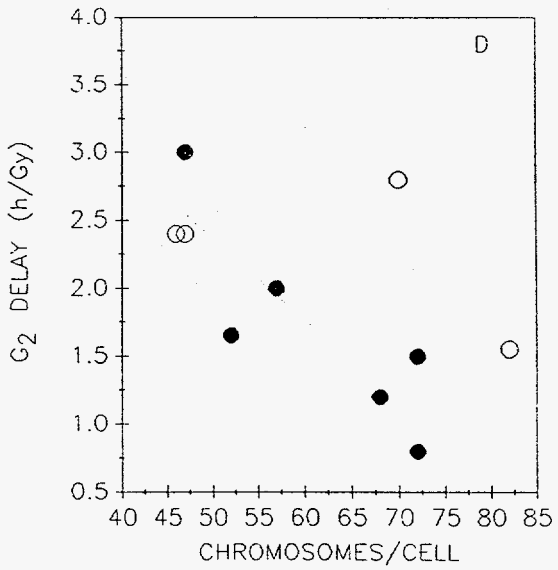
Figure Legends:

Figure 1. Comparisons of (a) radiation-induced G_2 delay and inherent radiosensitivity; (b) G_2 delay and chromosome terminal deletion induction; (c) survival and chromosome aberration induction (dicentrics, rings, and interstitial deletions); and (d) G_2 delay and mean chromosome number per cell in *p53*-mutant (●) and *p53*-normal (○) human tumor cell lines. The dotted lines are the best fit regression lines.


A
G_2 DELAY (h/Gy)
RADIOSENSITIVITY (D_0)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.5 1.0 1.5 2.0 2.5 3.0 3.5


B
G_2 DELAY (h/Gy)
TERMINAL DELETIONS/CELL*Gy
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.00 0.05 0.10 0.15 0.20 0.25


C
SURVIVAL (%)
CHROMOSOME ABERRATIONS/CELL
100
90
80
70
60
50
40
30
20
10
0.0 0.5 1.0 1.5 2.0 2.5


D
G_2 DELAY (h/Gy)
CHROMOSOMES/CELL
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
40 45 50 55 60 65 70 75 80 85