

UCSF

UC San Francisco Electronic Theses and Dissertations

Title

Consequences of cell cycle perturbations in human glioma cells

Permalink

<https://escholarship.org/uc/item/7hr9p94f>

Author

Gupta, N

Publication Date

1996

Peer reviewed|Thesis/dissertation

Consequences of Cell Cycle Perturbations in Human Glioma Cells

by

Nalin Gupta

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

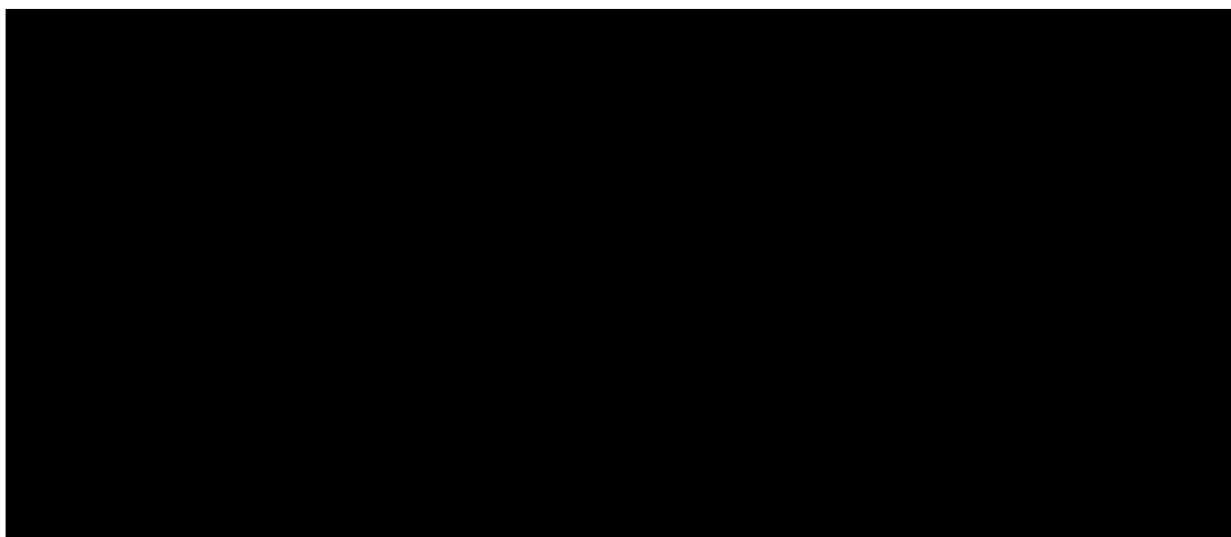
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date

University Librarian

Degree Conferred:

Copyright 1996

by

Nalin Gupta

Preface

ACKNOWLEDGMENTS

The emotions associated with this thesis are a curious mixture of nostalgia, trepidation and optimism. Graduate school is the most recent episode of my formal educational experience, characterized as it is by a certain nomadic flavor. Each new city I moved to has meant leaving behind familiar surroundings. At the same time, starting from scratch and building the structure of day-to-day life has been an immensely enriching experience. In San Francisco, a particular confluence of people and events created an environment where I was able to learn easily, cultivate my strengths, and measure my weaknesses. These years were, I believe, defining ones for me.

The contributions of a very large number of individuals are not explicitly noted elsewhere in this thesis. I would like to mention a few of these people here.

The members of the Division of Neurosurgery at the University of Toronto, in particular Charles Tator, were persistently supportive and indulged me while I pursued, what must have seemed to them, interests far removed from my previous training. Financially, I was supported by a generous fellowship from the Medical Research Council of Canada which gave me the the freedom to pursue my research interests. Jim Rutka, a UCSF alumnus, encouraged me from the outset to come to San Francisco and experience the flavor and color of the west coast. Since those days, Jim has remained a mentor but also has become a close friend.

Probably because of favorable weather, I have had the pleasure of hosting many of my friends from Canada here in San Francisco. Through the years, they have been my

source of cheer and support. In no particular order, they are: Paven, Howard, Rob, Lynn, Anita, Kaz, Sandra, Elisa, Alan, Debbie, Kamal, Hazel, Russ, Shaun, Simon, Katie, and Roger.

The cast of characters at UCSF seems too numerous to mention without omissions but I will try. The Biophysics students of the '92 crop, Sarah, Ted, Bernard, Anton, Javier and Kevin, shepherded me through the shoals of first year and beyond. Julie Ransom, a rock of Gibraltar, has been a quiet oasis of rationality. The lab where I have spent most of the past five years, like all labs, has seen many faces. Those in the beginning, Jolly, Lupe and CZ, created a welcoming environment that made me feel accepted immediately. Since then, Tomoko and Jingli have added to an already wonderful lab. I would like to single out Lily Hu for teaching me all I have learned from her through the years. Her patience and good humor seemed inexhaustible. The members of the BTRC extended family, Theresa, Glenn, Chris, Dolores, Ruth, Shabina, Garret, and Daphne, have tolerated all variety of eccentricities. Laleh Daneshvar, originally a member of the BTRC and more recently a Biophysics graduate student, has been a great friend and teacher. The stalwarts of the Morgan lab; CC, Charlie, Angel, and Mark kept a smile on my face. Other individuals deserve mention for their support and encouragement: Aviva Abosch, Susan Eastwood, Phil Gutin, Michael McDermott, and Diane Ralston

I owe a debt of gratitude to my qualifying committee, Joe Gray, John Fike, Ira Herskowitz, and Bill Morgan, who divined some positive aspects to my research proposal and subsequently passed me! I cannot thank John Murnane and Bill Morgan enough for being on my thesis committee and having time to listen, particularly on Friday afternoons.

I would like to recognize the enormous contribution of my thesis advisor, Dennis Deen. Dennis has been an advocate and an outstanding mentor to me. His interest and curiosity in science, his integrity and fairness, and the respect with which he treats those who work with him are the standards to which I aspire.

Finally, I would like to thank my parents and my sister Mona. There are no words that can do justice for all they have done.

STATEMENT ON CO-AUTHORS AND PUBLISHED MATERIAL

This thesis contains material that is or will be published in the scientific literature.

Chapter 1 (Introduction) contains excerpts from a book chapter, titled *Molecular Effects of Ionizing Radiation*, written by N. Gupta. This chapter will appear in **The Molecular Biology of Neurosurgical Disease** (Eds. C. Raffel and G. Harsh), Williams and Wilkins, Baltimore, 1996. Dennis F. Deen, Ph.D. (Professor, Depts. of Neurological Surgery and Radiation Oncology) and Philip H. Gutin, M.D. (Professor, Dept. of Neurological Surgery) are the senior authors.

Chapter 2 contains the full text of the manuscript, *Cytokinetic Damage and the Radiation-Induced G₁ Phase Checkpoint*, **Radiation Research**, Vol. 145, pp. 289-298, 1996. Experimental design, cell culture, cell purification, clonogenic assay, flow cytometry and manuscript preparation were done by N. Gupta. Rohini Vij (medical student, UCSF) performed the fluorescent *in situ* hybridization and chromosomal scoring as part of a summer research project under the direction of William F. Morgan Ph.D. (Professor, Dept. of Radiation Oncology, and Laboratory of Radiobiology and Environmental Health). Daphne Haas-Kogan M.D. (post-doctoral fellow, Dept. of Neurological Surgery) and Mark A. Israel M.D. (Professor, Dept. of Neurological Surgery and Pediatrics) provided the cell lines used in these studies.

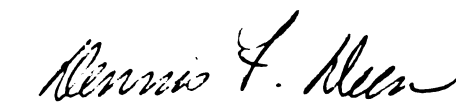
Chapter 3 contains the full text of the manuscript, *Radiation Age Response of Human Glioma Cells Differing in p53 Status*, that was submitted for publication.

Experimental design, clonogenic assays, flow cytometry and manuscript preparation were done by N. Gupta. Assistance with cell culture was provided by Guadalupe Gonzalez, Ph.D. (post-doctoral fellow, Dept. of Neurological Surgery) and Tomoko Ozawa, M.D. (post-doctoral fellow, Dept. of Neurological Surgery).

Chapter 4 contains the full text of the manuscript, *Cytotoxicity and Cell Cycle Effects of Paclitaxel When Used as a Single Agent and in Combination with Ionizing Radiation*, that is in press with the **International Journal of Radiation Oncology, Biology, Physics**. Experimental design, data analysis, flow cytometry and manuscript preparation was done by N. Gupta. N. Gupta was assisted in the cell culture and clonogenic assay by Lily Hu (SRA IV, Deen Laboratory).

Chapter 5 contains the full text of the manuscript, *A Statistical Approach for the Analysis of Clonogenic Data*, **Radiation Research**, Vol. 145, pp. 636-640, 1996. Conversion of data analysis to the software spreadsheet and manuscript preparation was done by N. Gupta. Refinement of statistical methodology was done by Kathleen Lamborn.

Appendix A contains the full text of the letter to the editor, *Comments on the Use of Paclitaxel as a Radiation Sensitizer*, **International Journal of Radiation Biology, Oncology, Physics**, Vol. 33, pp. 239-240, 1995.

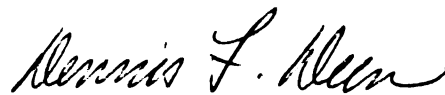
A handwritten signature in cursive script, reading "Dennis F. Deen", is positioned above a horizontal line.

Dennis F. Deen Ph.D.
Chairman, Thesis Committee

Abstract

Virtually all cytotoxic agents used to treat human malignancies selectively target dividing cells. Genetic characteristics unique to individual cell types are likely to modify cell survival and cell cycle perturbations after treatment. The p53 tumor suppressor gene regulates the G₁ checkpoint in response to radiation. Using human glioma cell lines differing in p53 function, cytokinetic changes, chromosomal aberrations, and cell survival were measured in irradiated G₁ cells. Although the presence of a G₁ checkpoint (wild-type p53) was associated with reduced clonogenic survival, it was not accompanied by a increase in the total number of chromosomal aberrations. Cells expressing a dominant negative p53 mutant had increased numbers of balanced translocations, but only at the highest radiation dose used (6 Gy). Additional results from these experiments suggest that wild-type p53 does not affect induction of DNA damage directly but, when present, may be involved in removing damaged cells from a cycling population. Cells wild-type for p53 have increased sensitivity to ionizing radiation in all phases of cell cycle compared to cells mutant for p53. The anaphase to metaphase transition during mitosis is believed to be the location of the checkpoint that is dependent upon accurate spindle assembly. Paclitaxel (Taxol™) is a natural product chemotherapeutic agent that stabilizes microtubules. Cell survival and cell cycle perturbations after paclitaxel treatment was studied in three human glioma cell lines: SF-126, U-87 MG and U-251 MG. Although, paclitaxel-induced cell killing plateaued above a threshold concentration, increasing treatment duration increased cell killing. U-251 MG cells showed a much larger degree of cell killing after palitaxel treatment as compared to the other two cell lines. Cell cycle analysis revealed that U-251 cells did not arrest in mitosis. In addition, a large fraction of U-87 MG cells appeared to

re-enter S phase without undergoing cytokinesis. These results suggest that the activity of the mitotic spindle checkpoint varies between tumor cell lines and its presence may contribute to the overall cellular response following paclitaxel treatment.

A handwritten signature in black ink, reading "Dennis F. Deen". The signature is written in a cursive style with a horizontal line underneath.

Dennis F. Deen Ph.D.

Chairman, Thesis Committee

Table of Contents

Preface	iii
Abstract	vii
Table of Contents	ix
List of Tables	xii
List of Figures	xiii
Abbreviations Used	xv
CHAPTER 1	INTRODUCTION
Overview	1
Radiation Biophysics	3
DNA as the Target for Ionizing Radiation	4
Cytokinetic Changes Following Irradiation	6
Cell Cycle Checkpoints	8
p53 Structure and Function	10
p53 and the G ₁ Checkpoint	12
p53 Regulated Genes	13
p53 and Apoptosis	15
Microtubule Function and Paclitaxel	17

CHAPTER 2	CYTOGENETIC DAMAGE AND THE RADIATION-INDUCED G₁ CHECKPOINT	
	Summary	19
	Introduction	20
	Materials and Methods	22
	Results	28
	Discussion	30
CHAPTER 3	RADIATION AGE RESPONSE OF HUMAN GLIOMA CELLS DIFFERING IN P53 FUNCTION	
	Summary	45
	Introduction	46
	Materials and Methods	48
	Results	51
	Discussion	53
CHAPTER 4	CYTOTOXICITY AND CELL CYCLE EFFECTS OF PACLITAXEL USED ALONE AND IN COMBINATION WITH IONIZING RADIATION	
	Summary	60
	Introduction	61
	Materials and Methods	63
	Results	67
	Discussion	70

CHAPTER 5	A STATISTICAL APPROACH FOR ANALYZING CLONOGENIC DATA	
	Summary	84
	Introduction	84
	Materials and Methods	86
	Results and Discussion	90
 CHAPTER 6	 CONCLUSION	
	General	98
	The G ₁ Checkpoint	98
	Paclitaxel-Induced Cell Cycle Perturbations	100
	Future Directions	102
 REFERENCES		103
 APPENDIX A	 COMMENTS ON THE USE OF PACLITAXEL AS A RADIATION SENSITIZER	129

List of Tables

2.1	Chromosomal aberration yields in U-87-lux.8 and U-87-175.4 cells after exposure to ionizing radiation in G ₁ .	34
2.2	Chromosomal rearrangments determined by fluorescence in situ hybridization of a biotinylated chromosome 4-specific DNA library.	37
3.1	Survival curve parameters.	56
3.2	Cell cycle phase percentages of cells in log phase growth as determined by analysis of dual-parameter flow cytometry plots.	57
4.1	Cell cycle phase fractions for control and treated cells after 48 and 8 h paclitaxel treatment.	75
4.2	Surviving fraction (SF) of log and plateau phase cells following 24 h paclitaxel treatment with cell cycle phase fractions at start of treatment.	76
4.3	Plating efficiency (PE) of asynchronous cells compared to mitotic cells following 8 h treatment with paclitaxel.	77
5.1	Z-scores for selected p-values.	94

List of Figures

1.1	The cell cycle.	7
1.2	p53 and the cell cycle.	14
2.1	Flow chart describing the analysis of purified G ₁ cells obtained by elutriation.	38
2.2	Cytokinetic results for asynchronous U-87-lux.8 cells after irradiation.	39
2.3	Cytokinetic results for asynchronous U-87-175.4 cells after irradiation.	40
2.4	Percentage of purified G ₁ cells remaining in G ₁ 48 h after irradiation.	41
2.5	Survival curves for U-87-lux.8 and U-87-175.4 cells.	42
2.6	Metaphase chromosomes from control U-87-lux.8 cells.	43
2.7	Metaphase chromosomes from irradiated U-87-lux.8 cells.	44
3.1	Survival curves for the human glioma cells studied.	58
3.2	Surviving fractions (SFs) for each elutriated fraction and corresponding cell cycle phase distributions for U-87 MG, U-87-lux.8, and U-87-175.4 cells.	59
4.1	The dose response for paclitaxel for SF-126, U-87 MG, and U-251 MG cells.	78
4.2	The effect of 48 h paclitaxel treatment and irradiation on survival of SF-126, U-87 MG, and U-251 MG cells.	79
4.3	The BrdU/PI contour plots for control and paclitaxel treated samples (48 h) before irradiation for SF-126, U-87 MG, and U-251 MG cells.	80
4.4	The effect of drug treatment times less than 24 h in SF-126, U-87 MG and U-251 MG cells.	81
4.5	The effect of 8 h paclitaxel treatment and irradiation on survival of SF-126, U-87 MG and U-251 MG cells.	82

4.6	The BrdU/PI contour plots for control and paclitaxel treated samples (8 h) before irradiation for SF-126, U-87 MG and U-251 MG cells.	83
5.1	Page 1 of an Excel spreadsheet demonstrating experimental data and calculation of Z-scores using both binomial and Poisson probability theory.	95
5.2	Page 2 of an Excel spreadsheet with estimated plating efficiency ("estPE" in figure), standard error, variance, estimated surviving fraction ("estSF" in figure) calculated using both binomial and Poisson probability theory.	96
5.3	Radiation survival curve for the cell line U-87-175.4.	97

Abbreviations Used

bp	base pairs
AT	ataxia telangiectasia
BrdU	5-bromodeoxyuridine
CFE	colony forming efficiency
CMEM	complete minimal essential media
DAPI	diamidine phenylindole
DPC	DNA-protein crosslink
DSB	double-strand break
FITC	fluorescein isothiocyanate
Gy	Gray
MPF	mitosis promoting factor
NER	nucleotide excision repair
PCNA	proliferating cell nuclear antigen
PE	plating efficiency
PI	propidium iodide
SSB	single-strand break
SF	surviving fraction
UV	ultraviolet
wt	wild-type

Chapter 1 Introduction

OVERVIEW

Normal eukaryotic somatic cells undergo a predictable series of events culminating in cytokinesis and the formation of two daughter cells. The regular progression of these events is defined as the cell cycle. In multi-cellular organisms, cell division occurs most rapidly during development, repair of tissue damage, and in organs that require continual cell renewal, i.e. bone marrow and epithelium. At the most fundamental level, uncontrolled cell division can be viewed as a necessary feature of tumor progression (71, 72, 79). Cell cycle research is not only important for understanding oncogenesis but is a prerequisite to the design of successful therapeutic strategies.

The use of modern molecular biological techniques to answer basic genetic and biochemical questions has led to a more detailed understanding of the cell cycle (138). An important milestone was the biochemical purification from frog embryos of a protein complex, called MPF, that regulates the cell cycle (54, 68, 91, 122). One component of MPF is a protein kinase, a member of a family of proteins called cell division kinases, or CDKs, that are required for progression from one cell cycle phase to the next. These kinases control the activity of other proteins by phosphorylating specific amino acids, usually serine, threonine or tyrosine residues (141, 185). There are at least six CDKs in human cells, which may allow for more refined control of cell cycle progression. Additional specificity is provided by a large family of proteins called cyclins that associate with and are necessary for CDK activity.

There are several lines of research that have led to the realization that oncogenesis and abnormalities in cell cycle regulation occur together. First, it is accepted that an

accumulation of genetic changes in different loci are required before a fully established malignant phenotype emerges. Some of the genes that are commonly mutated are direct participants in the cell cycle (85, 86). As tumors progress through stages of neoplasia in-step with these genetic changes, their proliferative potential and malignant features also increase (46, 208). Second, many oncogenes, regardless of their own function, eventually affect cell cycle progression. As an example, the RAS mediated TGF β signal transduction pathway ultimately regulates the progression of cells through late G₁. Transfection of dominant oncogenes into normal cells usually leads to increased growth rates and loss of contact inhibition. Finally, it appears that most cellular mechanisms activated in response to cellular and genetic damage cause cell cycle perturbations. The best known and most thoroughly documented example of this is the tumor suppressor gene, p53. Loss of wt p53 function results in the absence of a G₁ checkpoint and contributes to further genetic instability in certain cell types.

This thesis describes the effects of ionizing radiation and paclitaxel, two cancer therapeutic agents, upon human glioma cells grown *in vitro*. In particular, experiments were designed to test if a relationship exists between cell cycle perturbations and cell survival. Ionizing radiation produces chromosomal aberrations in a dose dependent manner which can be detected in mitotic cells. Using isogenic cell lines that differed in p53 function, experiments were done which measured the G₁ arrest, cell survival, and the formation of chromosomal aberrations following ionizing radiation. This work is described in Chapter 2. Another observation associated with cell killing after irradiation is the age response, i.e. cells irradiated in different phases of the cell cycle have different sensitivities to ionizing radiation. Typically, S phase cells are most resistant and G₂/M phase cells are most sensitive. The magnitude of the age response in cell lines that differ in p53 function is described in Chapter 3. To assess cell cycle perturbations occurring later in the cell cycle, the effect of paclitaxel upon cell survival and cell cycle progression was

investigated. Paclitaxel is a specific anti-microtubule agent that causes cells to arrest in mitosis. Cells were treated with paclitaxel alone and in combination with ionizing radiation. These experiments are described in Chapter 4. Finally, a statistical technique to analyze data obtained from the clonogenic assay, used extensively in these experiments, was developed and is described in Chapter 5.

RADIATION BIOPHYSICS

Interaction of ionizing radiation with biological material results in energy deposition unevenly through the sample. Electromagnetic radiation, i.e. an x-ray beam, can be regarded as a wave function as well as a stream of photons carrying discrete packets, or quanta, of energy. If the energy carried by a photon either breaks or modifies covalent bonds in intra-cellular molecules, biological effects may then ensue. The energy of individual quanta is determined by $h\nu$, where h is Planck's constant and ν is the frequency of the radiation.¹ Quanta of non-ionizing radiations have insufficient energy, in general, to modify covalent bonds. Energy deposition causes electrons to be raised to higher energy levels and may result in ejection of electrons if the photon energy exceeds the electron binding energy. Accelerated particles, protons and neutrons, are also used in therapeutic settings. By virtue of their particulate nature, these radiations deposit energy in discrete areas and set in motion other particles such as protons and α -particles. A specific type of radiation has biological effects only if some of its energy is absorbed by the medium it passes through. This is reflected in the physical unit of absorbed dose, the Gray (Gy)²,

¹ Photon energy (E) is related to wavelength (λ) by: $E(\text{keV}) = \frac{12.4}{\lambda(\text{\AA})}$. One kiloelectron volt (keV) is the energy acquired by an electron accelerated through 1000 volts.

² One Gy is equal to 100 rads.

which is equivalent to 1 joule/kg of tissue. Different radiations, e.g. neutrons *versus* x-rays, produce dramatically different effects upon tissue.

X-rays, γ -rays and accelerated particles are all ionizing radiations. Electromagnetic radiations are considered to be ionizing if they have photon energies in excess of 124 eV. Any form of ionizing radiation, however, may interact with a vital target, e.g. DNA, creating ions and subsequent chemical modifications. This is the *direct* action of radiation which predominates for particulate radiation (protons, neutrons or α -particles). For example, a neutron will collide with the nucleus of an atom in the absorbing material to produce nuclear products such as recoil protons. Some authors support the theory that direct action causes most of the biologically significant damage (102). Conversely, another mechanism that is believed to be more important is the interaction of a photon with orbital electrons of the absorbing intra-cellular medium, of which water molecules are the most common. This interaction ejects fast electrons from outer shells that create multiple ionizations along their tracks. This mechanism, the *indirect* action of radiation, is the predominant one for x-rays and γ -rays. The time course of these early events is extremely rapid. The initial ionization occurs in 10^{-15} seconds. Ion radicals and free radicals exist for only 10^{-10} to 10^{-5} seconds. The consequences of radiation-induced chemical alterations may not be expressed for hours or days, if the parameter is cell killing; and potentially years if genetic changes are not manifest until following generations.

DNA AS THE TARGET FOR IONIZING RADIATION

While radiation produces ionizations randomly throughout the cell, it has been assumed that DNA damage determines cell survival. Damage to other cellular components, such as proteins, mRNA, and lipids, are not believed to be significant because many copies

of these components exist and damaged molecules are presumably replaced by normal turnover.

Initial experiments, using ionizing particles with a limited range, selectively irradiated the nucleus and spared the cytoplasm (133). Using survival as an endpoint, cells whose cytoplasm was selectively irradiated tolerated ten times the radiation dose compared to cells that received mainly nuclear irradiation. This suggested that a nuclear target determined the degree of cell killing. A more specific result was obtained from experiments where cells that incorporated a radioactive DNA precursor, ^{125}I -iododeoxyuridine, were two orders more sensitive to decay events when compared to cells that were treated with a compound that localized to the cell membrane, ^{125}I -labeled concavalin A (214). Finally, other investigators observed radiation sensitization after allowing cultured cells to incorporate halogenated pyrimidines into newly synthesized DNA (195).

The major types of DNA lesions caused by x-rays and γ -rays are base damage, DNA-DNA or DNA-protein crosslinks (DPCs), and scissions of the phosphodiester backbone leading to single strand breaks (SSBs) or double strand breaks (DSBs) (51, 80, 164, 209). Lesions are characterized as being simple, i.e. with no damage to neighboring groups, or complex, where combinations of lesions or multiple lesions exist (211, 212). These represent only broad categories of lesions since the exact chemical changes are extremely diverse. It is unlikely that these different types of damage are repaired with equal efficiency or fidelity.

The majority of data acquired so far support the theory that DNA DSBs are the significant lesions that cause cell killing following ionizing radiation. Theoretically, a DSB is formed either by cooperation of two SSBs or a single event involving both strands. It appears that a single event involving both strands, defined as a *local multiply damaged site* by Ward (211, 213), is the most important type of lesion. Results from experiments using a variety of assays to study non-rejoined DNA breaks, unrepaired DSBs, and chromosomal

aberrations all demonstrate a strong correlation between DNA DSBs and cell killing (1, 51, 132, 142, 143, 150, 165, 199). Other lesions do not appear to be as important. DNA SSBs do not correlate with cell killing (211, 213). DPCs are caused by the formation of covalent bonds between DNA and nuclear matrix proteins (24, 223). Evidence suggests that DPCs do not play a role in radiation-induced cell killing (147).

CYTOKINETIC CHANGES FOLLOWING IRRADIATION

Cells do not arrest randomly in the cell cycle in response to ionizing radiation (99, 134, 149, 151, 152, 224, 228). Failure of progression commonly occurs during late G₁ into S phase, during S phase, and from late G₂ into M phase (Figure 1.1). In a classic experiment done in Robert Painter's laboratory (151), an 8 hour division delay was demonstrated in HeLa cells by measuring the percentage of mitotic cells (the mitotic index) at a number of time points following a 5 Gy dose of ionizing radiation. In later experiments Painter showed that this delay was due to a reduction in the rate of DNA synthesis (149). Terasima and Tolmach found that the division delay depended on the cell cycle phase when irradiation was performed. It was longest for cells in G₂, shortest for cells in G₁ and intermediate for cells in S (198). These data were confirmed by Leeper and colleagues who proved that the duration of the G₂ "arrest" was dose-dependent (99). There is evidence that the G₁ arrest in some cell types may represent an irreversible block to progression (36). While the exact meaning of "arrest" versus "delay" can be confusing, at the experimental level what is usually measured is the change in proportion of cells in the four cell cycle phases, i.e. the percentage of cells in G₁, S, G₂ or M phase. Technically, the most difficult step in most cell cycle studies remains the isolation of synchronized cell populations that can then be studied further.

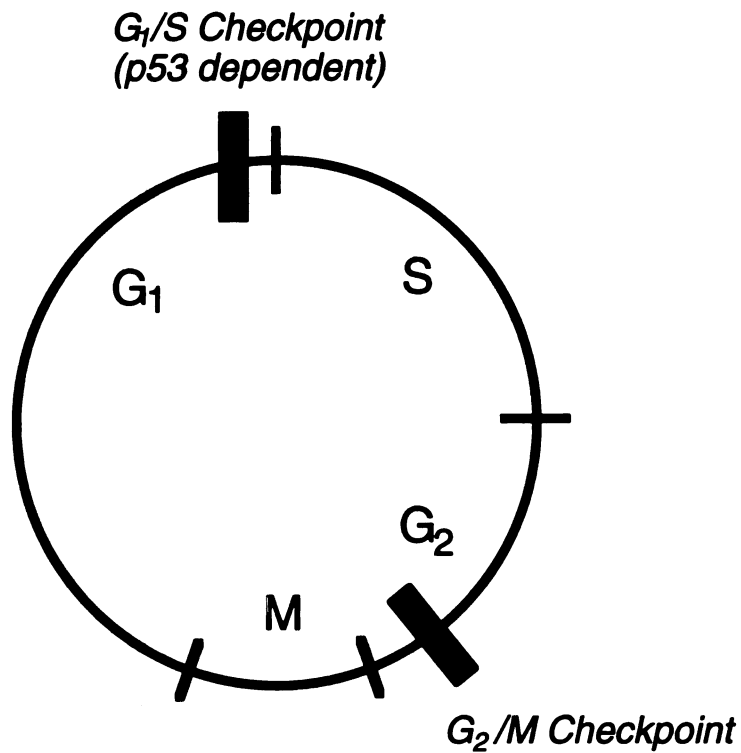


Figure 1.1. The cell cycle. A p53-dependent arrest in late G₁ occurs following DNA damage. A dose-dependent delay to progression after ionizing radiation occurs in late G₂. Mitosis (M phase) is the shortest while G₁ is the most variable.

CELL CYCLE CHECKPOINTS

Wild-type yeast cells (*S. cerevisiae*) arrest in G₂ following irradiation. These are identified as budded cells that have failed to enter mitosis. Those that repair DNA damage progress into M phase after a delay and remain viable. Evidence that attributed a specific gene to a delay in cell cycle progression following ionizing radiation was initially reported by Weinert and Hartwell (217). At that time a large number of mutants, identified by the designation *rad*,³ were known to be radiation sensitive. Weinert and Hartwell screened several of these mutants for failure to delay in G₂ following irradiation. They reasoned that the function of the G₂ delay was to allow repair to occur before progression into M phase so cells defective for a G₂ delay should be more sensitive to DNA damage. Using this strategy, one mutant, *rad9*, was identified that failed to arrest in G₂. This mutant was originally isolated in 1968 by Cox and Parry but its exact role at that time was unknown (32). Further characterization of this mutant confirmed that the absence of a G₂ delay led to increased radiation sensitivity. As well, an artificial 4 hour G₂ delay created by drug treatment in *rad9* mutant cells increased viability dramatically. From these experiments, Weinert and Hartwell coined the term "checkpoint" to indicate a decision point in the cell cycle where specific gene products that monitor cell cycle progression respond to cell damage by delaying the cell cycle thereby increasing the chances for repair.

This concept was expanded by Murray when he postulated that components of such a monitoring system (a "feedback control" system) consisted of a) a sensor to detect damage, b) a signaling pathway that communicates this information, and c) a component of the cell cycle machinery that mediates the actual arrest at a precise point, i.e. the checkpoint

³ Genetic nomenclature is inconsistent. In this thesis, genes are named by an italicized short form (*MDM2* or *p53*) and the corresponding protein by an un-italicized form (MDM2 or p53). Names for certain proteins, such as p21 or p53, refer to their measured molecular weight. Budding yeast (*S. cerevisiae*) nomenclature specifies a lower case italicized form for the mutant gene usually followed by a number. The wild-type gene is designated by upper-case letters.

(137). A large number of genes have now been identified in yeast that respond to a variety of stimuli such as unreplicated DNA, damaged DNA, and improper spindle assembly. The work done in yeast supports the concept that cellular damage or dysfunction is assessed and potentially repaired in G₂ prior to cell cycle progression.

The mammalian G₂/M CDK, CDC2, is required for cells to progress from G₂ into M phase. The activity of CDC2 is modulated, in turn, by other kinases (131). The total amount of CDC2 protein remains the same throughout the cell cycle but its association with cyclin B and phosphorylation of critical residues determines the timing of its activity. Rapid dephosphorylation of tyrosine residues at positions 14 and 15 on CDC2 causes activation of the CDC2-cyclin B1 complex. Phosphorylation of these proteins is thought to lead to mitotic events such as chromosome condensation and disappearance of the nuclear membrane. Rapid degradation of cyclin B1 subsequently triggers movement from metaphase into anaphase. Mutations of cyclin B1 that interfere with protein degradation effectively arrest cells at the metaphase/anaphase boundary.

As expected, the normal biochemistry of the cell cycle is affected by ionizing radiation (118). HeLa cells blocked at the G₁/S boundary pharmacologically and then released into drug-free media remain synchronized for enough time to study cell cycle phase specific events. Using this method, investigators found that cells irradiated shortly after release into S phase progressed slowly through S phase and demonstrated a prolonged G₂ delay (139). Accompanying this delay, cyclin B1 mRNA levels remained low. If cells were irradiated in early G₂, cyclin B1 mRNA levels rose but protein levels remained low. This indicated that radiation regulates cyclin B levels at both the mRNA and protein level, depending upon the cell cycle phase where irradiation occurs. As expected, the ability of CDC2 to phosphorylate histone H1 (a measure of its kinase activity) *in vitro* is inhibited rapidly after irradiation (113). There is some evidence that the length of the G₂ delay

correlates with increased survival (124, 193). This provides an explanation for the purpose of the cell cycle delay, i.e. allowing DNA repair to occur prior to mitosis.

P53 STRUCTURE AND FUNCTION

The p53 protein was first discovered as a cellular protein that co-precipitated with the SV-40 virus large T-antigen, which is involved in subverting the growth control mechanisms of quiescent cells (96). p53 belongs to a class of gene products, called tumor suppressors, that curb excessive proliferation (103, 206, 207). Some years passed before the realization that *p53* was one of the most commonly mutated genes in human cancer (59). p53 is, however, not an essential gene. Knockout mice lacking both copies of the p53 gene are normal at birth but a majority develop tumors, primarily lymphomas and sarcomas, before they reach 6 months of age (40).

The p53 protein is a transcription factor that has at least four domains: an amino terminal domain, a core domain, an oligomerization domain and a carboxy-terminal domain (155).⁴ The amino domain carries the site where interaction with transcriptional machinery occurs, the so-called transactivation domain. This site is the target for the cellular protein MDM2 and the adenovirus E1b protein (107). Both of these proteins interfere with the transcriptional activation function of p53. Indeed, MDM2 can function as an oncogene when overexpressed by interfering with p53 function (130, 148).

The core domain is required for sequence-specific binding of DNA. The 20 bp consensus binding site consists of two copies of a 10 bp sequence separated by up to 13 bp (42, 52). The common mutations in the core domain found in human tumors

⁴ Proteins often have discrete modules, or domains, that each perform one function. A single protein domain independently expressed will often retain its own function in the absence of the remainder of the protein.

inactivate growth suppression caused by p53, presumably by not allowing targeting of the protein to specific DNA sites (156). It should be noted that p53 also can bind to DNA in a non-specific manner and suppress a variety of promoters containing TATA elements (111, 116, 121, 162, 170, 177, 201, 204).

The oligomerization domain is required for p53 to form a tetramer, a complex with three other p53 molecules, that is required for efficient binding to DNA sequences. Mutations in this domain interfere with the ability of p53 molecules to form tetramers. When one allele in a cell contains a mutation in this domain and is expressed with a wild-type allele, p53 function is abrogated since p53 complexes contain both wild-type and mutant proteins and are, therefore, inactive. When this occurs the p53 mutant is said to be "dominant-negative" since the presence of only one mutant allele can result in a mutant phenotype. This is in contrast with the classical description of tumor suppressor genes that require inactivation of both alleles before a phenotype is evident.

The carboxy-terminal is involved in the non-specific association of p53 to free DNA ends (7, 145). This may represent the connection to DNA damage if p53 induction after ionizing radiation, for example, is found to be dependent upon DNA binding. There are clearly multiple functions of p53, since the carboxy-terminus can be deleted without an effect upon growth suppression (156).

There is an observed level of genomic instability in malignant cells. It is difficult to say whether this instability causes tumor progression or if it is a consequence of this process. Some studies have noted that X-irradiation causes ongoing chromosomal aberrations many generations after the initial DNA damaging event (21, 120). Loss of wild-type *p53* predisposes cells to gene amplification when grown in a certain selection medium (112, 225). These findings suggest that after an appropriate stimulus, cells with mutant *p53* should demonstrate greater genetic variation in later generations.

P53 AND THE G₁ CHECKPOINT

Early studies that showed p53 is a substrate of and associates with CDC2 hinted at the relationship between p53 and the cell cycle (13, 126). By studying wt p53 myeloid leukemia cells, Kastan was the first to demonstrate, with immunoprecipitation and Western blotting, that p53 protein levels rise after γ -irradiation (87). This response is rapid (one hour after irradiation) and occurs after relatively low radiation doses (105 cGy). Using flow cytometry to examine cell cycle progression, Kastan also noted that a percentage of G₁ cells failed to enter S phase following a 208 cGy radiation dose. Although Little demonstrated a G₁ arrest following irradiation as early as 1968 (109), the cause was unknown at that time. Kastan's results provide strong evidence that p53 is involved in the progression of cells from G₁ into S phase. The teleological reason reported for the purpose of the G₁ arrest is that it permits repair of DNA damage prior to attempting replication.

Experimental evidence from yeast supports the concept that a delay in cell cycle progression before the start of a critical process, such as DNA synthesis or mitosis, allows a period of time during which damage can be repaired (215, 217). There is no definitive proof in mammalian cells, as yet, that the G₁ delay leads to increased repair of DNA damage (134, 136). In fission yeast, the G₂ delay and radiosensitivity are genetically separate, thus providing a precedent for such a finding for the G₁ checkpoint in mammalian cells (9).

A number of reports in the past few years have shown that the presence of wt p53 leads to either no change or increased cell killing after irradiation (4, 14, 15, 98). This, of course, is opposite to the result one would expect if the G₁ checkpoint served a protective function. The activation of the G₁ checkpoint in normal human fibroblasts (wt p53) was shown in a recent paper to lead to a prolonged, and presumably permanent, G₁ arrest resembling senescence (36). This clearly suggests that in certain situations the function of

the G₁ checkpoint is not to allow repair prior to entry into S phase but to prevent the growth of damaged cells.

P53 REGULATED GENES

Because p53 is induced after irradiation, an obvious question is: Which genes are controlled by p53? Several genes have been discovered that are regulated by p53 including one that directly links p53 and the cell cycle. Using subtractive hybridization,⁵ a p53-inducible gene, *WAF1*, was identified that suppresses tumor growth *in vitro* (43, 64, 69). The protein, a phosphatase named p21^{WAF1/CIP1} or just WAF1/CIP1, is a potent inhibitor of several CDKs and delays progression through the cell cycle (Figure 1.2) (222). Accompanying p53 induction, WAF1/CIP1 is induced following ionizing radiation and precedes a G₁ arrest (41).

The *GADD* (Growth Arrest and DNA Damage-inducible) genes were isolated from a larger pool of clones by virtue of increased levels of mRNA expression after treatment with agents that caused DNA damage or conditions that led to growth arrest (50). One of these genes, *GADD45*, was strongly induced after X-irradiation (153). By studying a variety of cell lines, including those from ataxia telangiectasia (AT) patients,⁶ Kastan and colleagues determined that *GADD45* was not induced in cells that lacked the *AT* and/or *p53* genes (88). These results strongly suggested a connection leading from the AT protein to p53 to *GADD45*.

⁵ This technique allows the isolation of mRNA molecules that are expressed in one population of cells but not in another. cDNA is first made from poly (A) mRNA. cDNA molecules that are similar hybridize together and are subsequently excluded. Only those cDNA species that are different, therefore single-stranded, are left and are selected for further analysis.

⁶ Ataxia telangiectasia is an autosomal recessive disease with a complex phenotype that includes sensitivity to ionizing radiation.

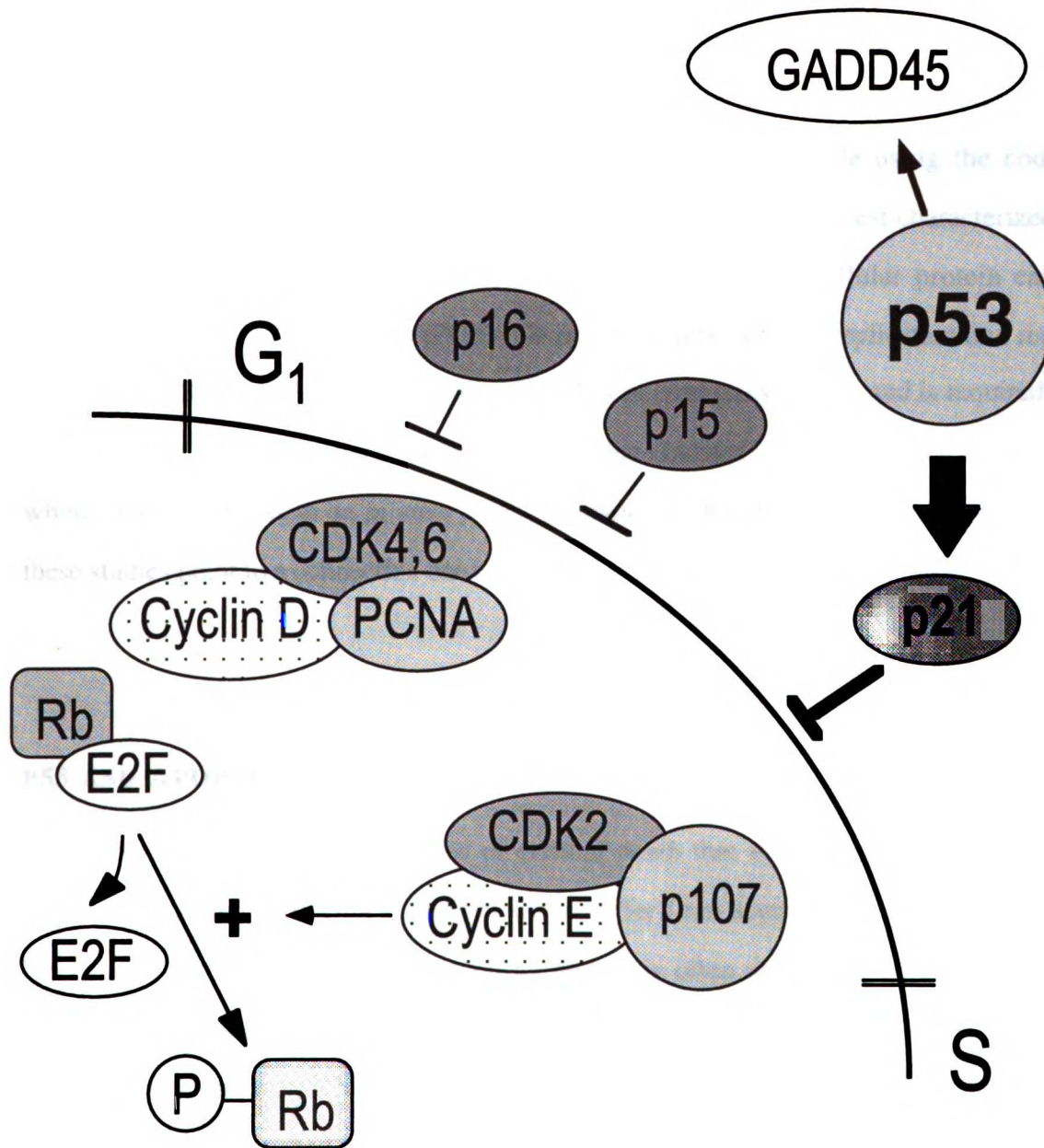


Figure 1.2. p53 and the cell cycle. The current model suggests that induction of wt p53 increases the expression of p21^{WAF1/CIP1} resulting in a G₁ cell cycle arrest.

The function of GADD45 remained unknown until *in vitro* experiments demonstrated an association with nucleotide excision repair (NER). NER is the dominant process involved in the removal of a damaged base or nucleotide using the coding information of the complementary strand (28, 169). This process is best characterized in the removal of UV-induced base damage. It is known that a cellular protein called proliferating cell nuclear antigen (PCNA) is involved in both DNA replication and repair (117, 157, 158). PCNA also associates with DNA polymerases δ and ϵ and is required for NER (179). Smith and colleagues have showed that GADD45 associates with PCNA and when added in excess to an *in vitro* assay, promotes NER (183). The conclusions from these studies point to a connection between the p53/GADD45 pathway and DNA NER.

P53 AND APOPTOSIS

Apoptosis refers to a process of cellular death that is characterized by specific morphological and biochemical features which differ from acute necrosis (89, 161, 221). Necrosis is distinguished by cell swelling and lysis often producing an inflammatory response. In apoptosis, there is cellular shrinkage, chromatin condensation, nuclear blebbing, non-random DNA fragmentation and rapid phagocytosis by macrophages. Apoptosis is distinguished from other types of cell killing because it requires specific gene products and can be attenuated by inhibitors of protein synthesis.

In older studies, dead irradiated cells were described as having undergone a "reproductive" death or an "interphase" death. Reproductive death is the inability of a single cell to produce a colony of at least 50 cells. This is usually measured by the standard clonogenic assay. This form of death is believed to be due to the formation of chromosomal aberrations that prevent successful mitoses (10, 159). Interphase death

refers to impairment of cellular function and loss of cell integrity prior to mitosis. This may be identical to the events that are now recognized as apoptosis.

Although wild-type p53 is known to have anti-proliferative effects when introduced into most cells in culture (8, 38, 125), introduction of the wild-type gene into leukemia or colon cancer cell lines causes apoptosis (178, 226). More convincing evidence about the role of p53 in apoptosis comes from studies using cells derived from knockout mice. Using mice homozygous for a *p53* deletion, two groups of investigators demonstrated that the absence of p53 protects thymocytes against apoptosis following ionizing radiation but not in response to stimuli that mimic T-cell receptor engagement (such as phorbol esters) or glucocorticoids (27, 115). This effect was dramatic since 50% of wild-type thymocytes lost viability (measured by exclusion of FITC) after 2.5 Gy, while only 20% of cells null for p53 lost viability after a much higher dose of radiation (20 Gy).

The actual initiation of apoptosis seems to depend upon the cell type involved. In an experiment using p53-null or p53-heterozygous knockout mice, expression of a mutated form of the SV40 large T antigen that interferes only with the Rb tumor suppressor protein, and not p53, produced aggressive choroid plexus tumors in the null mice (194). This is not surprising given that both tumor suppressor proteins are either non-functional (Rb) or absent (p53). In p53 wild-type mice, however, there was an increased level of apoptosis noted in the choroid plexus epithelium. This level of apoptosis was interpreted as representing the elimination of excessively proliferating epithelial cells. Focal tumors that arose in p53-heterozygous mice sustained a loss of the remaining p53 allele and demonstrated a decreased level of apoptosis. Fisher, in his review, makes the point that induction of apoptosis is a major reason why cancer therapy works in certain tumor types (49). Apoptosis may be a method by which an organism is able to remove damaged cells from the normal unaffected population.

MICROTUBULE FUNCTION AND PACLITAXEL

Microtubules are extended polymers of $\alpha\beta$ tubulin heterodimers (5, 119, 129). Assembled into 25 nm hollow tubes, microtubules serve important structural and functional roles in cells. Tubulin dimers are asymmetric which results in microtubules having clear polarity with one pole being the plus-end and the other the minus-end. Polarity is used by ATP-dependent motors to direct transport of sub-cellular organelles, anchor ends of microtubules, and segregate chromosomes.

Microtubules are not static structures. They continually undergo transitions between polymerization and depolymerization. This is termed *dynamic instability*. A kinetic balance towards polymerization (termed *rescue*) causes microtubule growth while greater rates of depolymerization (termed *catastrophe*) cause microtubule shrinkage. In animal cells, microtubules are nucleated at specific structures called centrosomes (84, 128). Prior to mitosis, the paired centrosomes separate and the microtubule array radically reorganizes and forms a bipolar spindle. Phosphorylation by MPF is believed to cause microtubule reorganization. Microtubules initially attach to one kinetochore causing the chromosome to move poleward (31, 92). Eventually, a microtubule from the other spindle pole attaches to the opposite side of the kinetochore, thereby pulling the chromosome to the equator and resulting in congression. The metaphase to anaphase transition, which is signaled by sister chromatid separation, is dependent upon chromosome congression. A chromosome that fails to reach the equator will cause a delay in the onset of anaphase. Relaxation of this checkpoint control may account for the frequent observation of aneuploidy in cancer cells.

A variety of chemotherapeutic drugs, including vinblastine and colchicine, target microtubules (81, 181). These drugs usually cause microtubule depolymerization. In contrast, paclitaxel, a natural product drug purified from the bark and needles of the Pacific

yew tree, stabilizes microtubules *in vitro*. Paclitaxel binds to tubulin tightly with a stoichiometry of one (37). Currently in use to treat a variety of malignancies (25, 76, 100, 123, 176, 200), the interaction of paclitaxel and microtubules has provoked considerable interest. In the presence of paclitaxel, abnormal microtubule dynamics affect mitosis and cytokinesis (82, 83). This leads to cells arrested in metaphase, inviable multi-nucleated cells, or apoptosis (114, 166).

Many clinical protocols use combination therapy to increase tumor response rates. The general principle of combination therapy is either to target different populations with several agents or to sensitize the tumor population to another agent without affecting normal tissues. One theoretical basis of using drugs as radiation sensitizers for oxic cells is the radiation age response. If the percentage of cells in a radiosensitive phase of the cell cycle (i.e. mitosis) can be increased, then cell killing should be greater following irradiation (187). It is unclear what the effect of simultaneously causing radiation and paclitaxel-induced cell cycle perturbations are in tumor cells. Cell cycle effects of paclitaxel and how they may influence cell survival are described in Chapter 4.

Chapter 2 Cytogenetic Damage and the Radiation-Induced G₁ Phase Checkpoint

SUMMARY

It is proposed that genomic integrity is preserved after DNA damage in a variety of ways. X-irradiation induces a p53-dependent G₁ cell cycle checkpoint which putatively allows time for repair of DNA damage. p53 is also involved in the initiation of apoptosis following radiation-induced DNA damage, presumably leading to the elimination of lethally damaged cells from the irradiated population. To test the hypothesis that the G₁ checkpoint allows additional time for repair, we investigated whether the presence of a G₁ arrest modified the frequency and type of chromosomal rearrangements at the first mitosis after irradiation. Isogenic cell lines derived from the same human glioma cell line, but differing in p53 status, were used. Purified G₁ cells, isolated by centrifugal elutriation and X-irradiated, were studied. The wild-type p53 cell line demonstrated a dose-dependent arrest during G₁, as determined by flow cytometry. These cells remained in G₁ as long as 48 hours after irradiation. Cells expressing a dominant-negative p53 mutation accumulated to a much lesser extent in G₁ after irradiation. Cells lacking the G₁ checkpoint showed increased survival at all radiation doses. There were no significant differences in the type or frequency of total chromosomal aberrations in mitotic cells from either cell line after 1, 2, 4 or 6 Gy X-rays, as measured by conventional cytogenetic analysis. There was an increase, however, in the number of reciprocal translocations in mitotic cells with mutant p53 (lacking a G₁ checkpoint), as measured by fluorescence *in situ* hybridization with a chromosome 4-specific DNA library, but only after 6 Gy. The results suggest that the presence of a well-defined p53-dependent G₁ arrest does not markedly reduce

chromosomal aberrations caused by low doses of ionizing radiation, but may modulate the overall degree of survival by inducing cells to undergo a prolonged G₁ arrest.

INTRODUCTION

Although a G₁ arrest following X-irradiation was first described in 1968 (109), its molecular basis remained obscure until evidence presented by Kastan and co-workers in 1991 revealed that p53 played an important role in the cell cycle (87). Kastan *et al.* observed accumulation of p53 protein following γ -irradiation as measured by flow cytometry and immunoprecipitation. In a subsequent report these investigators measured cell cycle perturbations by flow cytometric analyses of asynchronously growing cells, which differed in their p53 status, at a specific time point after irradiation (88). They recognized that the G₁ arrest represented activation of a cell cycle checkpoint and was dependent upon the presence of functional wt p53 protein. It should be noted that the p53 gene product, a transcription factor, also appears to have other cellular functions. In hematopoietic cells, wt p53 is required for radiation-induced apoptosis (27, 115, 220). The detection of a selectable marker amplified only in cell lines mutant for p53 suggested that wt p53 also prevents genomic instability (112, 225). Not surprisingly, the p53 tumor suppressor gene is frequently lost or mutated in many human tumors (103, 144, 196).

There is considerable speculation as to the function of the G₁ checkpoint. One hypothesis follows the attractive teleological argument that activation of the G₁ checkpoint allows for additional time to repair DNA damage before cells enter S phase (87, 88, 95). Experimental evidence from yeast supports the concept that a delay in cell cycle progression before the start of a critical process, such as DNA replication or mitosis, allows a period of time during which damage can be repaired (216-218). Without sufficient time for repair,

DNA replication could result in chromosome breakage and/or rearrangements that might ultimately lead to gene amplification, delayed mutation, genomic instability and even cell death. Additional support for this hypothesis comes from descriptions of the G₂ arrest in mammalian cells. Based on experiments with synchronized cells, a relationship was described between the dose of radiation and the duration of the G₂ arrest (99, 175, 193, 198, 210). For increasing doses of radiation, the time that cells remained in G₂ also increased. This implied that cells continued into mitosis once a certain period of time, a "delay," had elapsed. If this premise applies to the G₁ checkpoint in mammalian cells, two predictions can be made. First, the checkpoint must be temporary, so that it is truly a "delay" to progression, allowing cells to continue cycling after a period of time. Second, cells possessing a G₁ checkpoint should have more time to repair DNA damage and, therefore, show increased survival. It should be noted that there is no experimental proof, as yet, that the G₁ checkpoint in mammalian cells allows time for repair of DNA damage after ionizing radiation (134, 135). Indeed, evidence supporting a contrary viewpoint comes from a number of publications in the past few years that have shown, for the most part, that the presence of wt p53 leads to either no change or increased cell killing after irradiation (4, 14, 15, 98, 182, 192, 193). This, of course, is opposite to the result one would expect if the G₁ checkpoint served a protective function. The activation of the G₁ checkpoint in normal human fibroblasts (wt p53) was shown in a recent paper to lead to a prolonged, and presumably permanent, G₁ arrest resembling senescence (36). This suggests, that in certain situations, the activation of a cell cycle checkpoint is not always to allow repair prior to reinitiation of the cell cycle. It should be noted that the G₂ arrest and radiosensitivity were genetically separated in fission yeast, thus providing a precedent for such a finding for the G₁ checkpoint in mammalian cells (9).

A strong correlation exists between the dose of ionizing radiation delivered, the formation of chromosomal aberrations, and cell survival (30, 34, 60, 80). In this study we

examined if this relationship was maintained in the presence, or absence, of a radiation-induced G₁ checkpoint. In particular, in cells with a G₁ arrest, if there is increased time for DNA repair, we would expect fewer chromosomal aberrations after irradiation. Our experimental approach was to measure cell cycle changes, cell survival and, in detail, the type and frequency of chromosomal aberrations found at the first mitosis following X-irradiation in cells that differed in their ability to arrest during G₁.

MATERIALS AND METHODS

Cell Lines

A human glioma cell line, U-87 MG, wt for p53 gene function, was used as the parental cell line to construct isogenic cell lines differing in p53 status. The presence of functional p53 protein in this cell line was assessed by a) confirming the activation of the G₁ checkpoint after irradiation, b) demonstrating wt p53 protein by immunoblotting with wt-specific antibodies, and c) single strand conformation polymorphism analysis of exons 5-9 (data not shown). As described in detail elsewhere (23), recombinant retroviral constructs, encoding either the firefly luciferase (*lux*) gene as a control or a human p53 cDNA carrying a dominant-negative mutation (codon 175^{arg} → ^{his}) under the control of the Moloney MuLV long terminal repeat, were used to infect the parent cell line. Both constructs contained the neomycin-resistance gene with selection occurring in medium containing G418 (400 µg/ml). Colonies were expanded into cell lines and analyzed for the presence or absence of an X-ray-induced G₁ cell cycle arrest (227). Two cell lines were selected for further experiments. U87-175.4 carried a dominant-negative p53 construct and lacked an X-ray-induced G₁ cell cycle arrest; U87-lux.8 carried only wt p53 and demonstrated an X-ray-induced G₁ arrest. Both cell lines were cultured as monolayers in

CMEM, which consists of Eagle's MEM supplemented with nonessential amino acids, glutamine and 10% fetal calf serum, at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells from both lines were continuously maintained in medium containing G418 (400 µg/ml).

Cytokinetic Analysis

Two methods were used to analyze G₁ progression following X-irradiation. For purposes of simplicity, our initial experiments used log-phase cells. Cells were seeded into T-75 flasks 48 h before X-irradiation (doses ranging from 0 to 6 Gy). Colcemid was added at a final concentration of 0.2 µM to arrest cells in mitosis and to prevent entry into the next division cycle. Cell samples were collected at 3, 6, 9, 12, and 24 h, washed with phosphate-buffered saline, and then fixed in 70% ethanol. The percentage of cells in G₁ for each dose point was determined by measuring PI fluorescence on a Becton Dickinson FACScan (see below). The second method provided more detailed cell cycle phase information. Cells from the purified pool of G₁ cells were pulse-labeled for one hour with 10 µM BrdU 48 h after irradiation and then analyzed by flow cytometry (see below). These cells were also grown in the presence of Colcemid (0.2 µM). The number of time points was limited because of the small number of purified G₁ cells obtained from elutriation. As well, because our methods were tailored to assess G₁ progression, it was not possible to analyze perturbations in later cell cycle phases.

Centrifugal Elutriation

We selected centrifugal elutriation as the optimal method for isolation of G₁ cells, because all cells are in log-phase growth during harvest. The usual techniques for synchronizing cells in G₁ (or more accurately G₀), using drugs such as mimosine, or serum starvation, introduce complications when the goal is to obtain accurate cytokinetic or

survival information. Cells were separated by using a modified Beckman J2-21 centrifuge and a JE-6 elutriator rotor (57, 108). To obtain adequate numbers of exponentially growing cells, we seeded cells at low density into roller bottles containing 250 ml of MEM supplemented with 10% fetal calf serum and 400 $\mu\text{g/ml}$ of G418. The roller bottles were sealed after being gassed with air containing 5% CO_2 and incubated at 37°C for 3 days. Cells were trypsinized, disaggregated, filtered through a 70- μm filter to eliminate clumps, and counted. Approximately 5×10^7 cells were obtained for each experiment. Cells were centrifuged and resuspended into 10 ml of 0.05% trypsin to prevent aggregation. Before each run, the elutriator was sterilized with 400 ml of 70% ethanol and then rinsed with an equal amount of Eagle's MEM. Cells were injected into the elutriator while a constant flow rate of 30 ml/min of MEM was maintained through the chamber. Cell fractions were obtained by reducing the rotor speed by 100 rpm decrements and collecting 200 ml of medium at each speed setting. Fractions were centrifuged for 5-7 min at 1000 rpm, after which cells were resuspended in 10 ml CMEM and then counted. The three earliest fractions containing the purest content (~90%) of G_1 cells were used. The purity of the pooled fractions was confirmed by flow cytometric analysis. These fractions contained approximately 5×10^6 cells, ~10% of the original cell number. Cells from each run were divided so that all three assays (CFE, cytogenetic analysis, and flow cytometry) were performed on cells derived from a common pool (Figure 2.1).

X-irradiation and CFE Assay

Cells were irradiated on ice with a 150 kVp x-ray machine (Philips, Hamburg, Germany) at a dose rate of 1.2 Gy/min. The CFE assay has been optimized with respect to incubation time, feeder number, and number of cells seeded per plate. For all experiments, 6-well plates (35 mm) were prepared 24 h in advance by seeding an optimum number (5×10^4) of heavily irradiated (40 Gy) feeder cells into 4 ml of

CMEM. After cells were plated, the dishes were incubated for 2 weeks at 37°C. Colonies were then stained with methylene blue (0.66% solution in 95% ethanol) and counted. Plating efficiency was calculated as the ratio of the number of colonies formed to cells seeded. Surviving fraction was calculated as the plating efficiency of the irradiated cells divided by the plating efficiency of unirradiated cells. Data for each data point within each experiment were obtained by counting colonies from at least two separate 6-well plates. Each survival curve was performed at least twice.

Flow Cytometry

Determination of cell cycle phase fractions of samples obtained from elutriation experiments was by simultaneous measurement of PI fluorescence and fluorescence of a FITC-conjugated secondary antibody used to label a primary antibody specific for DNA. Cells were pulse labeled with 10 μ M BrdUrd for one hour immediately before collection. Cells were washed twice with cold phosphate-buffered saline, fixed in cold 70% ethanol, and then stored overnight at 4°C. Approximately 1×10^6 cells were required for each sample. Cells were centrifuged and then resuspended in 3 ml of 2.5 M HCl for 30 min, washed with 0.5% Tween 20 in phosphate-buffered saline, and then incubated for 1 h with a 1:100 dilution of mouse monoclonal anti-BrdUrd antibody (Caltag, South San Francisco, CA). After another two washes the cells were incubated for 30 min with a 1:500 dilution of goat FITC-conjugated anti-mouse antibody (Sigma Chemical Co., St. Louis, MO). This was followed by incubation with 0.5 ml of PI (10 mg/ml) for 30 min. Cells were filtered through a 37 μ m mesh and then analyzed under dual parameter (FITC *versus* PI) conditions with a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). Raw data were refined by plotting the area of the PI fluorescence signal *versus* its width and then selecting only the single cell population for further analysis. Phase fractions were determined by identifying and counting cell populations on a plot of FITC

fluorescence *versus* PI fluorescence using the CellQuest software package (Becton Dickinson). For the initial cytokinetic studies, cell cycle phase fractions were determined by measuring only PI fluorescence. For these analyses the incubation steps with anti-BrdUrd and FITC-conjugated anti-mouse antibodies were not performed. In the case of PI histogram analysis (for initial cytokinetic studies), cells within defined upper and lower limits for the G₁ population were counted.

Cytogenetic Analysis

One hour after X-irradiation, Colcemid was added at a final concentration of 0.2 μ M. Progression through the cell cycle was monitored microscopically. As soon as mitotic cells were observed, they were collected after gently shaking the flask. Chromosome preparations were made by treating cells for 20 min with hypotonic citrate (0.2% KCl, 0.2% Na citrate, 10% fetal bovine serum), fixing with 100% methanol for 10 min, washing with methanol:acetic acid (3:1), and dropping onto clean glass microscope slides. Because of potential radiation-induced cell cycle delays, multiple harvest times were used. Metaphase chromosomes were stained with 4% Giemsa for 7 min, and the frequency and type of chromosome aberrations were analyzed by examining 50 metaphase cells for each radiation dose, harvest time, and cell line.

Fluorescence In Situ Hybridization

A plasmid vector containing human chromosome 4-specific DNA sequences (pBS4) was kindly provided by J. Gray and D. Pinkel (University of California, San Francisco). The plasmid was amplified in *Escherichia coli* and purified with the Qiagen plasmid kit (Chatsworth, CA). Purified plasmid was labeled (1 μ g/reaction) with biotin by using the BioNick Labeling System (Gibco BRL, Gaithersburg, MD). Labeled DNA was separated from unincorporated nucleotides by using NucTrap Push Columns (Stratagene,

La Jolla, CA) and resuspended in 120 μ l of Nick Translation/Push Column buffer (Stratagene). The pBS4 probe was denatured with human Cot1 DNA (probe:Cot1 DNA::1:30) at 80°C for 5 min. The hybridization protocol and reagents were modified from a protocol obtained from a chromosome *in situ* kit (Oncor, Gaithersburg, MD). Briefly, slides were washed with 2X SSC (1X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) for 30 min at ambient temperature and dehydrated in an iced ethanol series (70%, 90%, 100% ethanol, 2 min/concentration). After drying, chromosomes were denatured in 70% formamide/2X SSC at 80°C for 30 s and dehydrated in an iced ethanol series (70%, 90%, 100% ethanol, 2 min/concentration). Thirty-five microliters of hybridization mix (50% formamide, 2X SSC, 10% dextran sulfate, HuCot1 DNA, and 35 ng labeled pBS4) were applied to slides under a 24 x 50-mm glass coverslip and sealed with rubber cement. After overnight hybridization at 50°C in a humidified chamber, slides were rinsed and then washed in two changes of phosphate-buffered detergent for 10 min at ambient temperature. Slides were then incubated with FITC-conjugated avidin for 20 min, also at ambient temperature. Slides were washed in three changes of phosphate-buffered detergent, and, if necessary, the fluorescent signal was amplified by subsequent incubation with anti-avidin and FITC-conjugated avidin (Oncor). In general, one round of amplification was sufficient. After FITC treatment, Antifade (Sigma) and PI were applied to slides under a glass coverslip. Metaphase chromosomes were analyzed on a Zeiss Axiomat 50 equipped with the Epi-fluorescence condenser IIIRS and a standard FITC filter set. One hundred independent metaphase cells for each radiation dose, harvest time, and cell line were analyzed for chromosomal rearrangements involving chromosome 4.

RESULTS

G₁ Growth Arrest

A time and radiation dose analysis of the percentage of cells remaining in G₁ was used to determine the rate of progression from G₁ to S phase. Flow cytometry profiles of asynchronous cells irradiated with various doses revealed that the percentage of U87-lux.8 cells (wt p53) remaining in G₁ at 24 h increased as a function of radiation dose up to 4 Gy (Figure 2.3). For these cells the rate of exit from G₁ was initially fast, followed by a slower component. Primarily, it was this later movement of cells into S phase that was inhibited after irradiation. Sixty percent of U87-lux.8 cells (normalized to the 0 h G₁ percentage) remained in G₁ 24 h after irradiation with 4 or 6 Gy. U87-175.4 cells (with a dominant-negative p53 mutation) had a narrower range of cells remaining in G₁ after irradiation (Figure 2.3): Most U87-175.4 cells remained in G₁ for 3 h and then rapidly entered S phase over the next 6 h with similar kinetics regardless of whether or not they were irradiated. Of note, approximately 25% of the unirradiated population from both cell lines remained in G₁ 24 h after irradiation.

Because these cytokinetic experiments utilized cells that were asynchronous with respect to cell cycle position, we confirmed our results by using G₁ cells purified by elutriation. After the last harvest time for mitotic cells (approximately 48 h), cells were pulse-labeled with 10 μ M BrdU, collected, and analyzed by flow cytometry. Dual-parameter plots (PI and BrdU) confirmed the conclusions of the asynchronous cytokinetic experiments (Figure 2.4A). Most U87-lux.8 cells (74%) remained in G₁ 48 h after irradiation with 6 Gy (Figure 2.4B), whereas only 9% of unirradiated cells remained in G₁. Doses < 6 Gy resulted in a progressively smaller percentage of cells remaining in G₁ after plating. This large difference between irradiated and unirradiated cells was not observed in U87-175.4 cells, for which the final percentage of cells in G₁ was 8% in the

unirradiated sample and 19% after 6 Gy. It should be noted that Colcemid (0.2 μ M) arrested virtually all cells at metaphase. Cells were prevented from entering the subsequent division cycle which would have greatly complicated the interpretation of results.

Cell Survival After Irradiation

Clonogenic assays were performed to obtain a quantitative assessment of the ability of irradiated cells to survive (Figure 2.5). The survival curves for these two cell lines clearly revealed that the presence of a functional G_1 checkpoint resulted in reduced clonogenicity after irradiation. The D_q 's for U87-lux.8 and U87-175.4, the dose at which back-extrapolation of the straight portion of the curve intersects the ordinate at the 100% survival line, were 0.16 and 1.35 Gy, respectively. The D_0 's for U87-lux.8 and U87-175.4, the dose required to achieve 63% cell killing at any point along the straight portion of the curve, were 0.90 and 0.94 Gy, respectively. The D_q and D_0 values reflect the qualitative impression that the cell line U87-175.4 possesses a shoulder in comparison to U87-lux.8, whereas the final slopes are much closer in magnitude.

Chromosomal Aberrations Induced After Irradiation

The frequency of aberrant metaphase cells and the types of chromosomal aberrations observed in metaphase chromosomes, as measured by Giemsa staining, are given in Table 2.1. Aberration frequency increased in a dose-dependent manner, but there were no qualitative or quantitative differences in aberration yield between the two cell lines. The total chromosome number was similar for each cell line with a range of 69-83 chromosomes per metaphase. Exchange-type aberrations (rings and dicentric chromosomes) are thought to represent misjoining events between broken chromosomes, and interstitial deletions are misjoining events between acentric breaks on the same chromosome. Both these types of aberrations involve rejoining (repair) of DNA breaks,

albeit incorrect repair. Terminal deletions, on the other hand, are thought to represent unrepaired chromosomal breaks. There was no difference in the yield of these types of aberrations between the two cell lines. Cells with these types of aberrations generally die because of abnormal segregation of chromosome material at mitosis.

Because many chromosomal rearrangements that are not necessarily lethal cannot be analyzed by conventional assays, e.g., reciprocal translocations, insertions, and inversions, chromosomal rearrangements in chromosome 4 were investigated by means of fluorescence *in situ* hybridization. Generally, there were three copies of chromosome 4 per metaphase in unirradiated cells (Figure 2.6). Insertions and reciprocal translocations, as well as dicentrics and deletions, involving chromosome 4 were observed after irradiation (Figure 2.7). There was a dose-dependent increase in chromosomal rearrangements at doses up to and including 4 Gy (Table 2.2). The yield of rearrangements was similar in both cell lines. As expected, equal numbers of translocations and dicentrics were formed. At 6 Gy, however, more rearrangements were observed in the U87-175.4 cells, and most of those were reciprocal translocations. It should be stressed that all cytogenetic data are from cells that reached mitosis of the first division cycle after irradiation. Colcemid prevented cells from entering the second division cycle.

DISCUSSION

In this study, we examined whether activation of a specific cell cycle checkpoint (manifested as a G₁ arrest) modulated cell survival by affecting the degree of DNA damage, as measured by chromosomal aberrations. The presence of wt p53 function (U87-lux.8) resulted in a well-defined G₁ arrest that was commensurate with the radiation dose

delivered to a population of G₁ cells. In addition, the total number of chromosomal aberrations (Table 2.1), was the same in cells that possessed the G₁ checkpoint compared to those that did not. Yet the presence of the G₁ checkpoint did not serve a protective function, because U87-lux.8 cells demonstrated a greater degree of cell killing at all radiation doses (Figure 2.5). Our conclusion is that the presence of a G₁ checkpoint, in the cells studied, did not affect radiation survival by a mechanism that reduces the amount of DNA damage. Indeed, reciprocal translocations in U87-175.4 cells, as measured by chromosome 4 fluorescence *in situ* hybridization, were *increased* after 6 Gy.

If the presence of a G₁ checkpoint reduces cell survival without a corresponding increase in chromosomal aberrations, then are there other possibilities to account for these results? The response of G₁ cells to irradiation is demonstrated by flow cytometry profiles of purified G₁ U87-lux.8 cells 48 h after irradiation (Figure 2.4): Increasing doses of radiation resulted in larger percentages of cells remaining in the G₁ phase of the cell cycle. These data are consistent with the findings of Di Leonardo *et al*, that a p53-dependent terminal G₁ arrest is responsible for increased cell killing after X-irradiation and may represent the final stage of a pathway that eliminates damaged cells from the dividing population (36). This may be analogous to the p53-dependent apoptotic response observed in thymocytes after irradiation (27, 115). It should be noted that because the parent cells used for transfection were derived from human glioma cells, apoptotic pathways may be deficient in these cells. It is clear that this model is restricted by our experimental technique to those cells in G₁. How cells in other phases of the cell cycle respond to irradiation is unknown, but if differences can be established, then a role for p53 may be proposed at other locations in the cell cycle.

The radiation survival curves were fit by means of the multitarget single-hit theory for radiation damage (3). The survival curve for U87-lux.8 demonstrated increased cell killing (i.e., reduced clonogenic survival) for a given dose of radiation. This is the

opposite of what one would expect if activation of the G₁ checkpoint allowed time for repair. The reduced cell survival for cells with a G₁ checkpoint (wt p53) suggests that a given amount of DNA damage is translated to a higher degree of cell killing compared to the dominant-negative clone. There are two possible explanations for this. Either cellular repair is being hindered, or another pathway, such as apoptosis or senescence, is responsible for removing damaged cells from the cycling pool. Because overall clonogenic survival is reduced, it would seem that this alternative pathway removes a greater percentage of cells than when the G₁ checkpoint is absent. The absolute sensitivity of cells to DNA damage sustained is reflected in the higher cell survival of U87-175.4 cells, which do not demonstrate a large G₁ arrest. In these two cell lines only a small minority of cells undergo apoptosis after radiation treatment (227), so it is plausible that the G₁ arrest observed at the first division cycle in U87-lux.8 cells may account, in part, for the reduced clonogenicity after irradiation. The effect of an intact G₁ checkpoint may also affect subsequent cell cycles although this was not tested by our methodology.

The relationship between cell killing and the formation of chromosomal aberrations is well established (10, 19, 20, 110, 163). It is believed that ionizing radiation causes DNA double-strand breaks, whose misrepair leads to chromosomal aberrations such as translocations and dicentrics. Measurement of chromosomal aberrations provides a direct assessment of the degree of DNA damage and the extent of repair that has occurred. In our cell lines the frequency and type of total chromosomal aberrations (measured by analyzing Giemsa stained chromosomes) were similar for all doses of radiation used (Table 2.1). Based on the times of collection, the maximal number of mitotic cells was collected at times slightly later than those of unirradiated control cells. This may represent a true G₁ delay or delay through S phase. Distinguishing these two options was beyond the scope of this study. However, there were no differences in chromosomal aberrations in mitotic cells collected from sequential time points, as determined by conventional cytogenetic analysis.

Of interest, however, were the results obtained by fluorescence *in situ* hybridization. At the highest dose of radiation used, 6 Gy, there was a large increase in reciprocal translocations over dicentric chromosomes and their associated fragments in those cells lacking functional p53 protein (U87-175.4). Genomic instability is associated with cells that lack p53, and the G₁ checkpoint (112). Because translocations are generally considered compatible with cell survival, and dicentrics are not, this raises the interesting possibility that increased reciprocal translocations may contribute to ongoing genomic instability in following generations. It also suggests that the p53-dependent pathway activated after X-irradiation may have greater sensitivity for certain types of chromosomal aberrations. There is also the possibility that rearrangements observed after 6 Gy may have conferred a survival advantage for U87-175.4 cells. This does not explain, however, why these cells showed increased cell survival at the lower doses where chromosomal aberrations were similar in type. It can be speculated that cells that sustain DNA damage, yet still remain viable, are susceptible to genomic instability and possibly, tumor initiation or progression.

In summary, by using glioma cell lines that differed in functional p53 status, we demonstrated that X-irradiation causes a prolonged G₁ arrest in cells with wt p53. As well, cells that possess a G₁ checkpoint show increased cell killing by the clonogenic assay. These results do not support the model that the function of the G₁ checkpoint is to allow increased time for repair of radiation-induced DNA damage. Those cells that are able to reach the first mitosis after irradiation do not have increased frequency of chromosomal aberrations after 1, 2 or 4 Gy irradiation. In fact, there is an increase in reciprocal translocations in cells that lack a G₁ checkpoint, but only after 6 Gy.

Table 2.1. Chromosomal aberration yields in U87-lux.8 and U87-175.4 cells after exposure to ionizing radiation in G₁

Cell line & Treatment	Time ^a (h)	% Aberrant	Exchanges	Acentric rings; IDs ^b	Terminals ^c
U87-lux.8 0 Gy	18	4	2	0	0
	18	2	0	0	1
	27	2	0	0	1
	27	0	0	0	0
	40	0	0	0	0
	40	2	0	0	1
	Mean	1.7	0.3	0	0.5
	16.5	2	2	0	1
	18	0	0	0	1
	27	6	3	0	0
U87-175.4 0 Gy	28	4	1	0	1
	40.5	0	0	0	0
	41.5	2	0	0	1
	Mean	2.3	1	0	0.7
U87-lux.8 1 Gy	18	36	12	2	6
	18	24	8	0	5
	27	26	9	2	9
	27	38	10	3	11
	40	42	15	3	8
	40	32	10	3	8
	Mean	33	9	2.2	7.8
	16.5	28	9	2	7
	18	30	9	3	6
	27	38	11	1	7
U87-175.4 1 Gy	28	38	9	1	11
	40.5	32	9	1	6
	41.5	40	11	7	9
	Mean	34.3	9.7	2.5	7.7
					cont'd

Cell line & Treatment		Time (h)	% Aberrant	Exchanges	Acentric rings; IDs	Terminals
U87-lux.8	2 Gy	18	56	28	11	21
		18	68	28	8	17
		27	54	22	7	16
		27	60	27	7	23
		40	70	29	20	29
		40	56	23	14	18
		Mean	52.3	26.2	11.2	20.7
U87-175.4	2 Gy	16.5	52	20	10	16
		18	48	21	7	16
		27	64	24	13	20
		28	56	23	9	22
		40.5	54	27	4	9
		41.5	64	26	11	19
		Mean	56.3	23.5	9	17
U87-lux.8	4 Gy	18	94	72	24	34
		18	84	53	39	38
		27	84	55	35	33
		27	86	58	39	44
		40	96	71	45	51
		40	94	75	38	42
		Mean	89.7	64.0	36.7	40.3
U87-175.4	4 Gy	16.5	90	63	30	36
		18	90	55	38	39
		27	92	70	26	45
		28	80	60	28	43
		40.5	96	80	41	43
		41.5	94	75	44	41
		Mean	90.3	67.2	34.5	42.8

cont'd

Cell line & Treatment	Time (h)	% Aberrant	Exchanges	Acentric rings IDs	Terminals
U87-lux.8 6 Gy	18 ^d				
	18 ^d				
	27	98	78	46	51
	27	96	78	45	43
	40	100	119	90	72
	40	100	108	78	61
	Mean	98.5	95.8	64.8	56.8
U87-175.4 6 Gy	16.5	100	115	40	55
	18	100	85	45	57
	27	100	93	57	48
	28	100	104	57	64
	40.5	96	97	49	43
	41.5	98	129	83	64
	Mean	99	103.8	55.2	55.2

^a Time after X-irradiation that mitotic cells were collected. The pairs of time points for each cell line at each dose point are the collection times from separate duplicate experiments. (For example, mitotic U87-175.4 cells, after 6 Gy, were collected at 16.5, 27 and 40.5 h after irradiation for the first experiment and at 18, 28 and 41.5 h for the repeat experiment.)

^b Interstitial deletions.

^c Terminal deletions were defined as acentric fragments greater in size than the width of a chromatid arm. Each asymmetric exchange was arbitrarily assigned one terminal deletion which was subtracted from the total number of acentric fragments counted. Those acentric fragments remaining were grouped as terminal deletions.

^d Insufficient metaphase cells obtained for cytogenetic analysis.

Table 2.2. Chromosomal rearrangements determined by fluorescence in situ hybridization of a biotinylated chromosome 4-specific DNA library ^a

Cell Line & Treatment ^b	Time (h)	% Aberrant	Normal B	Translocation Ba	Ab	Dicentrics AB	ab	Fragment b	Complex aBa, AAb aBA
U87-lux.8	40	0	377	0	0	0	0	0	0
0 Gy	40	1.1	369	2	2	0	0	0	0
U87-175.4	40	0	355	0	0	0	0	0	0
0 Gy	40.5	0.3	397	1	0	0	0	0	0
U87-lux.8	40	1.9	259	2	0	2	1	0	0
1 Gy	40	2.8	288	1	1	2	0	4	0
U87-175.4	40	2.0	346	2	3	1	1	0	0
1 Gy	40.5	1.5	339	2	0	0	0	3	0
U87-lux.8	40	2.4	294	2	2	2	1	0	0
2 Gy	40	3.2	283	3	3	1	1	1	0
U87-175.4	40	5.9	324	6	4	5	3	1	0
2 Gy	40.5	4.8	399	4	8	3	2	2	0
U87-lux.8	40	7.6	301	8	5	4	3	3	0
4 Gy	40	8.8	182	6	4	3	2	1	0
U87-175.4	40	8.2	331	6	6	9	6	0	0
4 Gy	40.5	16.2	308	14	12	12	8	1	3
U87-lux.8	40	9.3	258	5	4	6	5	4	0
6 Gy	40	10.5	133	5	4	3	0	1	1
U87-175.4	40	20.7	294	21	21	9	3	4	3
6 Gy	40.5	24.8	322	31	33	8	8	0	0

^a Chromosome rearrangements observed after fluorescence *in situ* hybridization were classified according to the PAINT terminology (205).

^b Data are presented for replicate experiments done on different days.

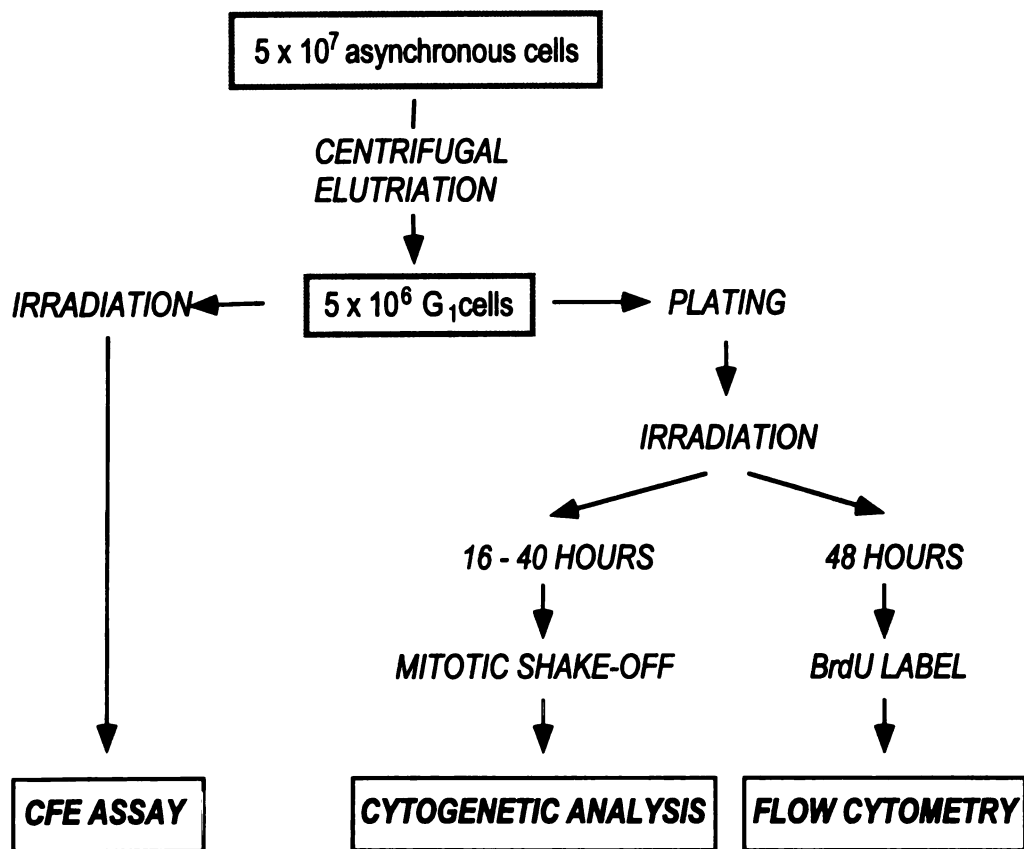


Figure 2.1. Flow chart describing the analysis of purified G₁ cells obtained by elutriation. The purity ranged from 85% to 95% G₁ cells.

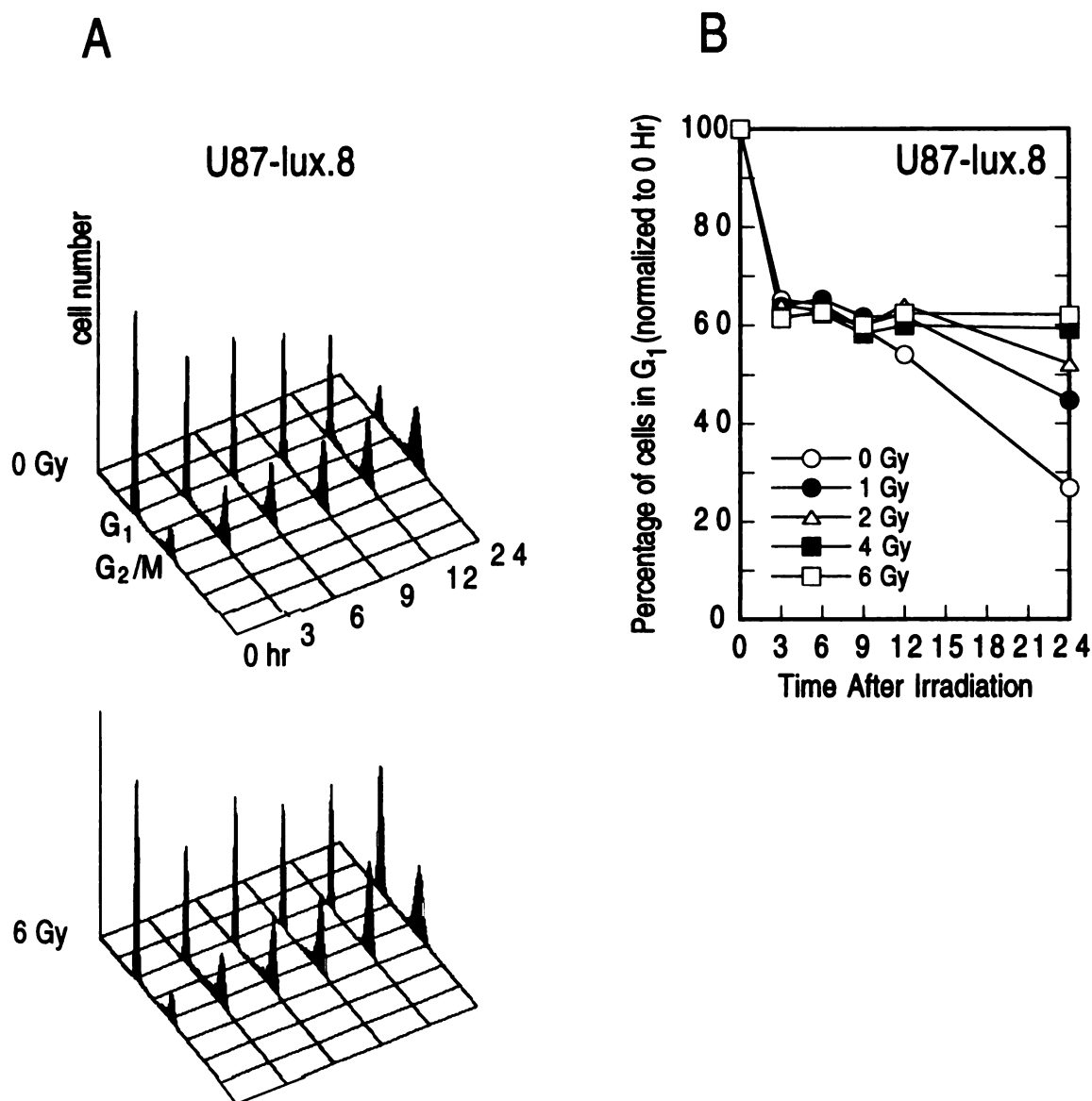


Figure 2.2. Cytokinetic results for asynchronous U87-lux.8 cells after irradiation. (A) For purposes of clarity, PI fluorescence histograms for only the control and 6 Gy samples are shown. Colcemid was added to cells at 0 h to prevent mitotic cells from dividing. (B) The percentage of cells in G₁ was measured and normalized to 0 hr and 0 Gy control samples for radiation doses of 0, 1, 2, 4, and 6 Gy and at times 3, 6, 9, 12, and 24 h after irradiation. The depletion of G₁ cells is shown as a function of time.

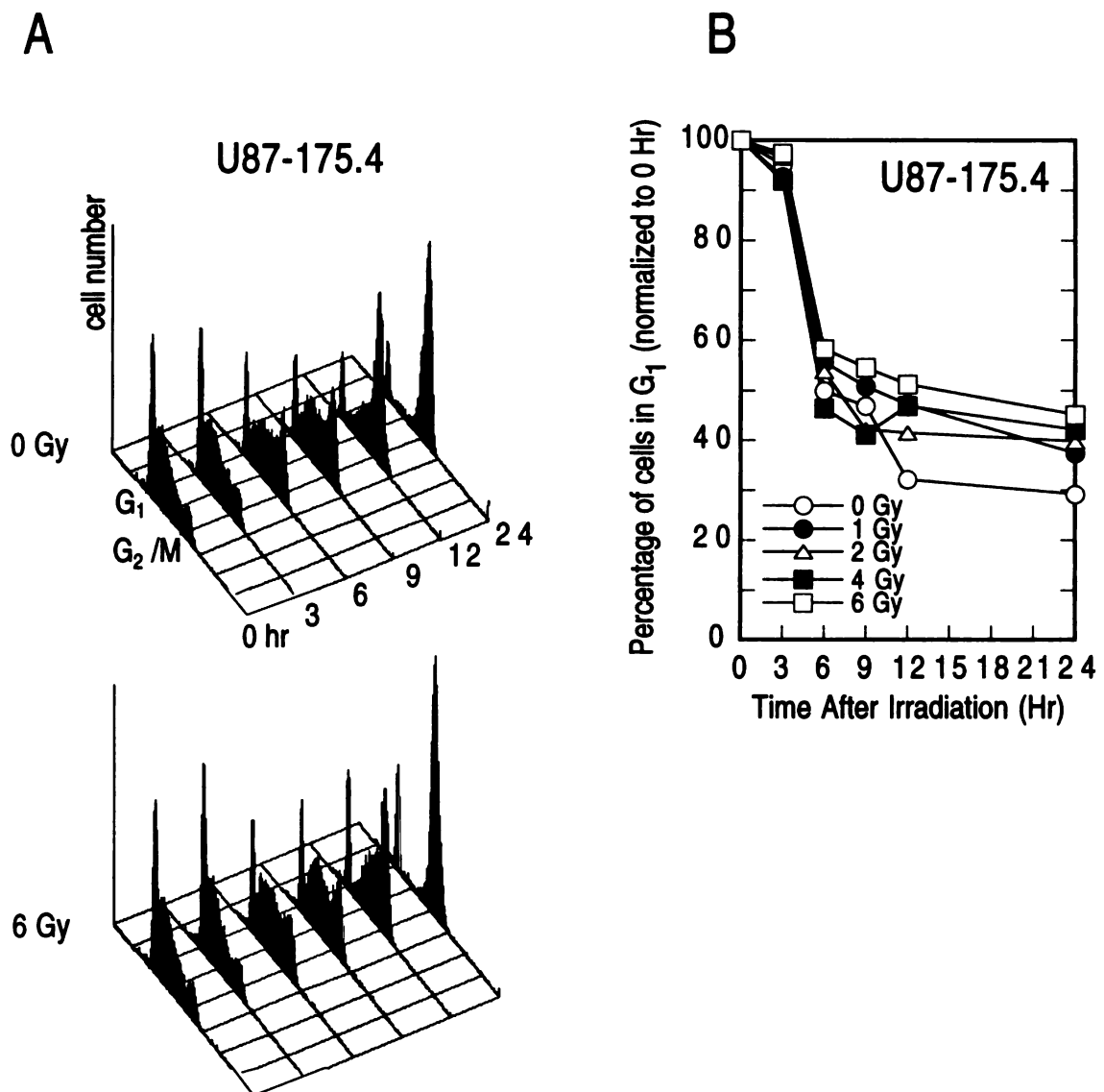


Figure 2.3. Cytokinetic results for asynchronous U87-175.4 cells after irradiation. (A) For purposes of clarity, PI fluorescence histograms for only the control and 6 Gy samples are shown. Colcemid was added to cells at 0 h to prevent mitotic cells from dividing. (B) The percentage of cells in G_1 was measured and normalized to 0 hr and 0 Gy control samples for radiation doses of 0, 1, 2, 4, and 6 Gy and at times 3, 6, 9, 12, and 24 h after irradiation. The depletion of G_1 cells is shown as a function of time.

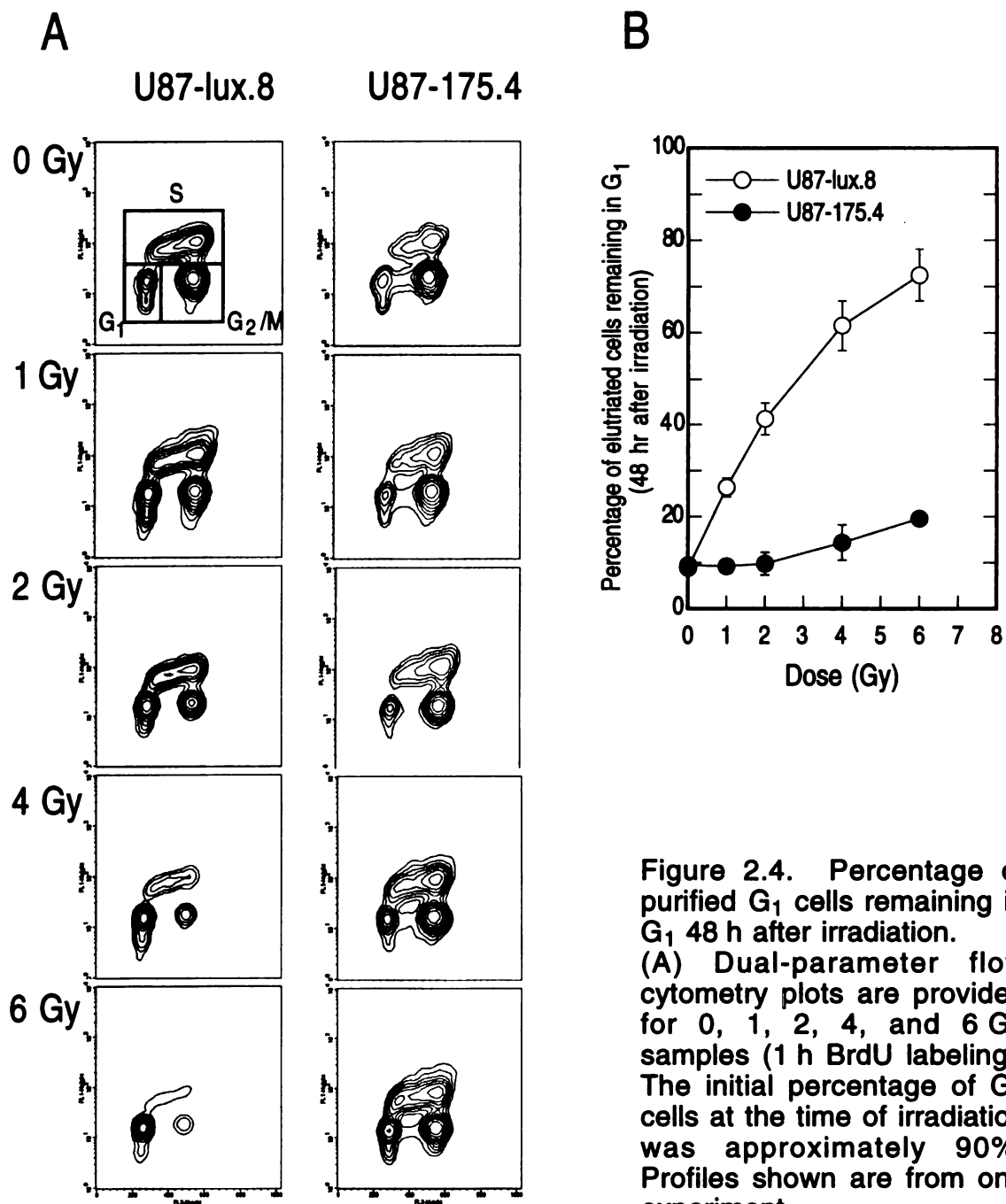


Figure 2.4. Percentage of purified G₁ cells remaining in G₁ 48 h after irradiation.

(A) Dual-parameter flow cytometry plots are provided for 0, 1, 2, 4, and 6 Gy samples (1 h BrdU labeling). The initial percentage of G₁ cells at the time of irradiation was approximately 90%. Profiles shown are from one experiment.

(B) The percentages of G₁ cells from the flow cytometry profiles from (A) are presented as a function of dose for both cell lines. Error bars represent the standard error of the mean of data obtained from two separate experiments.

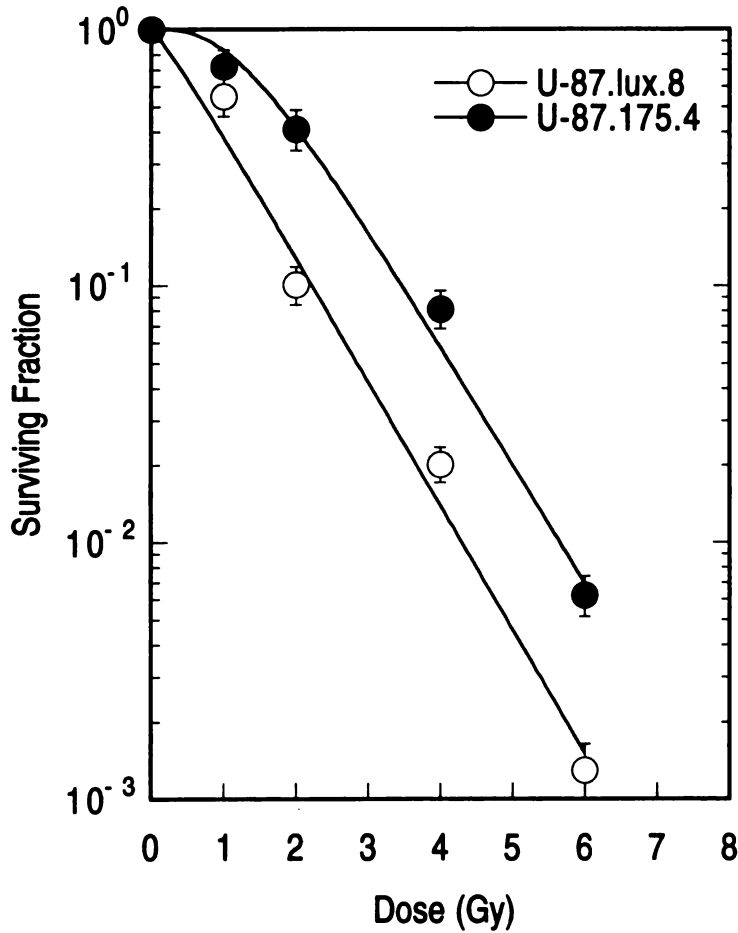


Figure 2.5. Survival curves for U87-lux.8 and U87-175.4 cells. Data are from irradiation of G_1 cells obtained by elutriation and are representative of at least two separate experiments. Curves were fit according to the multitarget single-hit formulation: $SF = 1 - (1 - e^{-qD})^n$; where SF is the surviving fraction, q is the inactivation coefficient, D is the dose of radiation, and n is the target multiplicity (3). The quasi-threshold dose (D_q) is given by the formula: $D_q = D_0 \ln n$. Error bars represent the upper and lower bounds of a 95% confidence interval for normalized data, as calculated by Fieller's Theorem (48, 56).

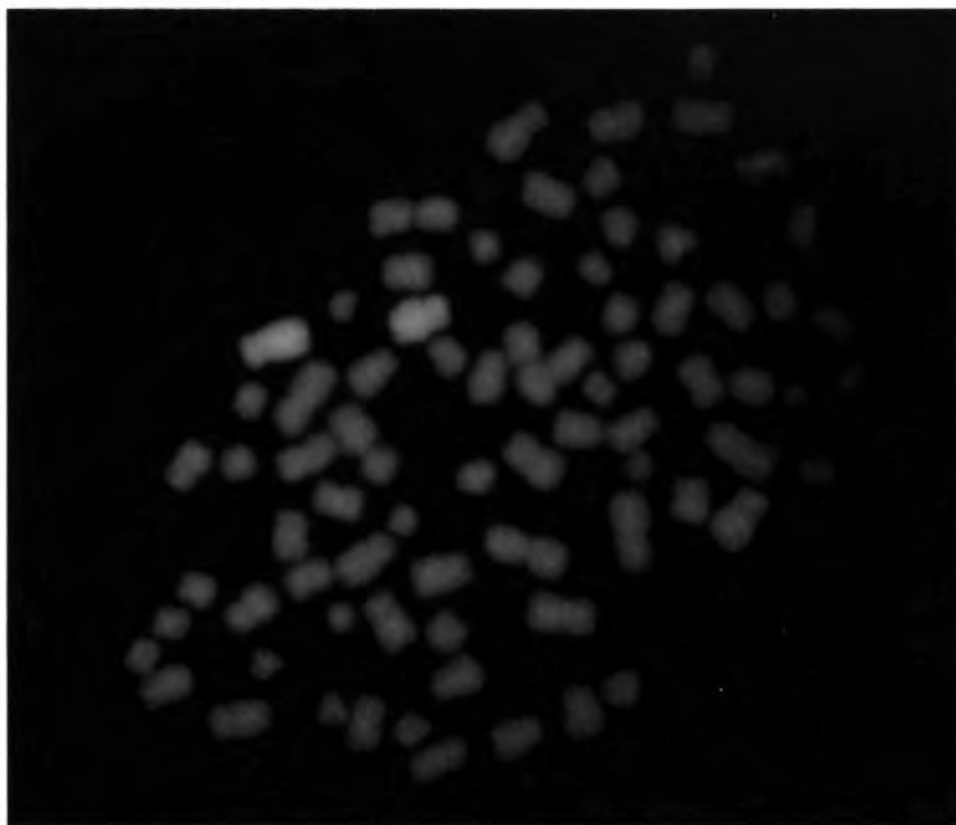


Figure 2.6. Metaphase chromosomes from control U87-lux.8 cells. Fluorescence *in situ* hybridization of a chromosome 4-specific DNA library (yellow fluorescence) to metaphase chromosomes from an unirradiated cell. Three copies of chromosome 4 are present. The other chromosomes are counterstained with PI (orange fluorescence).

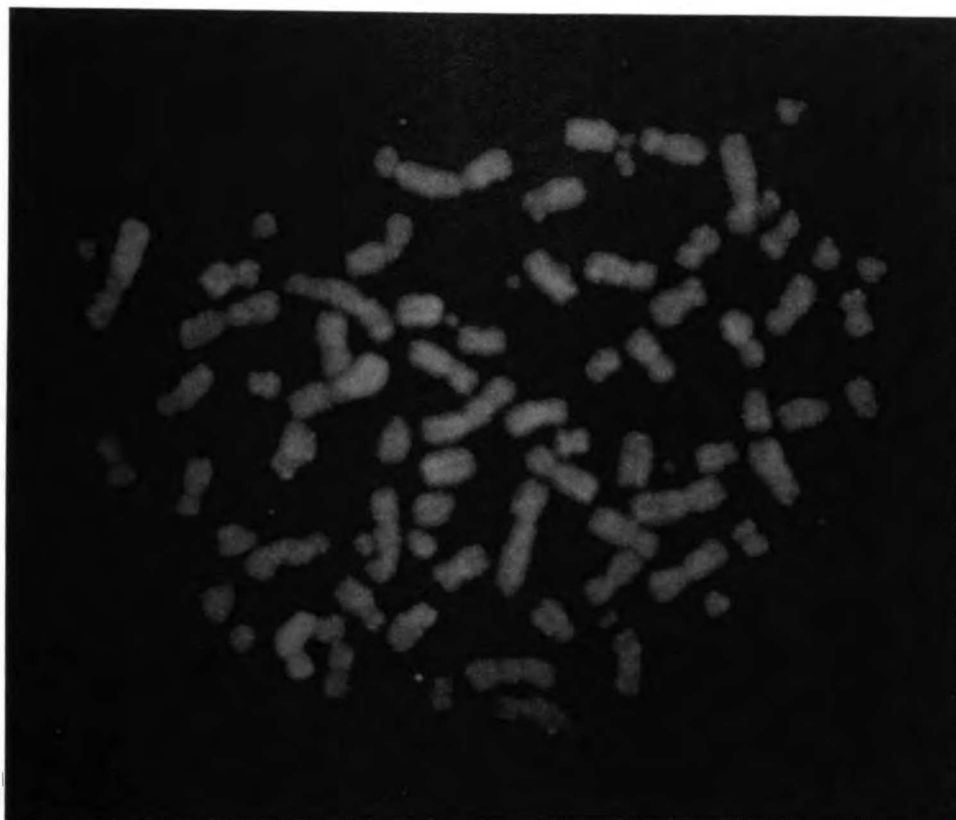


Figure 2.7. Metaphase chromosomes from irradiated U87-lux.8 cells. Fluorescence *in situ* hybridization of a chromosome 4-specific DNA library to metaphase chromosomes from a cell irradiated with 6 Gy. Two rearrangements involving chromosome 4 and dicentrics involving other chromosomes are obvious in this metaphase.

Chapter 3 The Radiation Age Response of Human Glioma Cells Differing in p53 Function

SUMMARY

The clonogenic potential of an irradiated population of cells is affected by numerous factors. Previous reports have demonstrated that cells with wild-type p53 have increased radiation sensitivity compared to cells with mutant p53. Survival also varies in most mammalian cells depending upon the position of cells in the cell cycle during irradiation, a phenomenon known as the age response. To determine if p53 affects the radiation age response we studied isogenic glioma cell lines which differ in p53 function. Measuring the age response is important for understanding mechanisms underlying how cycling cells, particularly those in human tumors, are effected by ionizing radiation. Using the standard clonogenic assay to measure surviving fraction, centrifugal elutriation to isolate cell populations with varying percentages of cell cycle phases, and flow cytometric analysis of cell cycle phases, we determined the age response of three human glioma cell lines. Although pure S phase populations were not obtained, fractions with the highest S phase percentage had the highest surviving fraction. Our results suggest there is a general increase in radiation sensitivity of wild-type p53 cells across all cell cycle phases. Other reasons contributing to the increased sensitivity of wild-type p53 cells include, *i*) a large fraction of cells in G₁, a radiosensitive phase of the cell cycle, *ii*) a reduced fraction of cells in S phase, a radioresistant phase, and *iii*) a narrower range of clonogenic survival from the most sensitive to most resistant cells as compared to mutant p53 cells.

INTRODUCTION

Cell survival after ionizing radiation is modified not only by environmental factors but also by the genetic background of the irradiated cells. For example, cells with certain mutations of the p53 tumor suppressor gene have decreased sensitivity to radiation compared to cells expressing wt p53 (14, 15, 63, 98, 182). It has been proposed that the function of wt p53 is to prevent damaged cells (i.e. following X-irradiation, treatment with chemotherapeutic agents, etc.) from continuing in the cell cycle thereby causing reproductive death (72, 95). Conversely, mutations in genes such as ATM (ataxia-telangiectasia mutated) and SCID (severe combined immunodeficiency) cause increased sensitivity to ionizing radiation (12, 18, 29, 70, 90, 97, 140, 171). All of these genes express proteins which are believed to be components of signal transduction pathways that are involved in the recognition and/or repair of cellular damage. We are particularly interested in the relationship between p53 function and radiation because this gene is, *a*) frequently mutated in human tumors and, *b*) is a member of a signal transduction pathway that is well-understood.

There are probably several reasons why wt p53 cells are more resistant to radiation. In some cases, irradiation triggers p53-dependent apoptosis which decreases overall survival (27, 115, 226). In addition, it was recently shown that DNA DSBs directly increase p53 protein levels eventually leading to a cell cycle arrest in late G₁ (88, 93, 143). The actual cell cycle arrest is mediated through transcriptional activation of p21, a cyclin-dependent kinase inhibitor (CKI) (64, 222, 229). In certain cell types irradiated cells do not re-enter the cell cycle but remain in G₁ in a senescence-like state (36, 227).

It is well known that cells in different phases of the cell cycle have varying sensitivities to ionizing radiation. Typically, mitotic and G₂ phase cells are the most sensitive to ionizing radiation, S phase cells are the most resistant, and G₁ phase cells have

varying sensitivities depending upon the length of G_1 . This phenomenon, the radiation age response (66), was originally described by investigators using rodent cells (35, 146, 198), and more recently in human cells (16, 160, 197, 219). While the exact biological basis of the age response is not known, it is probably closely related to biochemical events during the cell cycle. It was recently reported that components of transcription machinery participate in nucleotide excision repair (28, 47, 172). By analogy, it is possible that proteins capable of repairing DNA strand breaks may be associated with DNA polymerase and are more active during S phase. Conversely, the increased radiosensitivity of mitotic cells may be explained by the absence of active DNA metabolism, i.e replication, transcription, and repair. Other factors, such as chromatin conformation, may also affect cell survival (44).

The extent of the radiation age response plays an important role during clinical radiotherapy when reassortment occurs (66). Irradiating asynchronously cycling cells causes limited synchronization by killing a larger proportion of cells in sensitive phases of the cell cycle, leaving a surviving population enriched with cells in resistant cell cycle phases. By affecting the extent of reassortment, the age response may influence clinical response to a timed dosage schedule, i.e. with fractionation.

The experiments described in this study extend work previously reported by our group (63, 65, 227) and address a specific question: Does p53 function affect the radiation age response? By using centrifugal elutriation to fractionate asynchronous cell populations, we determined the magnitude of the radiation age response in three human glioma cell lines. In addition, by comparing the age response, clonogenic survival of cells in plateau and log phase growth, and cell cycle distributions, we attempt to explain why irradiated wt p53 cells show increased clonogenic survival compared to mutant p53 cells.

MATERIALS AND METHODS

Cell Lines

The cell line U-87 MG was originally derived from a malignant human glioma. U-87 MG cells express wt p53 protein and demonstrate a G₁ arrest. As described elsewhere, U-87 MG cells were used to develop isogenic cell lines differing in p53 status (227). Briefly, recombinant retroviral constructs, encoding either the firefly luciferase (lux) gene as a control or a human p53 cDNA carrying a dominant-negative mutation (codon 175^{arg→his}) under the control of the Moloney MuLV long terminal repeat (23), were used to infect U-87 MG cells. Both constructs contain the neomycin-resistance gene with selection occurring in medium containing the antibiotic G418 (400 µg/ml). Two clones, U-87-lux.8 and U-87-175.4, were selected for further studies. U-87-lux.8 cells (transfected with the vector and *neo* gene only) demonstrate a dose-dependent X-ray-induced G₁ arrest while U-87-175.4 cells (transfected with a dominant-negative p53 mutant) lack a G₁ arrest (63, 227). Stock cultures of U-87 MG cells were maintained as exponentially-growing monolayer cells in CMEM consisting of Eagle's medium supplemented with nonessential amino acids, glutamine, and 10% fetal calf serum. Cultures were incubated at 37°C in an humidified atmosphere containing 5% CO₂ and 95% air. U-87-lux.8 and U-87-175.4 cells were grown under the same conditions except that media contained G418.

Cell Survival

Survival curves for cells in log phase growth were generated by seeding exponentially growing cells into T-75 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) two days before irradiation. Irradiation of cells in plateau phase growth was done six days after seeding cells into tissue culture flasks when they had reached

confluency. Cells were irradiated on ice in tissue culture flasks, trypsinized, and counted with a Coulter Counter (Hialeah, FL) prior to plating. Clonogenic survival was measured by the CFE assay which was optimized with respect to incubation time, feeder number, and number of cells seeded per plate. For all experiments, six-well plates (35 mm) were prepared 24 h in advance by seeding an optimum number (5×10^4) of heavily irradiated (40 Gy) feeder cells into 4 ml of CMEM. After cells were plated, the dishes were incubated at 37°C in an humidified atmosphere containing 5% CO₂ and 95% air. Incubation of the colonies continued for 2 to 3 weeks; the actual time required to form at least 50-cell colonies depended on the cell line. The colonies were then stained with methylene blue (0.66% solution in 95% ethanol) and counted.

The PE was calculated as the ratio of the number of colonies formed to cells seeded. The SF was obtained by dividing the PE of the irradiated cells by the control PE (48, 62). For elutriated cells, control PEs from all fractions were averaged to obtain a single control PE that was then used to calculate the SF for each fraction.

Centrifugal Elutriation

A Beckman (Palo Alto, CA) J2-21 centrifuge housing a JE-9 elutriation rotor was used to elutriate cells. To obtain adequate numbers of exponentially growing cells, they were seeded at low density into roller bottles containing 250 ml of CMEM. Roller bottles were gassed with air containing 5% CO₂, sealed, and kept at 37°C for three days. On the day of the experiment, cells were labeled with 10 µM BrdU for 1 h, trypsinized, disaggregated, and filtered through a 40 micron filter to eliminate clumps. For control plating efficiencies (PEs), cells were immediately centrifuged, resuspended into 10 ml of 0.25% trypsin to prevent aggregation, and then elutriated. For irradiated PEs, cells were irradiated prior to elutriation. Before each run the elutriator was sterilized with 400 ml of 70% ethanol and then rinsed with an equal amount of Eagle's MEM. Cells were injected

into the elutriator while a constant flow rate of 30 ml/min of media was maintained through the rotor. The initial rotor speed was individualized for each cell line. Fractions of enriched cells were obtained by reducing the rotor speed by 100 rpm decrements and collecting approximately 200 ml of medium at each rpm setting. Cells were centrifuged for 5 min at 1000 rpm, resuspended in 10 ml CMEM, counted, and plated into six-well plates as described above.

X-irradiation

For survival curve experiments (Figure 3.1), cells were irradiated on ice while still in tissue culture flasks using a 150 kVp X-ray machine (Philips, Hamburg, Germany) at a dose rate of 1.2 Gy/min.. For elutriation experiments (Figure 3.2), cells incubated in roller bottles were trypsinized, placed on ice in 50 ml tubes, and then irradiated; a single radiation dose was chosen for each cell line that produced approximately the same degree of cell survival. Cells not labeled with BrdU had similar plating efficiencies as labeled cells.

Flow Cytometry

Determination of cell cycle phase fractions was by simultaneous measurement of PI fluorescence and fluorescence of a FITC conjugated secondary antibody used to label a primary antibody specific for BrdU. After elutriation cells were collected, washed twice with cold phosphate-buffered saline, resuspended in cold 70% ethanol, and then stored at 4°C. Approximately 1.5×10^6 cells were collected for each sample. Cells were centrifuged and then resuspended in 3 ml of 2.5 M HCl for 30 min, washed with 0.5% Tween 20 in phosphate-buffered saline, and then incubated for 1 h with a 1:100 dilution of mouse monoclonal anti-BrdU antibody (Caltag, South San Francisco, CA). After another two washes, the cells were incubated for 30 min with a 1:500 dilution of goat FITC-conjugated anti-mouse antibody (Sigma, St. Louis, MO). This was followed by

incubation with 0.5 ml of PI (10 mg/ml) for 30 min. Cells were filtered through a 37 μ m mesh and then analyzed under dual parameter (FITC *versus* PI) conditions with a Becton Dickinson FACScan (San Jose, CA). Raw data were refined by plotting the area of the PI fluorescence signal *versus* its width and then selecting only the single cell population for further analysis. Phase fractions were determined by identifying and counting cell populations on a plot of FITC fluorescence *versus* PI fluorescence using the CellQuest software package (Becton Dickinson).

RESULTS

The largest qualitative difference in clonogenic survival is between cells irradiated in plateau phase growth compared to cells irradiated in log phase growth (Figure 3.1). U-87-lux.8 cells in log phase are more radiation sensitive than the other two cell lines in log phase, whose survival curves are very similar. This is reflected in the α and β values provided in Table 3.1. The survival curve parameters for U-87-lux.8 log and plateau phase cells differ in both their α and β components whereas the β component accounts for most of the difference between U-87-175.4 log and plateau phase cells. Although both log phase U-87 MG (the parental cell line) and log phase U-87-lux.8 cells express wt p53, the transfected cells are more radiation sensitive. While there is no conclusive explanation why U-87 MG cells are more radiation resistant than U-87-lux.8 cells (both wt p53), the presence of the *neo* resistance gene and/or G418-containing medium may reduce the radiation survival of transfected cells. (U-87 MG cells detach from the culture dish and die in media containing 400 μ g/ml of G418.) Similarly, the cell cycle distributions shown in Table 3.2 also differ between U-87 MG and U-87-lux.8 cells. Comparing the two isogenic cell lines, which are maintained under identical conditions, reveals that

U-87-lux.8 cells (wt p53) are more sensitive than U-87-175.4 cells (dominant negative p53 mutant) despite being in log or plateau phase growth.

When all three cell lines are compared during log phase growth (Table 3.2), there are differences in the fractions of cells in G₁, S and G₂/M phases. U-87-lux.8 cells have the largest percentage of cells in G₁ (65.0%) while U-87-175.4 cells have the largest S phase population (33.2%). All cell lines contain a population of cells whose DNA content is greater than G₂/M cells. These probably represent an aneuploid population. The separation of cells by centrifugal elutriation, based upon differences in cell shape and density, was consistent between experiments for each cell line. Because G₁ cells are generally the smallest and least dense (when compared to S and G₂/M phase cells), early elutriation fractions contain >90% G₁ cells (Figure 3.2). Later fractions contain increasing percentages of S and G₂/M phase cells. For U-87-lux.8 cells, however, a substantial percentage of G₁ cells persist into later fractions. By examining the side scattering flow cytometry profile of each fraction (data not shown), the size of U-87-lux.8 G₁ cells range from small to equal to G₂/M cells. This presumably accounts for the presence of G₁ cells in later fractions (Figure 3.2). One consequence is that the fraction with the maximal S phase population is 30% for U-87-lux.8 cells (fraction 5) compared to 52% for U-87-175.4 cells (fraction 6).

In general, for all three cell lines, the fractions with the largest S phase percentage have the highest SF. Comparing the SFs of individual elutriated fractions, however, revealed qualitative differences between the cell lines. For U-87 MG cells, the ratio of the maximum SF (3.4×10^{-3}) to the minimum SF (7.6×10^{-4}) is 4.3. For U-87-lux.8 cells (wt p53) the ratio of maximum SF (4.4×10^{-3}) to minimum SF (9.1×10^{-4}) is 4.8. For U-87-175.4 cells (mutant p53), this ratio is 12.7 (1.1×10^{-2} to 8.5×10^{-4}).

DISCUSSION

The goal of this study was to establish the range of the radiation age responses of glioma cell lines differing in p53 function. Consistent with previous reports, we found that cells in G₁ phase are much more radiation sensitive than those in S phase. The S phase percentage appears to have a major effect upon clonogenic survival with the most radiation resistant fractions being those with the largest number of cells in S phase. The ratio of the maximum to the minimum SF for U-87 cells (Figure 3.2) is 4.3. We can assume that a pure population of S phase cells would have an even higher ratio. For U-87-lux.8 and U-87-175.4 cells, the fractions with highest percentage of S phase cells also have the highest SF. The range appears to be greater for U-87-175.4 cells (mutant p53) compared to U-87-lux.8 cells (wt 53). The ratio of the maximum to minimum SF is only 4.8 for U-87-lux.8 cells compared to 12.7 for U-87-175.4 cells. This difference is greater than expected if the difference was attributable to the an increased percentage of radioresistant S phase cells in fraction 6 for U-87-175.4 cells compared to fraction 5 for U-87-lux.8 cells (52% *versus* 30%). Interestingly, U-87-lux.8 and U-87-175.4 cells in early elutriation fractions, which are mostly cycling G₁ cells, have similar SFs to cells in plateau phase. This suggests that despite physiological differences between confluent and cycling G₁ cells, clonogenic survival is not affected, at least in the cells we studied.

A technical difficulty that exists with measuring the age response, and any cell cycle-specific parameter, is obtaining pure cell cycle phase populations. A variety of pharmacological agents can be used to synchronize cells but these agents usually cause additional cytotoxicity independent of subsequent irradiation. Because centrifugal elutriation separates cells based upon sedimentation rate, confounding variables from pharmacological agents are eliminated and interpretation of cell survival data is simplified. A disadvantage of the elutriation technique is that while early fractions are relatively G₁

pure, later fractions have varying percentages of all three cell cycle phases. Therefore, determining the actual SFs of S, G₂, and M phase cells is possible, but usually only by indirect methods (58). In our case, an additional difficulty was the broad distribution of sizes of U-87-lux.8 G₁ cells, which presumably accounts for the persistence of a large percentage of G₁ cells in later fractions.

As mentioned earlier, an intriguing feature of wt p53 cells is their increased sensitivity to ionizing radiation (14, 15, 63, 98, 182). It should be noted that these cell lines do not undergo significant levels of p53-dependent radiation-induced apoptosis (65). There are other reasons, however, that may explain why our wt-p53 cells are more sensitive to radiation. First, at the isoeffect doses used in our elutriation studies, 6 Gy for U-87-lux.8 cells and 7 Gy for U-87-175.4 cells respectively, and taking into account of the differences in cell cycle phase distributions, the increased radiation sensitivity of wt p53 cells appears to be distributed through all cell cycle phases. This finding is important because it is consistent with evidence that supports a role for p53 in phases of the cell cycle other than G₁ (2, 33, 61, 67, 190). Second, the presence or absence of the G₁ cell cycle checkpoint may affect cell survival to varying degrees. Previous studies have shown that irradiation of G₁ cells in certain cell types (including U-87-lux.8 cells) leads to a permanent cell cycle arrest, i.e. these cells cannot re-enter S phase and are probably senescent (36, 63, 227). This process probably contributes to the reduced survival of G₁ cells. Third, asynchronous U-87-lux.8 cells have an increased percentage of cells in G₁, a relatively radiosensitive phase of the cell cycle, compared to U-87-175.4 cells (Table 3.2). The reason for this difference is unclear even though the cell cycle times for both cell lines are similar (approximately 30 hours).

Determining cell cycle phase distributions by dual-parameter flow cytometry, which relies upon measurement of cellular DNA content, has inherent limitations. For example, differences *within* cell cycle phases (i.e. early G₁ *versus* late G₁) cannot be determined

with confidence. For the same reason, because G₂ and M phase cells have the same DNA content these two phases are grouped together, and individual radiation sensitivities for these two phases cannot be determined. Another issue to be kept in mind is while the two isogenic cell lines used are genetically identical except for p53 function, there are bound to be other genetic differences between these cells and other tumor cells or non-transformed cells. Therefore, confirmatory results using other biological systems are required before conclusions regarding p53 function can be generalized.

In summary, we observe that wt p53 glioma cells and those expressing a dominant-negative p53 mutant have, within the limits of our assays, an easily demonstrable age response. The increased sensitivity of wt p53 cells is probably due to both a generalized increased sensitivity to radiation in all phases of the cell cycle as well as cytokinetic differences as compared to mutant p53 cells.

Table 3.1. Survival curve parameters.^a

Cell line		α	β
U-87 MG	(log phase)	0.18	0.040
U-87-lux.8	(log phase)	0.37	0.052
	(plateau phase)	1.019	0.025
U-87-175.4	(log phase)	0.070	0.059
	(plateau phase)	0.078	0.14

^a Survival curves shown in Figure 3.1 were fitted according to the linear quadratic model: $SF = e^{-\alpha D - \beta D^2}$.

Table 3.2. Cell cycle phase percentages of cells in log phase growth as determined by analysis of dual-parameter flow cytometry plots.

Cell Line	Cell Cycle Phase Percentages ^a			
	G ₁	S	G ₂ /M	other ^b
U-87 MG	42.6 (3.1)	27.8 (2.8)	19.5 (1.5)	9.9 (1.8)
U-87-lux.8	65.0 (3.6)	16.7 (1.1)	12.2 (0.9)	6.1 (1.6)
U-87-175.4	46.5 (8.2)	33.2 (11.6)	12.6 (0.8)	8.1 (2.9)

^a Mean values from at least three separate experiments are given with the standard deviation of the sample in parentheses. Totals do not sum exactly to 100 due to averaging.

^b Represents cells that did not fall within regions limiting G₁, S or G₂/M. The majority of these cells have DNA content greater than G₂/M cells with the minority having a random distribution. Doublets and subcellular debris were excluded prior to quantitation of cell cycle phases by gating on the FITC-fluorescence area *versus* width plot.

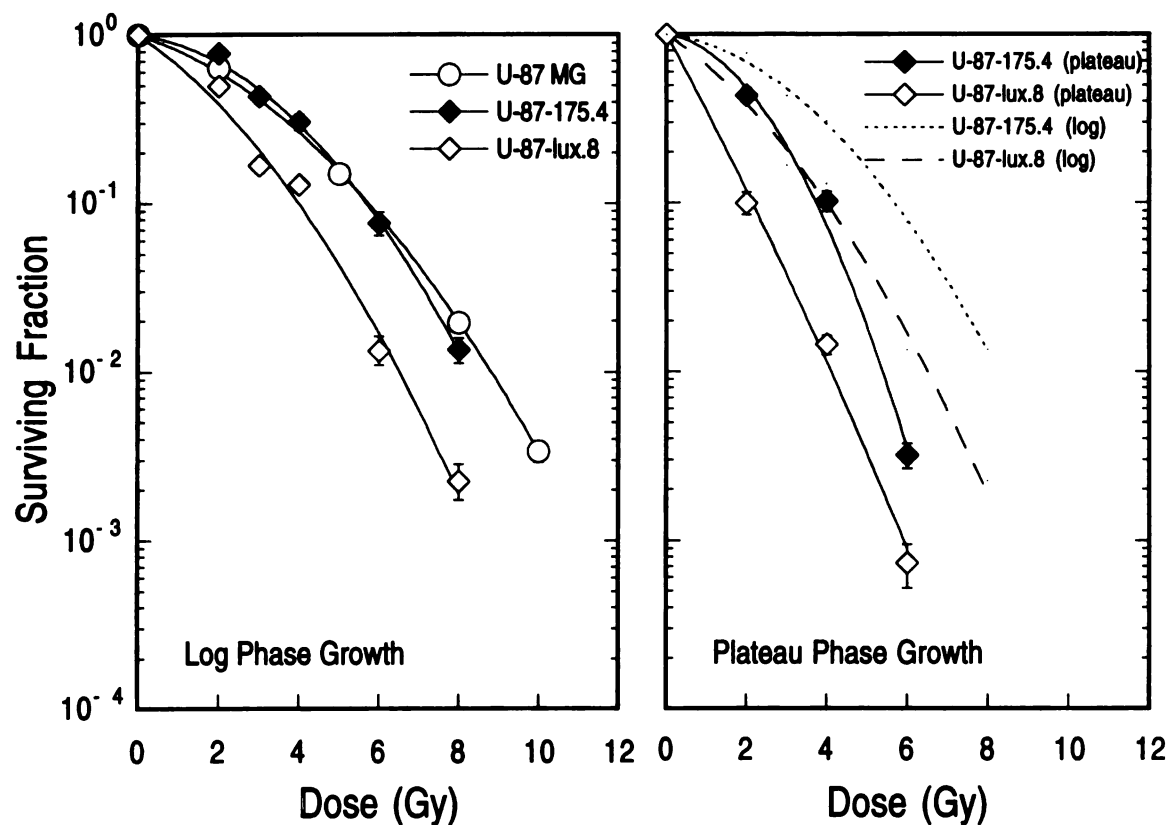


Figure 3.1. Survival curves for the human glioma cells studied. U-87-lux.8 and U-87-175.4 are isogenic cell lines derived from transfection of U-87 MG cells. The curve fits for U-87-lux.8 and U-87-175.4 cells in log phase growth are duplicated in the right-hand graph as dashed and dotted lines for purposes of comparison. Data were obtained after irradiation of asynchronous cells and are representative of at least two separate experiments. The curves were fitted according to the linear quadratic model: $SF = e^{-\alpha D - \beta D^2}$. The error bars represent the upper and lower limits of the 95% confidence interval for the point estimate as calculated by Fieller's Theorem, assuming Poisson probability (48, 62).

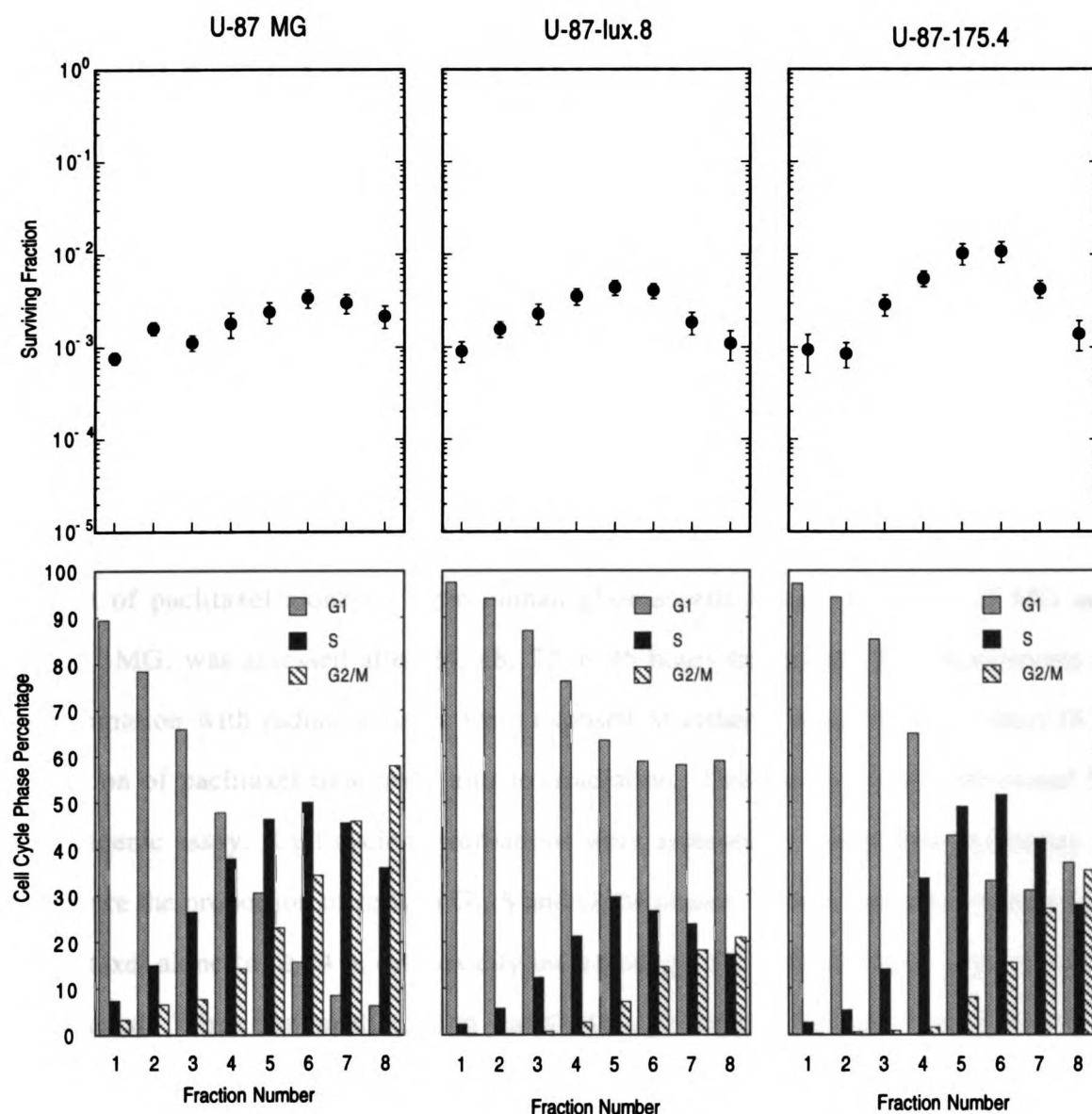


Figure 3.2. Surviving fractions (SFs) of each elutriated fraction (top) and corresponding cell cycle phase distributions (bottom) for U-87 MG cells (left, wt p53), U-87-lux.8 cells (middle, wt p53) and U-87-175.4 cells (right, dominant negative p53 mutant). U-87 MG cells were irradiated with 11 Gy, U-87-lux.8 cells with 6 Gy, and U-87-175.4 cells with 7 Gy. The mean control (unirradiated) plating efficiency used to calculate the individual SFs was 0.72 (SD: 0.092), 0.69 (SD: 0.091), and 0.41 (SD: 0.085) for U-87 MG cells, U-87-lux.8 and U-87-175.4 cells respectively. The error bars represent the upper and lower limits of the 95% confidence interval for the point estimate as calculated by Fieller's Theorem, assuming Poisson probability. Cell cycle phase distributions were measured by analyzing dual-parameter (FITC-BrdU *versus* PI) flow cytometry plots.

Chapter 4 Cytotoxicity and Cell Cycle Effects of Paclitaxel Used Alone and in Combination with Ionizing Radiation

SUMMARY

The extent of paclitaxel-induced cytotoxicity and cell cycle perturbations, when used alone and in combination with radiation, was determined in human glioma cells. The effect of paclitaxel alone on three human glioma cells lines, SF-126, U-87 MG and U-251 MG, was assessed after 24, 48, 72 or 96 hours treatment. For experiments in combination with radiation, cells were exposed to either a long (48 h) or short (8 h) duration of paclitaxel treatment prior to irradiation. Cell survival was determined by clonogenic assay. Cell cycle perturbations were assessed by using flow cytometry to measure the proportion of cells in G₁, S and G₂/M phases. When cells were treated with paclitaxel alone for ≥ 24 h, cytotoxicity increased up to a threshold dose, after which it plateaued. When treatment duration was ≤ 24 h, cytotoxicity was appreciably greater in U-251 MG cells than in SF-126 and U-87 MG cells. After 24 h of paclitaxel treatment, cells in plateau phase growth had increased survival compared to cells in log phase growth. In contrast, after 8 h paclitaxel treatment, mitotic cells had reduced survival when compared to cells from an asynchronous population. Cell cycle perturbations were consistent with the presence of a mitotic block after paclitaxel treatment although changes in other cell cycle phase fractions varied among cell lines. For experiments in combination with radiation, cytotoxicity was increased when cells were irradiated after 48 h of paclitaxel treatment but not after 8 h of treatment. The duration of paclitaxel treatment and the location of cells in the cell cycle modifies the degree of radiation cytotoxicity. The

mechanisms of paclitaxel cytotoxicity are likely to be multifactorial because varying effects are seen in different cell lines. Furthermore, it is clear that simply increasing the number of cells in G₂/M is insufficient in itself to increase the response of cells to radiation.

INTRODUCTION

Paclitaxel is a chemotherapeutic drug isolated from the bark or needles of the Pacific yew tree, *Taxus brevifolia*. It is being evaluated for clinical use in a variety of tumor types including ovarian cancer, breast cancer and malignant melanoma (25, 76, 100, 123, 176, 200). The cellular effects of paclitaxel are attributed to its strong stoichiometric binding of $\alpha\beta$ tubulin heterodimers (37, 154, 173, 174). It is postulated that as a result of paclitaxel binding, microtubule polymerization and mitotic spindle assembly proceeds incorrectly, leading to arrest in the mitotic phase of the cell cycle (82, 94). In particular, cells arrest at a metaphase to anaphase checkpoint that is activated in response to an abnormal spindle or misaligned chromosomes (55).

One reason for the interest in paclitaxel is that its effect upon microtubules differs from older agents, such as vinblastine and colchicine, which also bind tubulin molecules (81, 174, 181). Paclitaxel reportedly stabilizes or promotes microtubule polymerization while these other agents cause microtubule depolymerization. Jordan *et al*, however, reported no difference in microtubule dynamics after treatment with vinblastine or paclitaxel (82). These findings can be explained, in part, by examining the concentrations of paclitaxel used in these experiments. If paclitaxel is used at concentrations in the nanomolar range the morphological appearance of microtubules is unchanged from normal and the effect on microtubule function is the same for paclitaxel and drugs such as vinblastine (82). In this study, we examined cell cycle perturbations caused by microtubule

dysfunction using paclitaxel concentrations in the nanomolar range and treatment durations from 2 to 48 h.

Paclitaxel enhances the radiation response of a variety of cell types (26, 53, 74, 104, 188, 189, 202, 203). These results are usually explained by the observation that paclitaxel arrests cells in mitosis and mitotic cells are known to be more sensitive to radiation than cells in other phases of the cell cycle (180). This assumes that the drug-arrested cells are still viable. However, in nanomolar concentrations, paclitaxel by itself affects cellular viability to a significant degree, often producing > 90% cell killing. It is unclear if cells arrested in mitosis following paclitaxel treatment are able to re-enter the cell cycle or are irreversibly committed to permanent cell cycle arrest and cell death. Paclitaxel has been labeled as a radiation sensitizer (26, 53, 74, 104, 188, 189, 202, 203). However, strictly speaking, radiation sensitizers enhance the radiation effect without producing any cytotoxic effects by themselves (187). In most published reports of paclitaxel combined with radiation, paclitaxel by itself produces a >90% cell kill. Thus, the increased cell killing that is usually observed reflects the radiation response of a small fraction of the original cells (see Appendix B). In this study, we examined the dose response characteristics of three glioma cell lines to paclitaxel and the cytotoxicity of paclitaxel alone and in combination with irradiation. Because most experimental studies have used durations of paclitaxel treatment ≥ 24 h, the effects of shorter treatment durations upon cell cycle progression and radiation survival are not well defined. Our results using 8 and 48 h treatment durations suggest that the perturbations in cell cycle populations vary as a result of treatment duration, and may be a major factor that determines the efficacy of paclitaxel treatment when used in combination with radiation.

MATERIALS AND METHODS

Cell Culture

SF-126, U-87 MG and U-251 MG cell lines derived from human malignant glioma tumors were studied (75, 168). Stock cell cultures were maintained as exponentially-growing monolayer cells in CMEM, which consists of Eagle's medium supplemented with nonessential amino acids, glutamine, and 10% fetal calf serum. Cultures were incubated at 37°C in an humidified atmosphere containing 5% CO₂. Cells were removed from the plastic surface of the culture flasks by exposure to a mixture of saline A, trypsin (0.05%) and versene (0.02%) for 5-10 min at room temperature and then disaggregated by a sharp blow to the flask. Cells were then resuspended in CMEM and counted electronically with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Drug Treatment

Paclitaxel (Taxol™) was provided by the Bristol-Myers Squibb Company (Wallingford, CT). It was mixed as a stock solution in ethanol and used at the indicated concentrations by addition to media in flasks directly. The concentration of the stock solution was determined by measuring UV absorbance (227 nm). The concentration of ethanol in the treatment medium was < 1.0% (v:v) and did not affect plating efficiency of control cells or the radiation survival curves of cells not treated with paclitaxel (data not shown).

X-irradiation

Before irradiation, CMEM containing paclitaxel was removed, cells were rinsed once with fresh CMEM and then 15 ml of warm CMEM was placed in each flask. Flasks

containing cells were kept on ice for 10 min and then irradiated on ice, with a 150 kVp x-ray machine (Philips, Hamburg, Germany) at a dose rate of 1.2 Gy/min.

Cell Survival

Cell survival was determined with the CFE assay which was optimized with respect to incubation time, feeder number, and number of cells seeded per plate. For all experiments, 6-well plates (35 mm) were prepared 24 h in advance by seeding an optimum number (5×10^4) of heavily irradiated (40 Gy) feeder cells into 4 ml of CMEM. After cells were plated, the dishes were incubated at 37°C in an humidified atmosphere containing 5% CO₂ and 95% air. Incubation of the colonies continued for 2 to 3 weeks; the actual time required to form at least 50-cell colonies depended on the cell line. The colonies were then stained with methylene blue (0.66% solution in 95% ethanol) and counted. PE was calculated as the ratio of the number of colonies formed to cells seeded. The SF was calculated as the PE of the treated cells divided by the PE of the untreated cells. Error bars for all survival data represent the upper and lower bounds of a 95% confidence interval for normalized data points as calculated by Fieller's Theorem (48).

Calculation of the DER

The dose enhancement ratio (DER) was calculated by dividing the radiation dose causing 1 log cell kill by the radiation dose causing an equivalent cell kill after combined drug treatment and radiation.

SF of Cells in Log and Plateau Phase Growth

The SF of cells in log phase growth was measured by seeding cells into T-75 flasks at low density. After 24 h in culture, SF-126 and U-87 MG cells were treated with 100 nM paclitaxel and U-251 MG cells with 10.2 nM paclitaxel for 24 h. Cells in

plateau phase growth (confluence arrested) were obtained by maintaining cells 10-13 days in culture (depending upon the cell line). Cells were then treated with paclitaxel for 24 h with the same concentrations used for log phase cells. The PE was determined by the CFE assay. The SFs of cells in log and plateau phase growth were determined by normalizing the treated PEs to the control PEs of cells in log and plateau phase growth, respectively. Cell cycle phase fractions were measured at each data point by flow cytometry.

PE of Mitotic Cells

Cells were grown in T-175 tissue culture flasks and then treated with paclitaxel for 8 h. SF-126 and U-87 MG cells were treated with 100 nM paclitaxel and U-251 MG cells with 10.2 nM paclitaxel. The CMEM containing drug was removed and replaced with fresh CMEM. Mitotic cells were obtained by striking the flasks sharply and then collecting the floating cells with centrifugation. Cells were counted with a hemocytometer. PE of mitotic cells was determined by the CFE assay. To determine the mitotic index, an aliquot of sample was fixed in 70% ethanol, stained with diamidine phenylindole (DAPI) and examined by fluorescence microscopy. Two hundred cells were counted and scored as mitotic or interphase cells for each sample.

Flow Cytometry

Determination of cell cycle phase fractions was by simultaneous measurement of PI fluorescence and fluorescence of a FITC conjugated secondary antibody used to label a primary antibody specific for BrdU. Cells were pulse-labeled for 1 h with 10 μ M BrdU immediately before being collected, washed twice with cold phosphate-buffered saline, fixed in cold 70% ethanol, and then stored overnight at 4°C. Approximately 1×10^6 cells were collected for each sample. Cells were centrifuged and then resuspended in 3 ml of 2.5 M HCl for 30 min, washed with 0.5% Tween 20 in phosphate-buffered saline, and

then incubated for 1 h with a 1:100 dilution of mouse monoclonal anti-BrdU antibody (Caltag, South San Francisco, CA). After another two washes, the cells were incubated for 30 min with a 1:500 dilution of goat FITC-conjugated anti-mouse antibody (Sigma Chemical Co., St. Louis, MO). This was followed by incubation with 0.5 ml of PI (10 mg/ml) for 30 min. Cells were filtered through a 37 μ m mesh and then analyzed under dual parameter (FITC *versus* PI) conditions with a Becton Dickinson FACScan (San Jose, CA). Raw data were refined by plotting the area of the PI fluorescence signal *versus* its width and then selecting only the single cell population for further analysis. Phase fractions were determined by identifying and counting cell populations on a plot of FITC fluorescence *versus* PI fluorescence using the CellQuest software package (Becton Dickinson).

Treatment Protocols

First, to establish the toxicity of paclitaxel alone, we studied drug concentrations between 100 and 2000 nM to determine the optimal dose for subsequent experiments. Because U-251 MG cells proved to be extremely sensitive to this initial concentration range, we performed additional dose response experiments with drug concentrations in the 10-200 nM range for this cell line only.

To determine the effect of treatment time on drug-induced cytotoxicity, we studied treatment durations from 24 to 96 h (the cell lines studied have doubling times of 24 to 36 h under the growth conditions used). In a separate set of experiments, the combined cytotoxicity of paclitaxel and radiation was determined by pretreating cells with a concentration of paclitaxel that produced > 90% cell kill before irradiating them. Drug dose response experiments determined this to be 100 nM for SF-126 and U-87 MG cells and 10.2 nM for U-251 MG cells with a treatment duration of 48 h. Samples were collected for cell cycle analyses after 48 h paclitaxel treatment.

To determine survival for treatment durations less than 24 h, we performed clonogenic assays after 2, 6, 12, 18 and 24 h of paclitaxel treatment in all three cell lines. Samples from each time point were also examined by flow cytometry. Additional experiments were done combining shorter durations of paclitaxel treatment and radiation. For these studies we chose a treatment time (8 h) that resulted in less than 90% cell kill, yet still produced definite cell cycle perturbations. In U-251 MG cells, because 10.2 nM paclitaxel caused approximately 90% cell killing after 8 h treatment, we further reduced the drug concentration to 5.1 nM.

RESULTS

Cytotoxicity Produced by Paclitaxel

The drug dose response curves were biphasic; beyond a certain threshold concentration observed cytotoxicity plateaued (Figure 4.1). When drug exposure time was increased from 24-96 h, cytotoxicity increased in the plateau region. After 96 h treatment at a concentration of 200 nM, all cell lines studied displayed greater than 98% cell kill. SF-126 and U-87 MG cells were similarly sensitive to paclitaxel, while U-251 MG cells were more sensitive to the drug, prompting closer examination of concentrations below 200 nM.

Effect of 48 Hours Pretreatment by Paclitaxel Upon Radiation Survival and Cell Cycle Distribution

Cytotoxicity was enhanced slightly when cells were pretreated with paclitaxel before being irradiated (Figure 4.2). Because all three cell lines have doubling times between 24 and 36 h, a uniform treatment time of 48 h was chosen to ensure that all

cycling cells would reach mitosis. The DERs obtained from survival curves of SF-126, U-87 MG and U-251 MG cells were 1.2, 1.3 and 1.3, respectively.

The cell cycle phase fractions after 48 h of paclitaxel treatment (prior to irradiation) revealed different patterns for each cell type (Figure 4.3A-C). The flow cytometry profile for control SF-126 cells showed a rather typical cell cycle distribution for mammalian cells. In the treated sample, cells accumulated in the G₂/M region and the sub-G₁ region located to the left of the position of normal G₁ cells (Table 4.1). Using fluorescence microscopy, we observed that the sub-G₁ cells were actually cellular fragments of varying size, some of which contained nuclear material. We presumed they arose from fragile cells that had fragmented during preparation of cells for flow cytometry. The S phase population could not be distinguished clearly from the other populations. The population of cells with DNA content greater than G₂ cells increased in proportion from 4.4% of the control sample to 20.5% after paclitaxel treatment.

The flow cytometry profile for control U-87 MG cells was similar to SF-126 cells except that the population with greater than G₂ DNA content was larger (13.3% vs. 4.4%, Figure 4.3B, Table 4.1). For treated U-87 MG cells, 45.9% of the population arrested in G₂/M while a large population of cells (47.9%) continued to replicate their DNA without undergoing cytokinesis. This population was clearly visible to the right of the normal G₂ population. The normal S phase population completely disappeared. For paclitaxel treated U-87 MG cells (Figure 4.3B), the x-axis (DNA content) was compressed in order to bring all populations into view.

For control U-251 MG cells there was a typical cell cycle distribution although G₁ cells formed the majority of the overall population (Figure 4.3C, Table 4.1). After paclitaxel treatment normal cell cycle populations (i.e. G₁, S, G₂/M) could not be clearly identified. The majority of measured events were clustered in the sub-G₁ region. Fluorescence microscopy confirmed that these represented sub-cellular fragments. This

was presumably caused by increased fragility of U-251 MG cells after paclitaxel treatment. This phenomenon was much more prominent in U-251 MG cells than in SF-126 cells.

Effect of Shorter Treatment Times Upon Paclitaxel Cytotoxicity

We investigated cytotoxicity after shorter treatment times using the same concentrations of paclitaxel that were studied with 48 h treatment durations. Shorter treatment times produced a gradual and cumulative cell kill of approximately 90% cell kill in U-87 MG and SF-126 cells after 24 h (Figure 4.4A). U-251 MG cells had a quantitatively different response to 10.2 nM of paclitaxel; after 12 h of treatment, only 10% of U-251 MG cells retained clonogenic potential compared with 40% of SF-126 or U-87 MG cells. Flow cytometry profiles of samples from each time point (Figure 4.4B) revealed the evolution of cell cycle changes over 24 h of paclitaxel treatment. Generally, SF-126 and U-87 MG cells accumulated in G₂/M, whereas the profiles for U-251 MG cells revealed a time-dependent increase in cellular fragmentation. After 24 h, the flow cytometry profiles were virtually identical to those after 48 h (Figure 4.3). In U-251 MG cells, the accumulation of sub-G₁ events began 6 h after paclitaxel treatment.

Effect of 8 Hours Pretreatment by Paclitaxel Upon Radiation Survival and Cell Cycle Distribution

There was no change in radiation survival after paclitaxel treatment in either SF-126 or U-87 MG cells (Figure 4.5). In U-251 MG cells there was an increase in radiation survival following paclitaxel treatment. The flow cytometric profile of treated SF-126 cells showed an accumulation of G₂/M cells with an accompanying depletion of G₁ cells (Figure 4.6A, Table 4.1). Of note, the percentage of cells in S phase did not change, implying that cells continued to progress from S phase to G₂ as the G₁ population became smaller. In addition, a small population representing cellular debris (sub-G₁) was clearly

evident. The population of cells containing a DNA content greater than G_2 did not increase. In treated U-87 MG cells, the G_2/M population and the population with greater than G_2 DNA content increased. Although interpretation of the flow cytometric profile for U-251 MG cells is complicated by the presence of cellular fragments in the sub- G_1 region, there is an increase in the G_2/M population. The most dramatic change was the reduction of the G_1 population from 60.5% to 24.8%.

Non-cycling Cells are More Resistant to Paclitaxel than Cycling Cells

After 24 h paclitaxel treatment, the surviving fractions of plateau phase SF-126, U-87 MG and U-251 MG cells were 5.6, 8.5 and 5.7-fold greater than those of cycling cells, respectively (Table 4.2). This result demonstrates that paclitaxel cytotoxicity affects primarily the cycling population while quiescent cells are relatively protected. The cell cycle phase fractions of cells in plateau phase growth before paclitaxel treatment, determined by flow cytometry, show accumulation of G_0/G_1 cells with a marked reduction in the percentage of actively replicating (S phase) cells.

The PE of mitotic SF-126 cells after 8 h of paclitaxel treatment was ~16% the PE of asynchronous cells (Table 4.3); for U-87 MG and U-251 MG cells, the treated PE is approximately half the control PE. The percentage of cells in mitosis was > 90% for SF-126 and U-251 MG cells, and approximately 70% for U-87 MG.

DISCUSSION

This study highlights the cell line specificity of paclitaxel response, the importance of paclitaxel-induced cell cycle perturbations, and the varying effects observed when used in combination with radiation therapy. Our initial experiments confirm the characteristic

dose response relationship for paclitaxel reported by others (17, 104, 189). For a given drug exposure time there is a plateau in paclitaxel cytotoxicity as measured by clonogenic assay. This is true for all three cell lines examined and can be interpreted in at least two ways: first, above a certain drug concentration all available drug binding sites are occupied; and/or second, only a specific population of cycling cells is affected. Increasing the duration of paclitaxel exposure reduces clonogenicity (Figure 4.1). As well, a recent study showed that cells with longer doubling times have reduced paclitaxel cytotoxicity (6). These observations support the concept that while the majority of cells are affected during their initial transit through the cell cycle, a small number perform mitosis accurately and lose clonogenicity only if paclitaxel is present during subsequent mitoses.

While the general profiles of the dose response data are similar for all three cell lines, U-251 MG cells are clearly more sensitive. Higher paclitaxel concentrations, like those used for SF-126 or U-87 MG cells, results in very few surviving cells. Despite a 10-fold reduction in drug concentration for U-251 MG cells, survival is still markedly lower compared to the other two cell lines. A number of possible mechanisms may account for this difference in sensitivity to paclitaxel. Paclitaxel is known to be transported by P-glycoprotein, the membrane protein responsible for the multidrug resistance (MDR) phenotype (22, 101, 167). Failure to express P-glycoprotein or expression of a mutant protein would increase the sensitivity of cells to paclitaxel. Mutations in the target molecule (tubulin) can affect the response of cells to paclitaxel, presumably by altering binding affinity to paclitaxel (127). As well, under certain circumstances apoptosis is observed following treatment with paclitaxel (11, 39). It is unclear if the degree of apoptosis correlates with cellular response to paclitaxel treatment.

Others have shown that paclitaxel can enhance the radiation response of cells (26, 53, 74, 106, 188, 189, 202, 203). We therefore examined the effect of paclitaxel pre-treatment on cell cycle progression and overall survival following ionizing radiation.

Initially we chose paclitaxel concentrations that produced a quantitatively similar effect on clonogenicity (albeit in different cell lines), as those found by other investigators (26, 74, 188, 189, 202, 203). The survival curves for irradiated cells showed a modest increase in cell killing after pre-treatment for 48 h with paclitaxel, but 90 to 95% of clonogenic loss was accounted for by the action of paclitaxel alone. The small degree of radiation enhancement observed by ourselves and others (104), suggests that caution be used when extrapolating the usefulness of paclitaxel in multimodality treatment for various cancer types, particularly when radiation fractionation is being used. In a clinical situation, if the majority of cell kill is paclitaxel-related, substantial normal tissue toxicity may result.

With concentrations used in this study, 100 nM for SF-126 and U-87 MG cells and 10.2 nM for U-251 MG cells, we observed very clear cell cycle perturbations by flow cytometry. While there is evidence of a G_2/M block, the flow cytometry profiles suggest a variety of responses as a result of the presumed underlying abnormality in microtubule function. In U-87 MG and SF-126 cells, while the majority of cells are blocked in G_2/M , a sub-population continues to replicate DNA despite failing to undergo cytokinesis. This is most clearly demonstrated by the presence of a population of cells that incorporates BrdU and also has a DNA content greater than G_2 cells (Figure 4.3A and 4.3B, Figure 4.6A and 4.6B). In U-87 MG cells, after 48 h treatment, a substantial population appears to contain twice the DNA content of G_2 cells (Figure 4.3B), suggesting that initiation of DNA synthesis is not dependent upon completion of cell division. Indeed, in U-87 MG cells after 48 h of paclitaxel treatment, 48% of the cellular population continued DNA replication to some extent without cell division. While U-251 MG cells are the most sensitive to paclitaxel, fewer than 0.5% of these cells contain greater than G_2 DNA content. In U-251-MG cells there is a loss of cellular integrity in most cells as demonstrated by the predominance of many small subcellular fragments, indicating that paclitaxel treatment produced unusually fragile cells. Furthermore, the gradual increase in the sub- G_1

population from 6 to 24 h of paclitaxel treatment (Figure 4.4) accompanied by a fall in the G_2 population suggests that cell cycle progression is necessary for cellular fragmentation to occur. (Initially we suspected that this represented apoptosis of treated cells but direct microscopic examination did not reveal the characteristic nuclear appearance associated with apoptotic cells.) These results are consistent with a model where cells that can activate a mitotic checkpoint in response to an abnormal spindle (SF-126 or U-87 MG cells) have a survival advantage compared to those cells that are unable to arrest (U-251 MG).

Treatment for 8 h produced less cytotoxicity than did treatment for 48 h, but the 8 h treatment did not enhance cell killing by radiation in SF-126 and U-87 MG cells (Figure 4.5A and 4.5B), while it *increased* survival in U-251-MG cells (Figure 4.5C). These results (for U-251 MG cells) can be explained in the following manner. First, although paclitaxel treatment causes an accumulation of mitotic cells, these cells represent a population that has reduced clonogenic potential (Table 4.3). Second, there is a marked redistribution in the cell cycle after 8 h paclitaxel treatment with an increase in the G_2/M population and a large decrease in the G_1 population (Table 4.1). Third, the cell lines used in this study demonstrate a typical cell age response to radiation; where G_1 and M phase cells are more radiosensitive than S phase cells. Therefore, cell cycle redistribution may increase the number of cells in radioresistant phases of the cell cycle and consequently eliminate whatever radiation enhancement paclitaxel potentially causes.

An important cytokinetic feature of most malignant gliomas is a low labeling index ($< 10\%$) as measured by BrdU incorporation (77, 78). Based upon our results comparing cells in log *versus* plateau phase cells (Table 4.2), the non-cycling population in these tumors will be protected from paclitaxel cytotoxicity compared to actively proliferating cells. These results predict that a significant variation in tumor response can be expected in patients receiving paclitaxel depending upon the cell cycle distribution within the tumor. Furthermore, because tumors often display considerable heterogeneity, the optimal duration

of paclitaxel treatment, and timing with radiation, probably varies between tumor types and patients.

In summary, our study illustrates a wide variation in cellular response to paclitaxel, even in cell lines derived from the same tumor type. The major factors affecting paclitaxel cytotoxicity are the duration of treatment, the cell line studied, and the size of the cycling population. The use of paclitaxel as a radiation sensitizer must be regarded with some caution in view of the notable differences in cell cycle profiles and cellular survival that we and others (53, 105) have observed.

Table 4.1. Cell cycle phase fractions for control and treated cells after 48 and 8 h paclitaxel treatment

Cell Line	Treatment	Duration (h)	Cell Cycle Phase Distributions ^b			
			G ₁ (%)	S (%)	G ₂ /M (%)	>G ₂ (%)
SF-126	control	-	41.5	32.0	20.5	4.4
	100 nM paclitaxel	48	19.2	NM ^a	41.0	20.5
U-87 MG	control	-	29.6	35.5	21.0	13.3
	100 nM paclitaxel	48	1.4	NM	45.9	47.9
U-251 MG	control	-	65.3	21.4	10.8	<0.5
	10.2 nM paclitaxel	48	NM	NM	NM	NM
SF-126	control	-	31.0	34.3	22.1	12.0
	100 nM paclitaxel	8	12.1	36.0	36.7	11.8
U-87 MG	control	-	44.2	19.7	25.4	10.0
	100 nM paclitaxel	8	34.5	12.7	35.2	14.3
U-251 MG	control	-	60.5	27.2	10.9	<0.5
	10.2 nM paclitaxel	8	24.8	21.0	21.6	<0.5

^a NM (Not Measured). In these samples the indicated cell cycle population could not be clearly identified (Figure 4.3).

^b Cell cycle phase fractions were measured by counting cells located within specified regions of flow cytometric profiles as illustrated in Figures 4.3 and 4.6.

Table 4.2. Surviving fraction (SF) of log and plateau phase cells following 24 h paclitaxel treatment with cell cycle phase fractions at start of treatment

Cell line	growth phase ^a	SF ^b	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
SF-126	log phase	0.15 (0.029)	43.0	31.2	19.6
	plateau phase	0.85 (0.24)	63.8	9.19	21.5
U-87 MG	log phase	0.090 (0.011)	57.5	27.3	11.1
	plateau phase	0.77 (0.073)	79.0	2.0	14.3
U-251 MG	log phase	0.17 (0.025)	77.6	13.2	5.6
	plateau phase	0.97 (0.079)	80.1	4.0	11.3

^a Cells were treated with paclitaxel at 1 day (log phase) or 9-13 days after seeding (plateau phase). SF-126 and U-87 MG cells were treated with 100 nM paclitaxel and U-251 MG cells with 10.2 nM paclitaxel.

^b The number in parenthesis, added or subtracted from the SF, represents the upper and lower limits of the 95% confidence interval as calculated by Fieller's theorem.

Table 4.3. Plating efficiency (PE) of asynchronous cells compared to mitotic cells following 8 h treatment with paclitaxel^a

Cell line	PE (asynchronous cells) ^b	PE (mitotic cells)
SF-126	34.1 (2.6)	5.5 (0.7)
U-87 MG	40.3 (5.7)	18.5 (2.0)
U-251 MG	19.6 (4.3)	11.0 (0.7)

^a SF-126 and U-87 MG cells were treated with 100 nM paclitaxel and U-251 MG cells with 10.2 nM paclitaxel.

^b The value for the PE is the mean of two separate experiments. The number in parenthesis is the standard error of the mean.

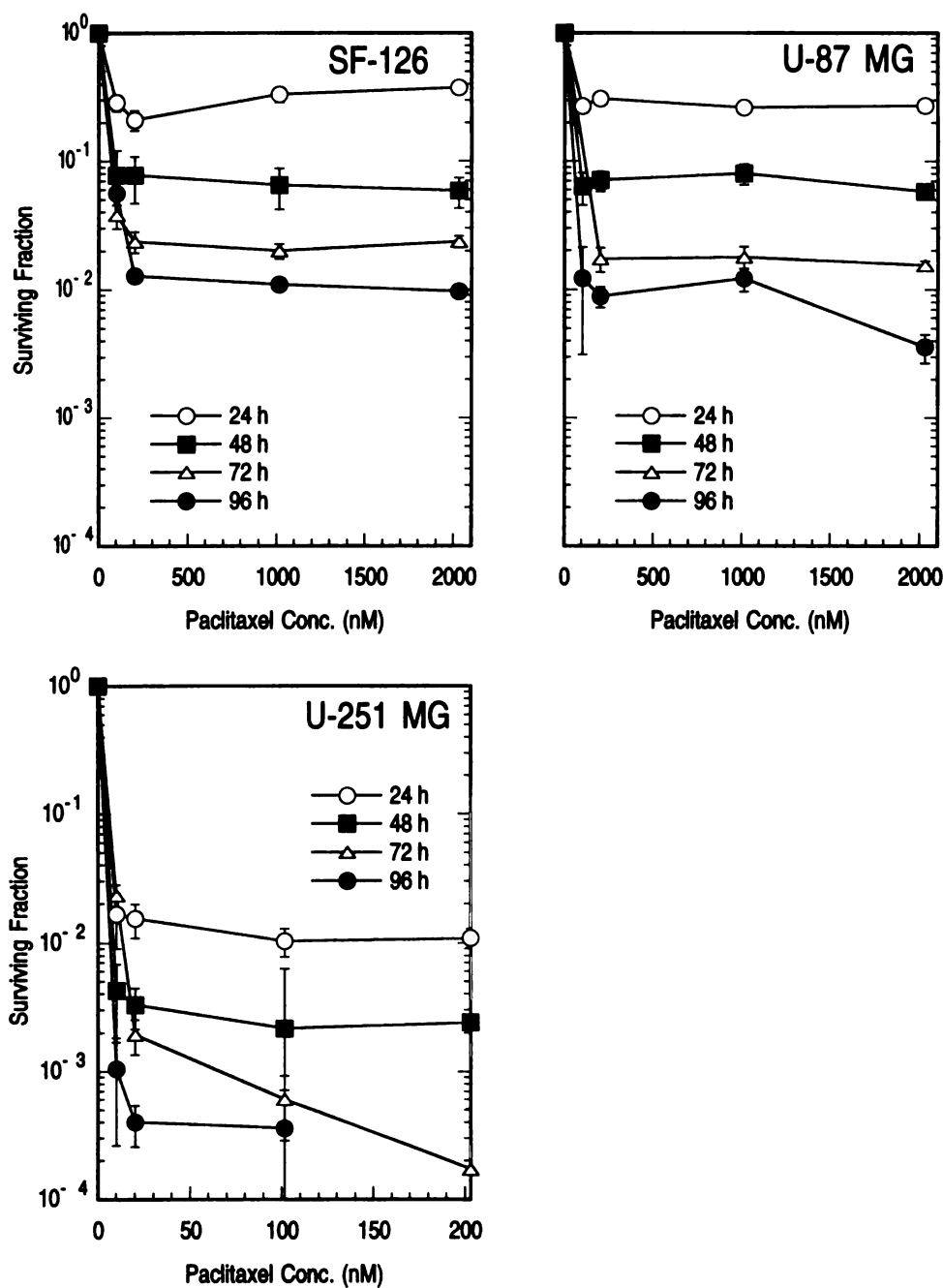


Figure 4.1. The dose response for paclitaxel for SF-126, U-87 MG and U-251 MG cells. Cells were assayed for survival after 24, 48, 72 or 96 h of treatment with graded paclitaxel concentrations. Data are shown for U-251 MG cells at drug concentrations only from 0 to 200 nM. In U-251 MG cells cytotoxicity at paclitaxel concentrations higher than 200 nM did not increase for 24, 48 or 72 h treatment (data not shown). Virtually no colonies were obtained for higher concentrations than 100 nM with a treatment duration of 96 h. All data shown are representative of at least two separate experiments.

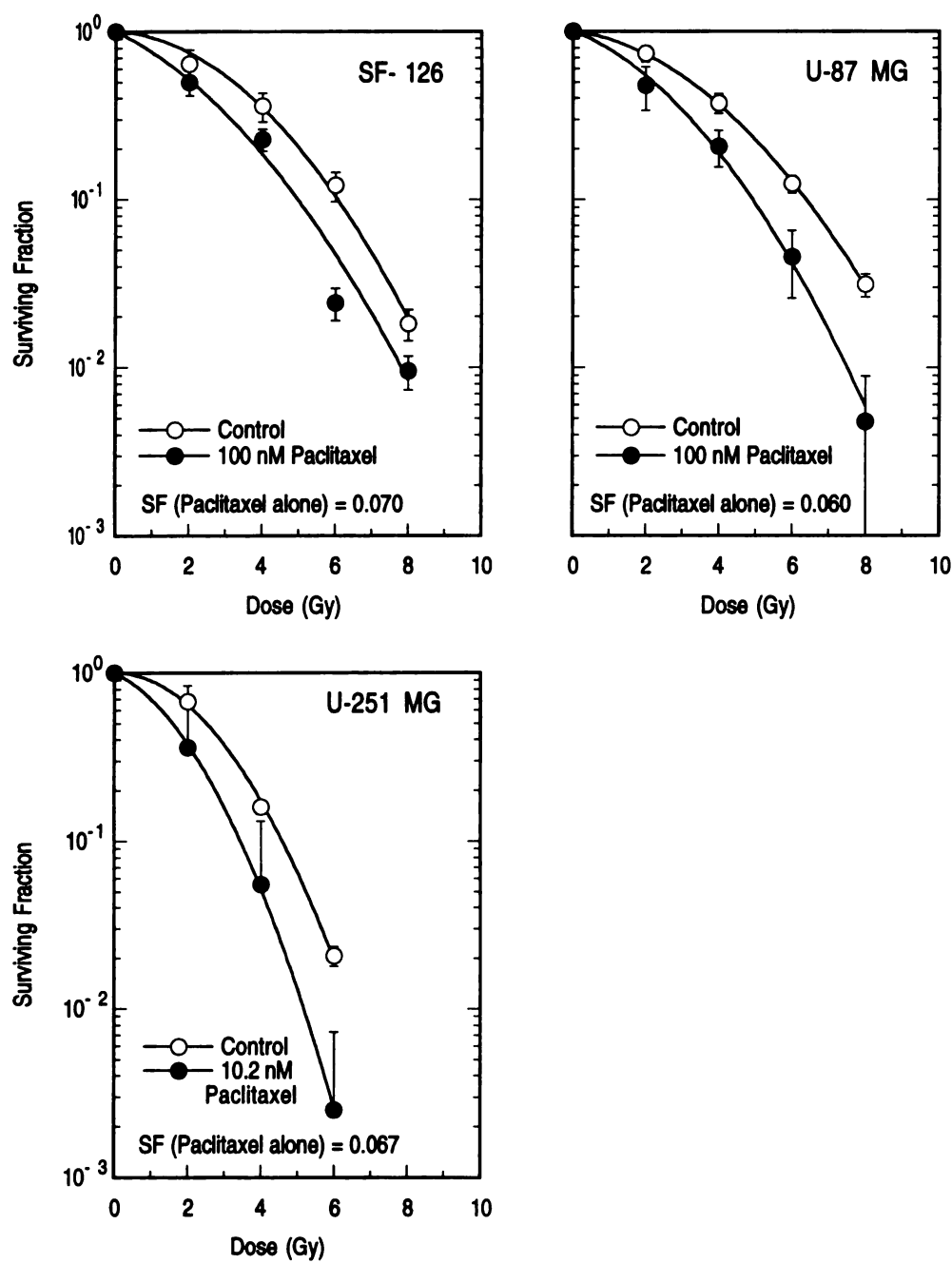


Figure 4.2. The effect of 48 h paclitaxel treatment and irradiation on survival of SF-126, U-87 MG and U-251 MG cells. All data shown are representative of at least two separate experiments.

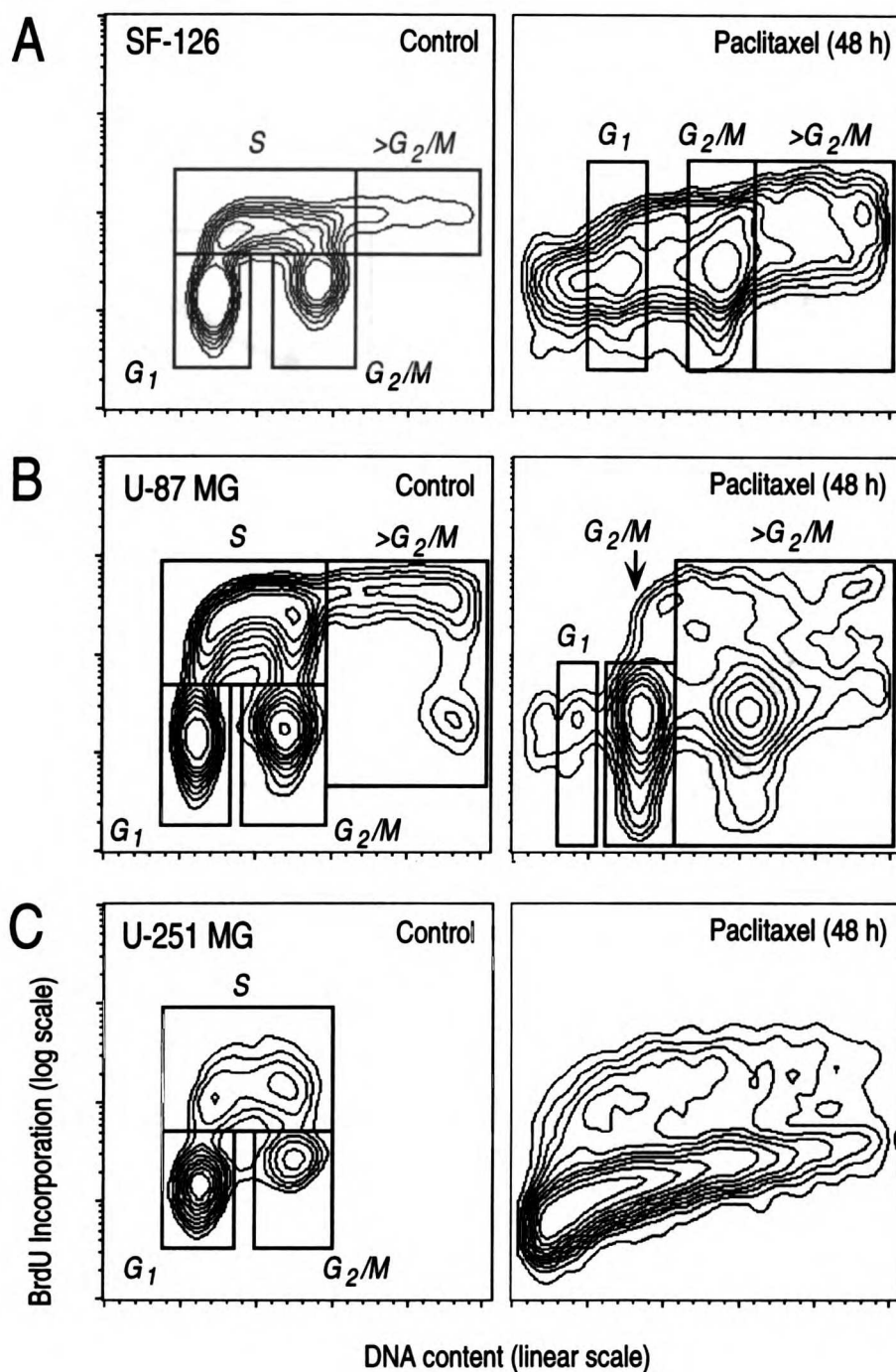


Figure 4.3. The BrdU/PI contour plots for control and paclitaxel treated samples (48 h) before irradiation for SF-126, U-87 MG and U-251 MG cells. The contour lines represent 10% probability increments with a 0.5% threshold applied to all data. The identifiable populations are surrounded by boxes. Percentages of cells located within each population are given in Table 4.1.

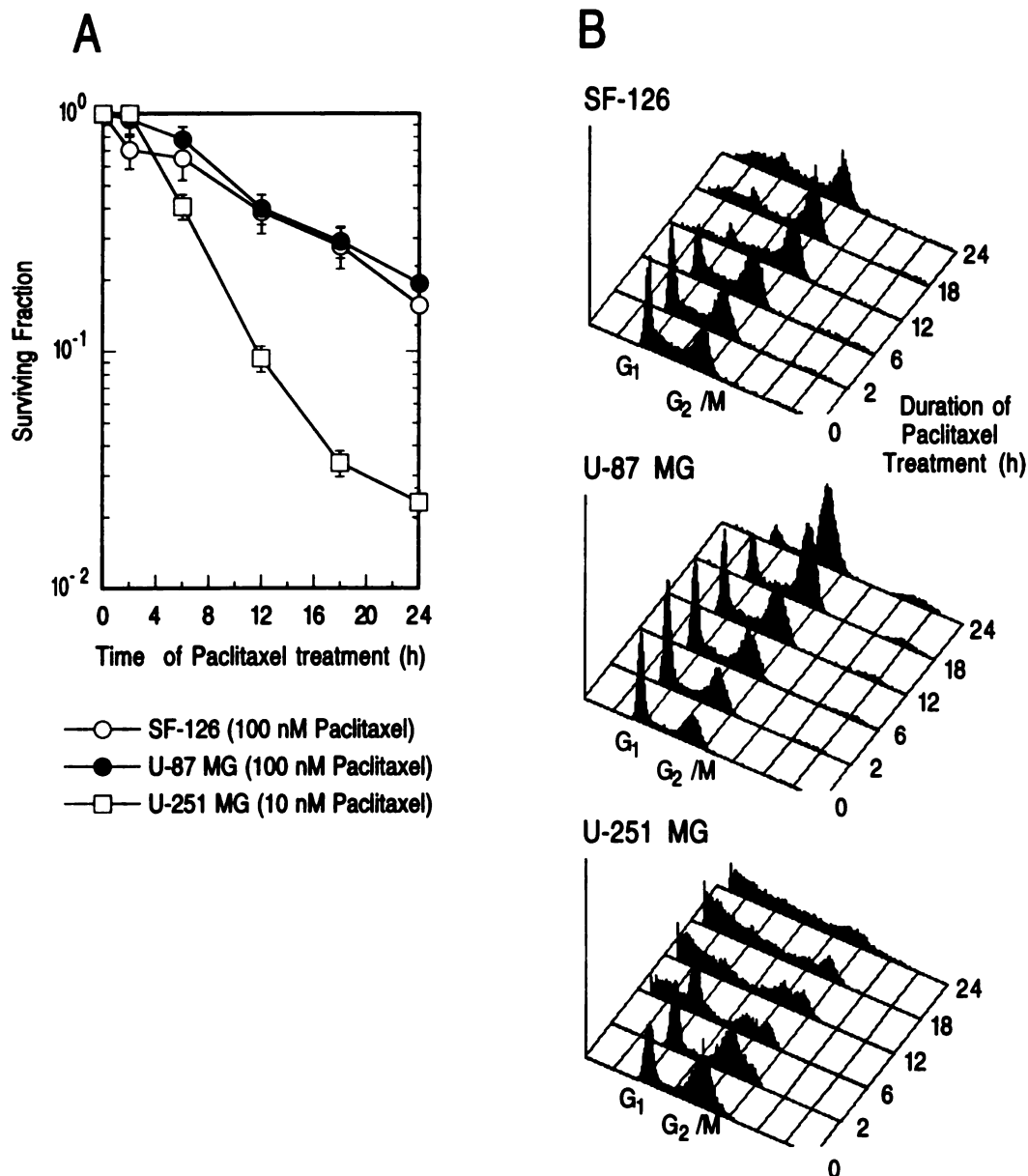


Figure 4.4. The effect of drug treatment times less than 24 h in SF-126, U-87 MG and U-251 MG cells. The PI histograms demonstrate that loss of clonogenicity over 24 h is accompanied by accumulation of cells in G₂/M and depletion of the G₁ and S phase populations. Data for all time points except at 18 h are representative of at least two experiments; the 18 h data were obtained in a single experiment.

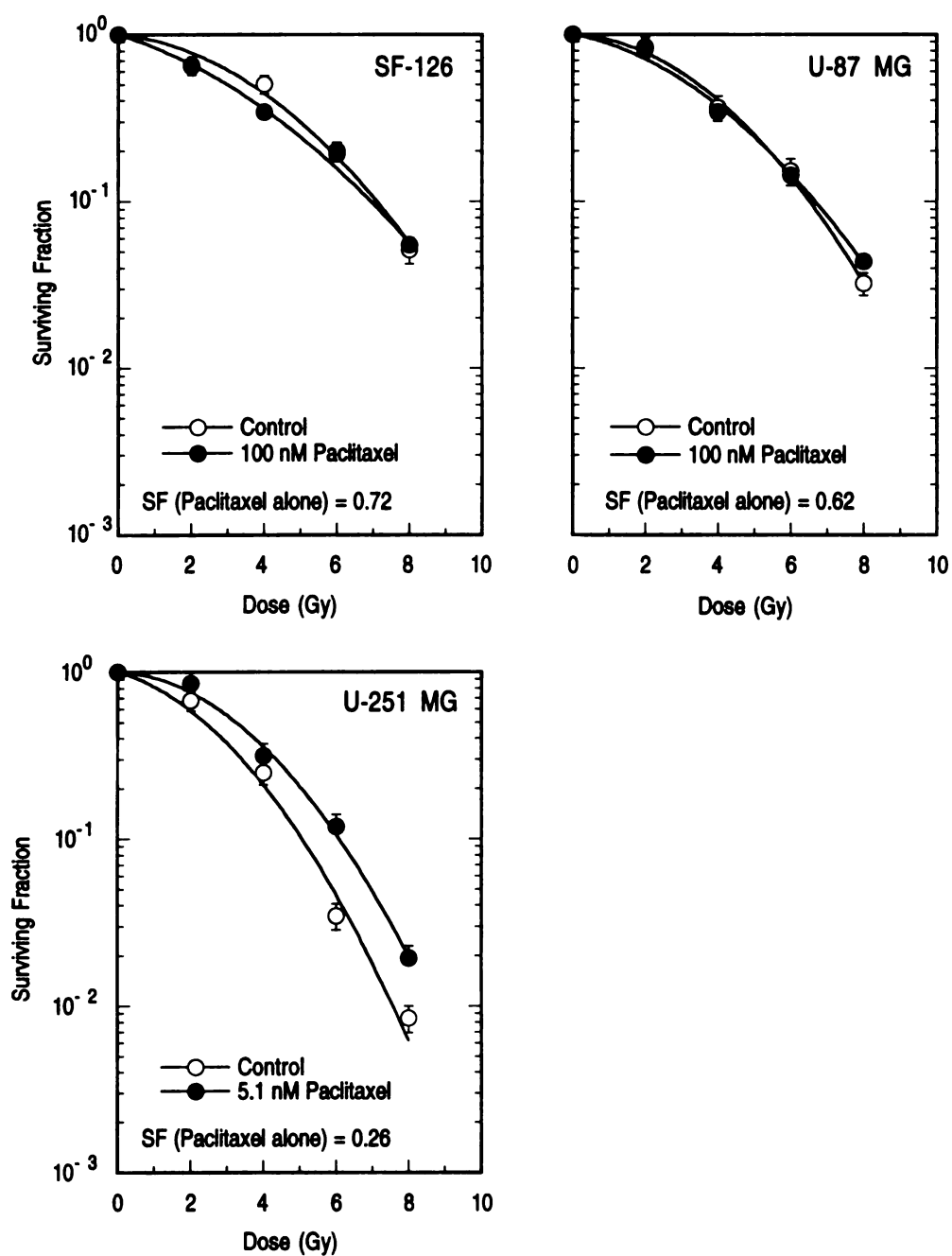


Figure 4.5. The effect of 8 h paclitaxel treatment and irradiation on survival of SF-126, U-87 MG and U-251 MG cells. All data shown are representative of two separate experiments.

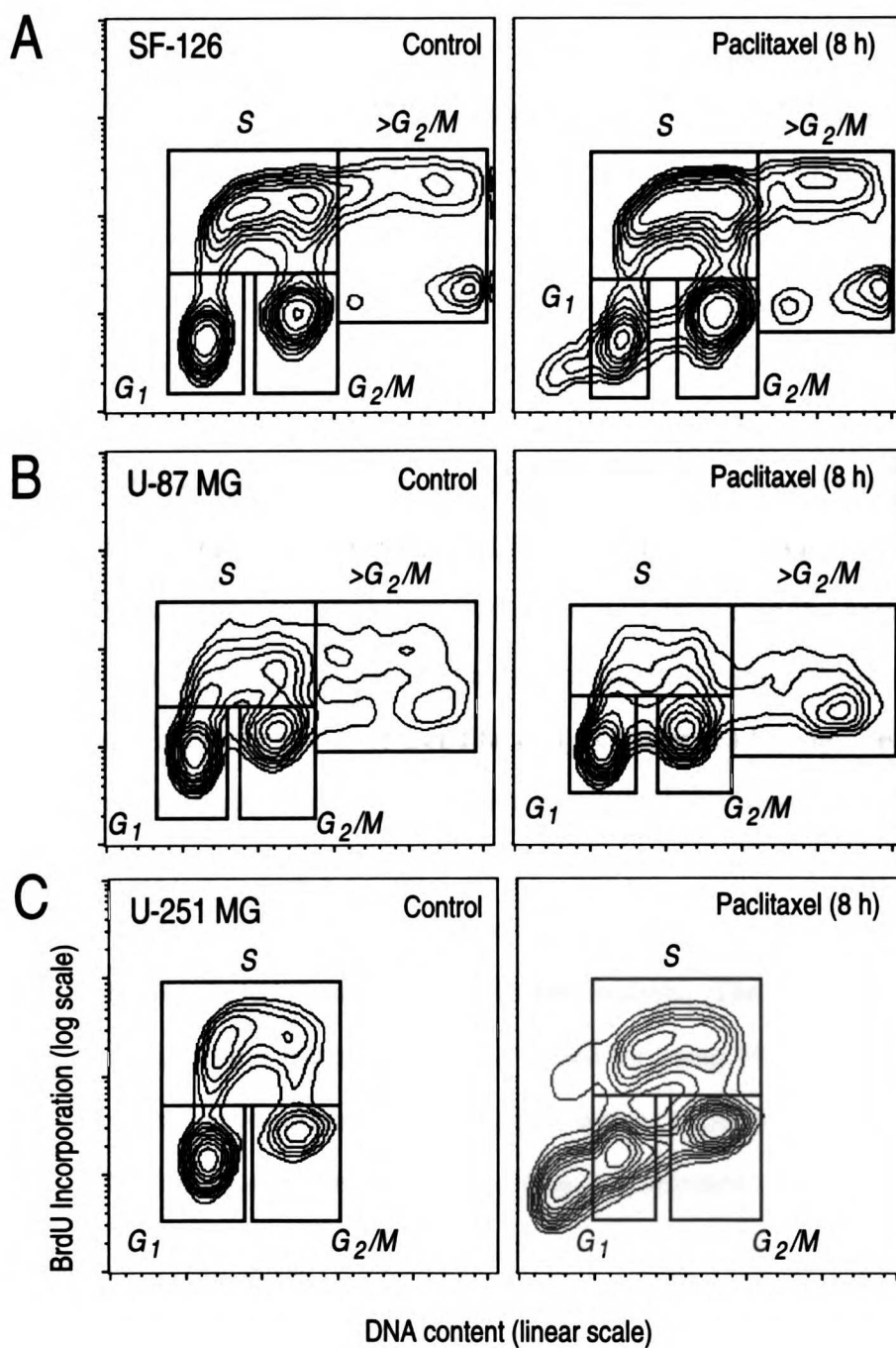


Figure 4.6. The BrdU/PI contour plots for control and paclitaxel treated samples (8 h) before irradiation for SF-126, U-87 MG and U-251 MG cells. The contour lines represent 10% probability increments with a 0.5% threshold applied to all data. The measured populations are surrounded by boxes. Percentages of cells located within each population are given in Table 4.1.

Chapter 5 A Statistical Approach for Analyzing Clonogenic Data

SUMMARY

The CFE assay is a mainstay in the measurement of cell response in vitro to many physical and chemical agents. Currently, CFE data can be calculated in a variety of ways. Authors rarely describe in detail the methods used to determine the extent of biological variation within experiments. Use of standard methods of data analysis and presentation would improve interpretation of data and facilitate comparison between laboratories. Here we propose such a method. Binomial and Poisson probability theory were used to increase the accuracy of the estimate of the surviving fraction and to create an objective criterion for determining whether data obtained from serial dilutions of cell numbers used in the CFE assay should be excluded or included for further analysis. The variability inherent in the calculation of surviving fraction was determined by using Fieller's theorem, a special statistical application for assessing ratios of estimates, to determine the 95% confidence interval. All calculations were done on a simple and commercially available spreadsheet program.

INTRODUCTION

In many fields of biology the dose-response relationship for a particular agent or protocol is initially evaluated in cell culture systems. The CFE assay is the gold standard for determining cytotoxicity of physical (e.g. radiation) and chemical agents (66).

Typically, the PE is calculated as the number of colonies per plate divided by the number of cells originally seeded. The SF is calculated as the PE of treated cells divided by the PE of untreated control cells. Thus, quantitative information is easily obtained from the CFE assay if the number of cells seeded and the number of colonies are known.

Classically, the clonogenic assay is performed by seeding single or multi-well culture plates with a known number of cells. Since it is not known beforehand what the PE will be, plates are usually seeded with a range of dilutions, e.g. each well of a six-well plate might be seeded with 25, 50, 100 or 200 cells. The observed PE is calculated for each plate of each dilution and the estimated plating efficiency ($\hat{PE}$) is then calculated by averaging the values of all plates. This average is then divided by the average of the control group, and the result is reported as the estimated surviving fraction ($\hat{SF}$) with an estimated standard deviation given by dividing the PE standard deviation across dilutions by the control $\hat{PE}$. This approach is limited since it does not take into account that data from plates with a higher number of seeded cells, within the range of non-overlapping colonies, are more reliable. Since a dilution series is usually prepared from a stock single-cell suspension, the observed numbers of colonies should increase linearly with the number of cells seeded per well or plate. In practice, due to overlapping colonies or media depletion, colony counts from plates with higher plating densities sometimes are lower than expected. In this case an arbitrary decision is made whether to include the data in the overall estimate. It would be preferable to have a clearly defined method of determining which data should be excluded. In addition, the actual final confidence interval for the surviving fraction based upon the method described above does not take into account the variability of the control $\hat{PE}$.

In this communication, we propose the use of basic principles of binomial and Poisson probability theory to improve the quality of the estimate of PE. In general, the actual numbers of cells plated after the preparation of a dilution series, i.e. counting,

pipetting and plating, will follow a Poisson distribution. Binomial probability theory is used in the special situation where the number of cells plated is precisely known. An example, using a radiation survival curve, is provided to demonstrate both methods of data analysis. In addition, we provide a readily accessible rule for determining when to delete data derived from those plates with more colonies, and show how using Fieller's (48, 56) theorem provides a more appropriate confidence interval for SF. Naturally, this approach may be used for any experiment using the clonogenic assay.

MATERIALS AND METHODS

Cell Culture and X-irradiation

Human glioma cells, U-87-175.4, were grown as a monolayer in CMEM supplemented G418 (400 $\mu\text{g/ml}$) at 37° C in an humidified atmosphere of 5% CO₂ in air. Cells were irradiated on ice with a 150 kVp x-ray machine (Philips, Hamburg, Germany) at a dose rate of 1.3 Gy/min.

Colony Forming Efficiency

The standard CFE assay was used with minor modifications. This assay has been optimized with respect to incubation time, and feeder number. (These variables affect final clonogenic potential if not optimized.) Six-well plates (35 mm) were prepared 24 h in advance by seeding 5×10^4 heavily irradiated (40 Gy) feeder cells into 4 ml of media. To ensure that colonies would be obtained at each radiation dose point, a range of plating densities was prepared by making specified dilutions from a stock single cell suspension. A different plating density was used in each six-well plate. After cells were seeded, the plates were incubated for 2 weeks at 37° C. Plates were then stained with methylene blue

(0.66% solution in 95% ethanol) for 10 minutes, dried, and colonies containing at least 50 cells were counted.

Data and Statistical Analyses

Using standard binomial or Poisson probability theory, the expected proportion of cells forming colonies is equal to the true PE. The variability of the observed plating efficiency (observed counts divided by number of cells plated) at any dilution is described by the standard error (SE), where

$$SE = \sqrt{PE \cdot \frac{(1 - PE)}{n}} , \quad (1a)$$

and n is the number of cells plated.

When n is an estimate, as it is when serial dilutions are used, the observed count has a Poisson distribution and the standard error of the observed plating efficiency is

$$SE = \sqrt{\frac{PE}{n}} . \quad (1b)$$

In either case, if the colonies are not overlapping, the plates with more colonies (i.e., seeded with lower cell dilutions) provide more accuracy (smaller SE) and should be weighted more heavily in an overall estimate of the PE ($\hat{PE}$). This can be achieved by adding all colonies counted across a range of plating densities and dividing by all cells seeded in all plates, and using the result as the $\hat{PE}$ rather than averaging the individual observed PE's.

To verify that a given plate should be included in the calculation, we begin with the plate with countable colonies that was seeded with a lower number of cells, on the assumption that the colonies will not be overlapping. For each higher plating density, the observed proportion of colonies between adjacent dilution levels is compared using the

normal approximation to the binomial or Poisson to determine the probability that a decrease at the higher seeding density at least as large as that observed would occur by chance. Specifically, a Z-score is calculated as follows:

$$Z = \frac{PE_L - PE_H}{\sqrt{\frac{PE_L(1 - PE_L)}{n_L} + \frac{PE_H(1 - PE_H)}{n_H}}}, \text{ under binomial theory or,} \quad (2a)$$

$$Z = \frac{PE_L - PE_H}{\sqrt{\frac{PE_L}{n_L} + \frac{PE_H}{n_H}}}, \text{ under Poisson theory;} \quad (2b)$$

where PE_L is the observed plating efficiency from the plate seeded with the lower plating density, PE_H is the observed plating efficiency from the plate seeded with the higher plating density, and n_L is the total number of cells seeded at the lower plating density, and n_H is the total number of cells seeded at the higher plating density. Both PE_L and PE_H are approximately normally distributed. This approximation is generally considered acceptable if $(n \times PE) > 25$ (binomial) or $(n \times PE) > 9$ (Poisson) (73). It follows that the Z scores as defined above are approximately normally distributed. The Z-score is compared to a table providing the probability distribution for standard normal (Z) scores to determine the p-value. Some standard Z-scores and p-values are given in Table 5.1 (184). A one-tailed hypothesis test can be done by comparing this p-value with a pre-specified p-value cutoff for inclusion of data.

For our sample data we selected a p-value of 0.05. Therefore, if the Z-score from a six-well plate with lower plating density as compared to the higher plating density was < 1.65 , the data were accepted and colony counts from that six-well dish were added to the overall data set. Conversely, if the Z-score was > 1.65 the data were rejected.

The following is a method for measuring the variability that accompanies the estimation of the SF by determining the 95% confidence interval. Initially, the estimated plating efficiencies for control and treated cells are calculated by

$$\hat{PE}_c = \frac{t_c}{n_c} \text{ and} \quad (3)$$

$$\hat{PE}_t = \frac{t_t}{n_t} \text{ respectively;} \quad (4)$$

where t_c is the total number of control colonies counted, t_t is the total number of treated colonies counted, n_c is the total number of control cells seeded, and n_t is the total number of treated cells seeded. The estimated surviving fraction is given by

$$\hat{SF} = \frac{\hat{PE}_t}{\hat{PE}_c} . \quad (5)$$

Therefore, $\hat{SF}$ is a ratio of two independent estimates. As stated above, each of these can be considered to be approximately normally distributed. Fieller's theorem, specifically designed to address ratios of estimates, can be used to determine the upper and lower limits of a confidence interval (48, 56) as follows:

$$\left\{ \hat{SF} + \left(\frac{g}{(1-g)} \right) \cdot \hat{SF} \right\} \pm \left(\frac{Z}{\hat{PE}_c(1-g)} \right) \cdot \sqrt{(\hat{SF}^2 V_c) + V_t(1-g)} ; \quad (6)$$

where,

$$g = \left(\frac{Z}{\hat{PE}_c} \right)^2 V_c , \quad (7)$$

$$V_{c,t} = \frac{PE_{c,t}(1 - PE_{c,t})}{n_{c,t}}, \text{ under binomial theory or,} \quad (8)$$

$$V_{c,t} = \frac{PE_{c,t}}{n_{c,t}} \text{ under Poisson theory.} \quad (9)$$

Z is the value from the normal distribution table for a two-tailed test (e.g., for 95% confidence interval, $Z = 1.96$), V_c is the variance of the control sample, and V_t is the variance of the treated sample. To calculate the confidence interval, the $\hat{PE}_c$ and $\hat{PE}_t$ are substituted for PE_c and PE_t respectively.

All calculations were performed on an Excel (Microsoft, Redmond, WA) spreadsheet, v. 4.0 for Macintosh. The Excel spreadsheet shown in Figures 5.1 and 5.2, and this manuscript can be obtained by anonymous ftp to [btrcsun.ucsf.edu](ftp://btrcsun.ucsf.edu) (IP#: 128.218.13.254). The spreadsheet is saved in SYLK format and the manuscript in RTF format (created with Microsoft Word, v. 5.1a for Macintosh) and are located in `/u/ftp/pub/deen/stats.95` as `./excelcfe.xls` and `./wordcfe.doc` respectively. These files when downloaded as plain text files can be converted into normal format by Macintosh or Windows versions of Excel and Word. The spreadsheet includes all equations described above and will analyze clonogenic data by the method used in this paper once colony counts are entered. The results were plotted using KaleidaGraph (Abelbeck and Synergy Software, Reading, PA), v. 3.0.4 for Macintosh.

RESULTS AND DISCUSSION

In this report we describe a statistical approach that improves the precision of the clonogenic survival assay and reduces the subjectivity of certain aspects of data interpretation. This approach is appropriate when colony counts differ due only to random

variation and dilution. In general, this assumption is met and the proposed approach should improve the usefulness of this assay in three ways. First, by accounting for the increased reliability of data from plates with higher numbers of cells seeded, the precision of the $\hat{SF}$ is improved. Second, while some individuals use strict criteria for accepting or rejecting primary data for further analysis, usually the decision is arbitrary. We propose a method to assess data variation and select data based upon binomial probability theory. Third, it is important to accurately reflect the variability of $\hat{SF}$ if the quality of the experiment is to be assessed. To do so requires consideration of the variability in the denominator (i.e. control $\hat{PE}$), which we have done by considering the control $\hat{PE}$ as a separate experimental observation. It should be noted that this approach is restricted to analysis of data obtained from one experiment. Analysis and reporting data from multiple experiments requires a conceptually different approach and is not addressed by this report.

In general terms, the confidence one has for a particular experimental observation is related to the number of events (in this case, the number of cells) that are being measured. For example, there is a greater degree of confidence in the colony count measured from a plate seeded with 1000 cells compared to a plate where only 10 cells are seeded (assuming that the treatment condition is the same and the colonies are not overlapping). Weighting the results from plates with higher densities is easily done by calculating the $\hat{PE}$ from the total number of colonies counted and cells seeded in all plates used for one experimental condition (Equations 3 and 4). In the example presented, if $\hat{PE}$ is calculated from data provided in Figure 5.1 by averaging PE 's from each plate, rather than the method described in this paper, the results are underestimated by 2-10% compared to the $\hat{PE}$'s shown in Figure 5.2.

A number of variables can affect the clonogenicity of cells independent of the treatment protocol. One that became apparent to us was a deviation from the expected colony number between various plating densities. At times, we observed that the number

of colonies in the plate with the highest plating density contained fewer colonies than expected. There are at least two reasons why higher plating densities may reduce colony forming efficiency: first, larger colony numbers may affect growth if competition for substrates occurs and second, colonies may overlap one another such that the number of distinct colonies is reduced. While there is expected biological variation, the obvious question is at what level does this variation exceed the probability that it occurred by chance? The results from calculations (Equation 2) testing sequentially lower dilutions are shown on Figure 1 in the column labeled "Z-score". For the sample data set, one six-well plate (1 Gy, 100 cells per well) was excluded for any further analysis based on a p-value of 0.05 (two-tailed test) as the cut-off whether to accept or reject data. The choice of 95% was arbitrary but one that is often used in biology, and the investigator may choose the stringency by which to analyze his/her data. We have assumed that the colonies obtained from the lowest plating density were the least affected by the two factors listed above.

The PE of control cells is generally less than 100%, usually in the vicinity of 30 to 60%. The SF value is therefore, a normalization of the PE of treated cells based upon an independently performed assessment of the clonogenic potential of untreated cells. It is apparent that the control PE is not a constant, as it is usually regarded, but an experimental observation subject to its own variation. This variation should be incorporated in the calculation of reported experimental error relative to the SF value. The upper and lower limits of the 95% confidence interval for the surviving fraction was determined using Fieller's theorem (Equations 6-9) and are depicted on page 2 of the Excel spreadsheet (cell P2, Figure 5.2). These data can be easily exported into a number of programs for plotting (Figure 5.3).

The accuracy to which n , the number of cells plated per well, is known determines whether binomial or Poisson probability theory is appropriate for data analysis. In assays where n is known exactly, binomial theory is most appropriate for the calculation of the

standard error and the 95% confidence interval (Equations 1, 2, 8 and 9). If cell preparation will result in a Poisson distribution of numbers in each plate (e.g. when preparing cells by serial dilutions), Poisson theory is more appropriate. In Figures 5.1 and 5.2, the same data set is analyzed using both binomial and Poisson probability theory based equations for purposes of comparison. The Poisson-based Z-scores, standard errors and confidence intervals varied from a binomial-based method at the lower values of n but did not affect the decision whether to include or exclude specific six-well plates.

The method outlined above is an informative way to calculate clonogenic data with a degree of statistical confidence using a commonly available software package. Since the CFE assay is used widely in many experimental situations, we reasoned that standardizing data analysis will facilitate comparison of results and hopefully encourage further use of statistical methods leading to stronger experimental design.

Table 5.1. Z-scores for selected p-values

p-value	Z-score	Z-score
	(one-tailed)	(two-tailed)
0.1	1.28	1.65
0.05	1.65	1.96
0.025	1.96	2.24
0.01	2.33	2.58

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Experiment: NG04-034												
2													
3		Sample	Cells	Wells	Total Cell	Number of Colonies						Z Score	
4			Seeded		Number							Binomial	Poisson
5			per Well										
6													
7		Control	50	6	300	21	24	21	24	23	33	1.07	0.74
8			25	6	150	14	13	8	14	18	14		
9													
10													
11													
12													
13		1 Gy	200										
14			100	6	600	20	15	19	15	18	19	2.49	2.18
15			50	6	300	11	10	14	11	15	14	1.25	1.06
16			25	6	150	6	7	7	9	9	8		
17													
18													
19													
20		2 Gy	1000										
21			500										
22			250	6	1500	25	16	25	24	19	24	-0.52	-0.50
23			100	6	600	10	9	8	6	6	10		
24													
25													
26													
27		4 Gy	5000										
28			2500	6	15000	21	24	23	26	24	25	1.62	1.61
29			1000	6	6000	14	12	14	14	7	12	-0.35	-0.35
30			500	6	3000	5	5	3	9	5	7		
31													
32													
33													
34		6 Gy	10000	6	60000	9	6	9	8	10	8	-0.17	-0.17
35			5000	6	30000	3	6	4	4	3	4	-0.50	-0.50
36			2500	6	15000	2	0	2	2	1	3		
37													
38													
39													
40													

Z > 1.65;
data
excluded

Figure 5.1. Page 1 of an Excel spreadsheet demonstrating experimental data and calculation of Z-scores using both binomial and Poisson probability theory. The higher and lower plating densities were compared, and those dishes with Z-scores > 1.65 were rejected from further analysis (in this case, only the dish plated with 100 cells per well for the 1 Gy sample was excluded). The rows under "Cells Seeded per Well" that do not contain any counts, i.e. 1 Gy, 200 cells per well, were plates where counting could not be done because high plating densities resulted in confluent colonies.

	N	O	P	Q	R	S	T	U
1	Experiment: NG04-034							
2	Confidence Interval:		1.96					
3	Constant(g) for binomial:		0.00839					
4								
5	BINOMIAL PROBABILITY							
6	Sample	Colony Number	estPE	Standard Error	Variance	estSF	dSF+	dSF-
7								
8								
9	Control	227	0.504	0.0236	0.00056	1	0.0000	0.0000
10	1 Gy	121	0.269	0.0137	0.00019	0.533	0.0771	0.0681
11	2 Gy	182	0.0867	0.00614	3.8E-05	0.172	0.0302	0.0273
12	4 Gy	250	0.0104	0.00066	4.3E-07	0.0206	0.0034	0.0030
13	6 Gy	84	0.0008	8.7E-05	7.6E-09	0.00159	0.0004	0.0004
14								
15								
16								
17	Constant (g) for Poisson:		0.01692					
18								
19	POISSON PROBABILITY							
20	Sample	Colony Number	estPE	Standard Error	Variance	estSF	dSF+	dSF-
21								
22								
23	Control	227	0.504	0.0335	0.00112	1	0.0000	0.0000
24	1 Gy	121	0.269	0.0160	0.00026	0.533	0.1036	0.0852
25	2 Gy	182	0.0867	0.00642	4.1E-05	0.172	0.0369	0.0310
26	4 Gy	250	0.0104	0.00066	4.3E-07	0.0206	0.0041	0.0034
27	6 Gy	84	0.0008	8.7E-05	7.6E-09	0.00159	0.0004	0.0004
28								

Figure 5.2. Page 2 of an Excel spreadsheet with estimated plating efficiency ("estPE" in figure), standard error, variance, estimated surviving fraction ("estSF" in figure) calculated using both binomial and Poisson probability theory. Upper and lower limits of the 95% confidence interval are calculated by adding and subtracting "dSF+" and "dSF-" from "estSF", respectively.

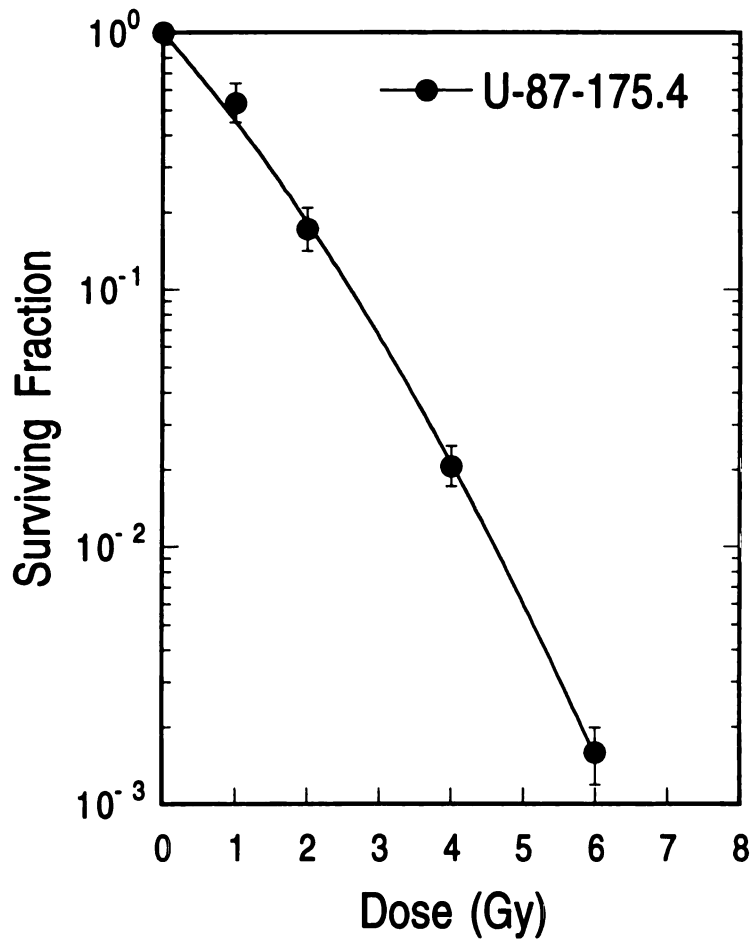


Figure 5.3. Radiation survival curve for the cell line U-87-175.4. The curve was derived from values shown in Figure 5.2 and fitted according to the linear quadratic model: $SF = e^{-\alpha D - \beta D^2}$; $\alpha = 0.22$; $\beta = 0.10$. The error bars represent the upper and lower limits of the 95% confidence interval for the point estimate using Poisson probability.

Chapter 6 Conclusion

GENERAL

Several features characterize the features of malignant cells: *i*) unregulated cell proliferation, *ii*) metastasis, and *iii*) tissue invasion. Many successful cancer treatment strategies target proliferating cells. Therefore, understanding the effect of cytotoxic agents upon cycling cells remains an important research goal. The experimental results described in this thesis clarify relationships between cellular injury caused by ionizing radiation or paclitaxel, cell cycle perturbations, and cell survival.

The major conclusions reached by this study are: *i*) the p53 tumor suppressor gene influences cellular response to ionizing radiation in several ways such as activation of a dose-dependent G₁ checkpoint, changes in cell cycle phase distributions, increasing radiation sensitivity in all phases of the cell cycle and, affecting the yield of certain chromosomal aberrations, and *ii*) treatment with an anti-microtubule drug (paclitaxel) produces cell line-specific cell cycle perturbations which, in turn, influence overall cell survival.

THE G₁ CHECKPOINT

As was pointed out in the discussion accompanying Chapter 2, it has been proposed that the function of the G₁ checkpoint is to allow DNA repair prior to S phase. The experiments described in Chapter 2 were designed to test this hypothesis. Because cells derived from naturally occurring tumors are genetically heterogenous, isogenic cell

lines which differ in p53 function were used. U-87 MG (the parent cell line), despite expressing wt p53, grows rapidly and is tumorigenic.⁷ The wt p53 cell line transfected with vector alone (U-87-lux.8) displays a dose-dependent G₁ arrest following ionizing radiation. This was clearly demonstrated by comparing the percentage of G₁ cells that arrest following 6 Gy (78%) *versus* 0 Gy (10%) (Figure 2.4). This is in contrast to cells expressing a dominant negative p53 mutant at high levels (U-87-175.4) (227), which do not arrest in G₁ after an equivalent dose.

Generally, the yield of DNA DSBs and chromosomal aberrations parallels the dose of ionizing radiation. For this reason, if radiation-induced DNA damage is reduced by the presence of a G₁ checkpoint, then the number of chromosomal aberrations measured at the first mitosis following irradiation should also be reduced. As shown in Table 2.1, the total number of aberrations scored was the same in both cell lines. Therefore, wt p53 does not appear to provide any advantage for U-87-lux.8 cells. One caveat of this result, however, is that while the total damage induced was the same for both cell lines, fewer U-87-lux.8 cells were collected at mitosis compared to U-87-175.4 cells. The apparent reason for this is that the radiation-induced G₁ arrest is probably permanent in these cells; a hypothesis supported by studies using rodent derived cell lines (36) and the same cell lines used in these experiments (227).

The reason for loss of cellular clonogenic potential is multifactorial. Chromosomal aberrations caused by DNA mis-repair, i.e. dicentrics and ring chromosomes, are believed to interfere with mitosis and result in failed cell division. Apoptosis and activation of the G₁ checkpoint leading to a permanent cell cycle arrest, as discussed above, may also reduce clonogenicity. Based upon the results described in Chapters 2 and 3, there are several

⁷ In contrast, expressing wt p53 exogenously in transformed cells usually stops or slows proliferation. This suggests that, at least in U-87 MG cells, tumorigenicity and the radiation-induced G₁ checkpoint are controlled through separate pathways.

mechanisms that may account for the increased radiation sensitivity of U-87-lux.8 cells. The most obvious is that wt p53 causes an increase in the percentage of G₁ cells, a radiosensitive phase of the cell cycle, while reducing the percentage of S phase cells, a radioresistant phase of the cell cycle (Table 3.2). The overall radiation survival of the cycling population is then reduced. It should be noted that wt p53 cells in all phases of the cell cycle are more radiation sensitive compared to mutant p53 cells (Chapter 3). Another possibility is that activation of the G₁ checkpoint prevents a number of cells with genetic damage from reaching mitosis which might otherwise retain the ability to form colonies. A quantitative problem with this argument is that while the percentage of wt p53 cells that arrest in G₁ after lower radiation doses is small (Figure 2.4), clonogenic survival is still substantially reduced when compared to cells with mutant p53 (Figure 2.5). In some cell types, p53-dependent apoptosis after ionizing radiation results in decreased cell survival. In U-87-lux.8 and U-87-175.4 cells, appreciable levels of apoptosis do not occur after irradiation (65).

PACLITAXEL-INDUCED CELL CYCLE PERTURBATIONS

Paclitaxel stabilizes microtubules *in vitro* by preventing depolymerization of tubulin subunits. As described by Jordan *et al* (82), microtubule dynamics are adversely affected by lower concentrations of paclitaxel without any noticeable increase in the mass of polymerized microtubules. At a functional level, while paclitaxel may interfere with microtubule dynamics, cell cycle progression prior to mitosis seems normal (Figure 4.4). Cells leave G₁ and enter S at the same rate as untreated cells. Furthermore, movement into mitosis seems to be required before most of the observed paclitaxel-induced cytotoxicity

occurs. Non-cycling plateau phase cells, as expected, are considerably more resistant to paclitaxel-induced toxicity than cycling cells.

As suggested by Kung *et al*, cell line-specific differences in cell cycle progression may be due to faulty cell cycle checkpoints (94). From the results described in Chapter 4, it is clear that U-251 MG cells are much more sensitive to paclitaxel than SF-126 and U-87 MG cells. The reason for this difference is suggested by the cell cycle profiles (Figures 4.3 and 4.6). For SF-126 and U-87 MG cells, some cells arrest in mitosis after treatment with paclitaxel; as represented by an increase in the number of cells with G₂/M DNA content. U-251 MG cells, in contrast, continue through mitosis and fragment (due to either paclitaxel-induced damage or vortexing of samples during preparation for flow cytometry). If the spindle checkpoint protects cells with an abnormal spindle from entering mitosis, its absence could greatly increase the number of inviable cells in a treated population.

Another interesting finding is that pretreatment with paclitaxel for a shorter duration (8 hours) may, in certain cases, increase the surviving fraction of a cycling population from irradiation. In experiments where U-251 MG cells were treated with paclitaxel for eight hours prior to irradiation, cell survival *increased* slightly compared to irradiation alone (Figure 4.5). A transient increase in the percentage of cells which are in S phase, and also radioresistant, that accompanies a mitotic block may account for this phenomenon. (An increase in the S phase percentage occurs secondary to a reduction in the G₁ fraction because repopulation of G₁ cannot occur from the pool of newly divided cells.) Therefore, this observations suggests that the timing of drug treatment is critical when delivered in combination with ionizing radiation.

FUTURE DIRECTIONS

An intriguing observation described in Chapter 2 is that chromosome translocations are increased following irradiation of mutant p53 G₁ cells with 6 Gy. Chromosome translocations, therefore, may be another indicator of a type of genomic instability also regulated by p53. An important question is whether chromosome instability is accelerated many generations after irradiation and if this is also dependent upon mutations in p53 or other genes. Examining chromosomal aberrations extended times after irradiation and using a variety of genetic backgrounds would address this question. In addition, studies examining the response of human xenograft tumors which differ in p53 function will be essential to see if findings from these experiments hold true *in vivo* or if they are specific only for cells in culture.

Reportedly, p53 is also involved in a G₂ checkpoint. This presents the obvious question of what effect p53 has upon paclitaxel-induced cytotoxicity, both alone and in combination with ionizing radiation. How these differing cell cycle effects interact is unknown and experiments designed to investigate these interactions would provide important information with clear clinical relevance.

The goal of the studies described in this thesis was to describe, in a well defined *in vitro* system, cell cycle events that occur after treatment with agents commonly used in patients. While predicting the response of human tumors to therapeutic agents is currently unfeasible, understanding the events at the cellular and molecular level might serve as a bridge from our present level of knowledge. Ultimately, the challenge for basic scientists and clinicians is to improve the efficacy of current treatment and also develop new strategies that will successfully address the problem of cancer. Only through a complete understanding of the basic biology of cancer will this be possible.

References

1. Abella Columna, E., Giaccia, A.J., Evans, J.W., Yates, B.L. and Morgan, W.F.: Analysis of restriction enzyme-induced chromosomal aberrations by fluorescence in situ hybridization. *Environ. Mol. Mutagen.*, **22**, 26-33 (1993).
2. Agarwal, M.L., Agarwal, A., Taylor, W.R. and Stark, G.R.: p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **92**, 8493-8497 (1995).
3. Alpen, E.L.: *Radiation Biophysics*, Prentice Hall, Englewood Cliffs, NJ, 1990.
4. Arlett, C.F., Green, M.H., Priestley, A., Harcourt, S.A. and Mayne, L.V.: Comparative human cellular radiosensitivity: I. The effect of SV40 transformation and immortalisation on the gamma-irradiation survival of skin derived fibroblasts from normal individuals and from ataxia-telangiectasia patients and heterozygotes. *Int. J. Radiat. Biol.*, **54**, 911-928 (1988).
5. Ault, J.G. and Rieder, C.L.: Centrosome and kinetochore movement during mitosis. *Curr. Opin. Cell Biol.*, **6**, 41-49 (1994).
6. Baguley, B.C., Marshall, E.S., Whittaker, J.R., Dotchin, M.C., Nixon, J., McCrystal, M.R., Finlay, G.J., Matthews, J.H.L., Holdaway, K.M. and Zijl, P.v.: Resistance mechanisms determining the *in vitro* sensitivity to paclitaxel of tumour cells cultured from patients with ovarian cancer. *Eur. J. Cancer*, **31A**, 230-237 (1995).
7. Bakalkin, G., Yakovleva, T., Selivanova, G., Magnusson, K.P., Szekely, L., Kiseleva, E., Klein, G., Terenius, L. and Wiman, K.G.: p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc. Natl. Acad. Sci. USA*, **91**, 413-417 (1994).

8. Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K. and Vogelstein, B.: Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science*, **249**, 912-915 (1990).
9. Barbet, N.C. and Carr, A.M.: Fission yeast *wee1* protein kinase is not required for DNA damage-dependent mitotic arrest. *Nature*, **364**, 824-827 (1993).
10. Bedford, J.S., Mitchell, J.B., Griggs, H.G. and Bender, M.A.: Radiation-induced cellular reproductive death and chromosome aberrations. *Radiat. Res.*, **76**, 573-586 (1978).
11. Bhalla, K., Ibrado, A.M., Tourkina, E., Tang, C., Mahoney, M.E. and Huang, Y.: Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia*, **7**, 563-568 (1994).
12. Biedermann, K.A., Sun, J.R., Giaccia, A.J., Tosto, L.M. and Brown, J.M.: SCID mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. *Proc. Natl. Acad. Sci. USA*, **88**, 1394-1397 (1991).
13. Bischoff, J.R., Friedman, P.N., Marshak, D.R., Prives, C. and Beach, D.: Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. *Proc. Natl. Acad. Sci. USA*, **87**, 4766-4770 (1990).
14. Brachman, D.G., Beckett, M., Graves, D., Haraf, D., Vokes, E. and Weichselbaum, R.R.: p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines. *Cancer Res.*, **53**, 3667-3669 (1993).
15. Bristow, R.G., Jang, A., Peacock, J., Chung, S., Benchimol, S. and Hill, R.P.: Mutant p53 increases radioresistance in rat embryo fibroblasts simultaneously transfected with HPV16-E7 and/or activated H-ras. *Oncogene*, **9**, 1527-1536 (1994).

16. Buchholz, D.J., Lepek, K.J., Rich, T.A. and Murray, D.: 5-Fluorouracil-radiation interactions in human colon adenocarcinoma cells. *Int. J. Radiat. Oncol. Biol. Phys.*, **32**, 1053-1058 (1995).
17. Cahan, M.A., Walter, K.A., Colvin, O.M. and Brem, H.: Cytotoxicity of taxol against human and rat malignant brain tumors. *Cancer Chemother. Pharmacol.*, **33**, 441-444 (1994).
18. Canman, C.E., Wolff, A.C., Chen, C.Y., Fornace, A.J., Jr. and Kastan, M.B.: The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia. *Cancer Res.*, **54**, 5054-5058 (1994).
19. Carrano, A.V.: Chromosome aberrations and radiation-induced cell death. I. Transmission and survival parameters of aberrations. *Mutat. Res.*, **17**, 341-353 (1973).
20. Carrano, A.V.: Chromosome aberrations and radiation-induced cell death. II. Predicted and observed cell survival. *Mutat. Res.*, **17**, 355-366 (1973).
21. Chang, W.P. and Little, J.B.: Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving x-irradiation: association with delayed reproductive death phenotype. *Mutat. Res.*, **270**, 191-199 (1992).
22. Chen, G., Jaffrezou, J.P., Fleming, W.H., Duran, G.E. and Sikic, B.I.: Prevalence of multidrug resistance related to activation of the *mdr1* gene in human sarcoma mutants derived by single-step doxorubicin selection. *Cancer Res.*, **54**, 4980-4987 (1994).
23. Cheng, J., Yee, J.-K., Yeargin, J., Friedmann, T. and Haas, M.: Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. *Cancer Res.*, **52**, 222-226 (1992).

24. Chiu, S.M., Xue, L.Y., Friedman, L.R. and Oleinick, N.L.: Copper ion-mediated sensitization of nuclear matrix attachment sites to ionizing radiation. *Biochemistry*, **32**, 6214-6219 (1993).
25. Choy, H., Akerley, W., Safran, H., Clark, J., Rege, V., Papa, A., Glantz, M., Puthawala, Y., Soderberg, C. and Leone, L.: Phase I trial of outpatient weekly paclitaxel and concurrent radiation therapy for advanced non-small-cell lung cancer. *J. Clin. Oncol.*, **12**, 2682-2686 (1994).
26. Choy, H., Rodriguez, F., Koester, S., Hilsenbeck, S. and Von Hoff, D.: Investigation of taxol as a potential radiation sensitizer. *Cancer*, **71**, 3774-3778 (1993).
27. Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L. and Wyllie, A.H.: Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849-852 (1993).
28. Cleaver, J.E.: It was a very good year for DNA repair. *Cell*, **76**, 1-4 (1994).
29. Cornforth, M.N. and Bedford, J.S.: On the nature of a defect in cells from individuals with ataxia-telangiectasia. *Science*, **227**, 1589-1591 (1985).
30. Cornforth, M.N. and Bedford, J.S.: A quantitative comparison of potentially lethal damage repair and the rejoining of interphase chromosome breaks in low passage normal human fibroblasts. *Radiat. Res.*, **111**, 385-405 (1987).
31. Coue, M., Lombillo, V.A. and McIntosh, J.R.: Microtubule depolymerization promotes particle and chromosome movement in vitro. *J. Cell Biol.*, **112**, 1165-1175 (1991).
32. Cox, B.S. and Parry, J.M.: The isolation, genetics and survival characteristics of ultraviolet light sensitive mutants in yeast. *Mutat. Res.*, **6**, 37-55 (1968).

33. Cross, S.M., Sanchez, C.A., Morgan, C.A., Schimke, M.K., Ramel, S., Idzerda, R.L., Raskind, W.H. and Reid, B.J.: A p53-dependent mouse spindle checkpoint. *Science*, **267**, 1353-1356 (1995).
34. Dewey, W.C., Miller, H.H. and Leeper, D.B.: Chromosomal aberrations and mortality of X-irradiated mammalian cells: Emphasis on repair. *Proc. Natl. Acad. Sci. USA*, **68**, 667-671 (1971).
35. Dewey, W.C., Noel, J.S. and Dettor, C.M.: Changes in radiosensitivity and dispersion of chromatin during the cell cycle of synchronous Chinese hamster cells. *Radiat. Res.*, **52**, 373-394 (1972).
36. Di Leonardo, A., Linke, S.P., Clarkin, K. and Wahl, G.M.: DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.*, **8**, 2540-2551 (1994).
37. Diaz, J.F. and Andreu, J.M.: Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: Reversibility, ligand stoichiometry, and competition. *Biochemistry*, **32**, 2747-2755 (1993).
38. Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B. and et al.: p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.*, **10**, 5772-5781 (1990).
39. Donaldson, K.L., Goolsby, G.L. and Wahl, A.F.: Cytotoxicity of the anticancer agents cisplatin and taxol during cell proliferation and the cell cycle. *Int. J. Cancer*, **57**, 847-855 (1994).
40. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S. and Bradley, A.: Mice deficient for p53 are developmentally normal but susceptible to tumors. *Nature*, **356**, 215-221 (1992).
41. el-Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y. and et al.:

- WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res*, **54**, 1169-1174 (1994).
42. el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W. and Vogelstein, B.: Definition of a consensus binding site for p53. *Nat. Genet.*, **1**, 45-49 (1992).
 43. el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B.: WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817-825 (1993).
 44. Elia, M.C. and Bradley, M.O.: Influence of chromatin structure on the induction of DNA double strand breaks by ionizing radiation. *Cancer Res.*, **52**, 1580-1586 (1992).
 45. Elomaa, L., Joensuu, H., Kulmala, J., Klemi, P. and Grenman, R.: Squamous cell carcinoma is highly sensitive to Taxol, a possible new radiation sensitizer. *Acta Otolaryngologica*, **115**, 340-344 (1995).
 46. Fearon, E.R. and Vogelstein, B.: A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767 (1990).
 47. Feaver, W.J., Svejstrup, J.Q., Bardwell, L., Bardwell, A.J., Buratowski, S., Gulyas, K.D., Donahue, T.F., Friedberg, E.C. and Kornberg, R.D.: Dual roles of a multiprotein complex from *S. cerevisiae* in transcription and DNA repair. *Cell*, **75**, 1379-1387 (1993).
 48. Finney, D.J.: *Statistical Method in Biological Assay*, 3rd ed., Charles Griffin and Company, London, 1978.
 49. Fisher, D.E.: Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539-542 (1994).
 50. Fornace, A.J., Jr., Nebert, D.W., Hollander, M.C., Luethy, J., Papathanasiou, M., Fargnoli, J. and Holbrook, N.J.: Mammalian genes coordinately regulated by

growth arrest signals and DNA-damaging agents. *Mol. Cell. Biol.*, **9**, 4196-4203 (1989).

51. Frankenberg-Schwager, M.: Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. *Radiat. Environ. Biophys.*, **29**, 273-292 (1990).
52. Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E. and Shay, J.W.: A transcriptionally active DNA-binding site for human p53 protein complexes. *Mol. Cell. Biol.*, **12**, 2866-2871 (1992).
53. Geard, C. and Jones, J.: Radiation and Taxol effects on synchronized human cervical carcinoma cells. *Int. J. Radiat. Oncol. Biol. Phys.*, **29**, 565-569 (1994).
54. Gerhart, J., Wu, M. and Kirschner, M.: Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.*, **98**, 1247-1255 (1984).
55. Gorbsky, G.J.: Kinetochores, microtubules and the metaphase checkpoint. *Trends in Cell Biology*, **5**, 143-148 (1995).
56. Govindarajulu, Z.: *Statistical Techniques in BioAssay*, Karger, Basel (Switzerland), 1988.
57. Grabske, R.J., Lake, S., Gledhill, B.L. and Meistrich, M.L.: Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. *J. Cell. Physiol.*, **86**, 177-190 (1975).
58. Gray, J.W.: Quantitative cytokinetics. *Pharmaceutical Therapeutics*, **22**, 163-197 (1983).
59. Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C.: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855-4878 (1994).

60. Grote, S.J., Joshi, G.P., Revell, S.H. and Shaw, C.A.: Observations of radiation-induced chromosome fragment loss in live mammalian cells in culture and its effect on colony-forming activity. *Int. J. Radiat. Biol.*, **38**, 395-408 (1981).
61. Guillouf, C., Rosselli, F., Krishnaraju, K., Moustacchi, E., Hoffman, B. and Liebermann, D.A.: p53 involvement in control of G2 exit of the cell cycle: role in DNA damage-induced apoptosis. *Oncogene*, **10**, 2263-2270 (1995).
62. Gupta, N., Lamborn, K. and Deen, D.F.: A statistical approach for analyzing clonogenic survival data. *Radiat. Res.*, (1996).
63. Gupta, N., Vij, R., Haas-Kogan, D.A., Israel, M.A., Deen, D.F. and Morgan, W.F.: Cytogenetic damage and the radiation-induced G1 phase checkpoint. *Radiat. Res.*, **145**, 289-298 (1996).
64. Gyuris, J., Golemis, E., Chertkov, H. and Brent, R.: Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791-803 (1993).
65. Haas-Kogan, D.A., Yount, G., Haas, M., Levi, D., Kogan, S.C., Hu, L., Vidair, C., Deen, D.F., Dewey, W.C. and Israel, M.A.: p53-dependent G1 arrest and p53-independent apoptosis influence the radiobiologic response of glioblastoma. *Int. J. Radiat. Oncol. Biol. Phys.*, **in press**(1996).
66. Hall, E.J.: *Radiobiology for the Radiologist*, 4th ed., J. B. Lippincott, Philadelphia, 1994.
67. Han, Z., Chatterjee, D., He, D.M., Early, J., Pantazis, P., Wyche, J.H. and Hendrickson, E.A.: Evidence for a G2 checkpoint in p53-independent apoptosis induction by X-irradiation. *Mol. Cell. Biol.*, **15**, 5849-5857 (1995).
68. Hara, K., Tydeman, P. and Kirschner, M.: A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA*, **77**, 462-466 (1980).

69. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J.: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805-816 (1993).
70. Hartley, K.O., Gell, D., Smith, G.C., Zhang, H., Divecha, N., Connelly, M.A., Admon, A., Lees-Miller, S.P., Anderson, C.W. and Jackson, S.P.: DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell*, **82**, 849-856 (1995).
71. Hartwell, L.: Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543-546 (1992).
72. Hartwell, L.H. and Kastan, M.B.: Cell cycle control and cancer. *Science*, **266**, 1821-1828 (1994).
73. Hastings, N.A.J. and Peacock, J.B.: *Statistical Distributions*, John Wiley & Sons, New York, 1975.
74. Hei, T.K., Piao, C.Q., Geard, C.R. and Hall, E.J.: Taxol and ionizing radiation: Interaction and mechanisms. *Int. J. Radiat. Oncol. Biol. Phys.*, **29**, 267-271 (1994).
75. Ho, J.T. and Deen, D.F.: Prolonged hyperthermia eliminates mycoplasma from cultured human and rat brain tumor cell lines. *Radiat. Res.*, **127**, 339-341 (1991).
76. Holmes, F.A., Walter, R.S., Theriault, R.L., Forman, A.D., Newton, L.K., Raber, M.N., Buzdar, A.U., Frye, D.K. and Hortobagyi, G.N.: Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J. Natl. Cancer Inst.*, **83**, 1797-1805 (1991).
77. Hoshino, T., Ahn, D., Prados, M., Lamborn, K. and Wilson, C.: Prognostic significance of the proliferative potential of intracranial gliomas measured by bromodeoxyuridine labeling. *Int. J. Cancer*, **53**, 550-555 (1993).

78. Hoshino, T., Ito, S., Asai, A., Shibuya, M., Prados, M., Dodson, B., Davis, R. and Wilson, C.: Cell kinetic analysis of human brain tumors by in situ double labeling with bromodeoxyuridine and iododeoxyuridine. *Int. J. Cancer*, **50**, 1-5 (1992).
79. Hunter, T. and Pines, J.: Cyclins and cancer. *Cell*, **66**, 1071-1074 (1991).
80. Iliakis, G.: The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *BioEssays*, **13**, 641-648 (1991).
81. Jordan, M.A., Margolis, R.L., Himes, R.H. and Wilson, L.: Identification of a distinct class of vinblastine binding sites on microtubules. *J. Mol. Biol.*, **187**, 61-73 (1986).
82. Jordan, M.A., Toso, R.T., Thrower, D. and Wilson, L.: Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA*, **90**, 9552-9556 (1993).
83. Jordan, M.A., Wendell, K., Gardiner, S., Derry, W.B., Copp, H. and Wilson, L.: Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res.*, **56**, 816-825 (1996).
84. Joshi, H.C.: Microtubule organizing centers and gamma-tubulin. *Curr. Opin. Cell Biol.*, **6**, 54-62 (1994).
85. Kamb, A.: Cell-cycle regulators and cancer. *Trends Genet.*, **11**, 136-140 (1995).
86. Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavitigian, S.V., Stockert, E., Day, R.S.r., Johnson, B.E. and Skolnick, M.H.: A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436-440 (1994).

87. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W.: Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304-6311 (1991).
88. Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B. and A.J. Fornace, J.: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587-597 (1992).
89. Kerr, J.F., Wyllie, A.H. and Currie, A.R.: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239-257 (1972).
90. Kirchgessner, C.U., Patil, C.K., Evans, J.W., Cuomo, C.A., Fried, L.M., Carter, T., Oettinger, M.A. and Brown, J.M.: DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science*, **267**, 1178-1183 (1995).
91. Kirschner, M.: The cell cycle then and now. *Trends Biochem. Sci.*, **17**, 281-285 (1992).
92. Koshland, D.E., Mitchison, T.J. and Kirschner, M.W.: Polewards chromosome movement driven by microtubule depolymerization in vitro. *Nature*, **331**, 499-504 (1988).
93. Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B.: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA*, **89**, 7491-7495 (1992).
94. Kung, A.L., Sherwood, S.W. and Schimke, R.T.: Cell line-specific differences in the control of cell cycle progression in the absence of mitosis. *Proc. Natl. Acad. Sci. USA*, **87**, 9553-9557 (1990).
95. Lane, D.P.: p53, guardian of the genome. *Nature*, **358**, 15-16 (1992).

96. Lane, D.P. and Crawford, L.V.: T antigen is bound to a host protein in SV40-transformed cells. *Nature*, **278**, 261-263 (1979).
97. Lavin, M.F., Khanna, K.K., Beamish, H., Teale, B., Hobson, K. and Watters, D.: Defect in radiation signal transduction in ataxia-telangiectasia. *Int. J. Radiat. Biol.*, **66**, S151-156 (1994).
98. Lee, J.M. and Bernstein, A.: p53 mutations increase resistance to ionizing radiation. *Proc. Natl. Acad. Sci. USA*, **90**, 5742-5746 (1993).
99. Leeper, D.B., Schneiderman, M.H. and Dewey, W.C.: Radiation-induced division delay in synchronized Chinese hamster ovary cells in monolayer culture. *Radiat. Res.*, **50**, 401-417 (1972).
100. Legha, S.S., Ring, S., Papadopoulos, N., Raber, M. and Benjamin, R.S.: A phase II trial of taxol in metastatic melanoma. *Cancer*, **65**, 2478-2481 (1990).
101. Lehnert, M., Emerson, S., Dalton, W.S., deGiuli, R. and Salmon, S.E.: In vitro evaluation of chemosensitizers for clinical reversal of p-glycoprotein-associated taxol resistance. *Monogr. Natl. Cancer Inst.*, **15**, 63-67 (1993).
102. Lett, J.T.: Damage to DNA and chromatin structure from ionizing radiations, and the radiation sensitivities of mammalian cells. *Prog. Nucleic Acid. Res. Mol. Biol.*, **39**, 305-352 (1990).
103. Levine, A.J., Momand, J. and Finlay, C.A.: The p53 tumour suppressor gene. *Nature*, **351**, 453-456 (1991).
104. Liebmann, J., Cook, J.A., Fisher, J., Teague, D. and Mitchell, J.B.: Changes in radiation survival curve parameters in human tumor and rodent cells exposed to paclitaxel (Taxol). *Int J Radiat Oncol Biol Phys*, **29**, 559-564 (1994).

105. Liebmann, J., Cook, J.A., Fisher, J., Teague, D. and Mitchell, J.B.: In vitro studies of Taxol as a radiation sensitizer in human tumor cells. *J. Natl. Cancer Inst.*, **86**, 441-446 (1994).
106. Liebmann, J.E., Cook, J.A., Lipschultz, C., Teague, D., Fisher, J. and Mitchell, J.B.: Cytotoxic studies of paclitaxel (taxol) in human tumour cell lines. *Br. J. Cancer*, **68**, 1104-1109 (1993).
107. Lin, J., Chen, J., Elenbaas, B. and Levine, A.J.: Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.*, **8**, 1235-1246 (1994).
108. Linfoot, P.A., Gray, J.W., Dean, P.N., Marton, L.J. and Deen, D.F.: Effect of cell cycle position on the survival of 9L cells treated with nitrosoureas that alkylate, cross-link, and carbamoylate. *Cancer Res.*, **46**, 2402-2406 (1986).
109. Little, J.B.: Delayed initiation of DNA synthesis in irradiated human diploid cells. *Nature*, **218**, 1064-1065 (1968).
110. Little, J.B. and Nagasawa, H.: Effect of confluent holding on potentially lethal damage repair, cell cycle progression, and chromosomal aberrations in human normal and ataxia-telangiectasia fibroblasts. *Radiat. Res.*, **101**, 81-93 (1985).
111. Liu, X., Miller, C.W., Koeffler, P.H. and Berk, A.J.: The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription. *Mol. Cell. Biol.*, **13**, 3291-3300 (1993).
112. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T. and Tlsty, T.D.: Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923-935 (1992).
113. Lock, R.B. and Ross, W.E.: Possible role for p34^{cdc2} kinase in etoposide-induced cell death of Chinese hamster ovary cells. *Cancer Res.*, **50**, 3767-3771 (1990).

114. Long, B.H. and Fairchild, C.R.: Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. *Cancer Res.*, **54**, 4355-4361 (1994).
115. Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. and Jacks, T.: p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847-849 (1993).
116. Mack, D.H., Vartikar, J., Pipas, J.M. and Laimins, L.A.: Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature*, **363**, 281-283 (1993).
117. Madsen, P. and Celis, J.E.: S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. A role for cyclin in DNA replication? *FEBS Lett.*, **193**, 5-11 (1985).
118. Maity, A., McKenna, W.G. and Muschel, R.J.: The molecular basis for cell cycle delays following ionizing radiation: a review. *Radiother. Oncol.*, **31**, 1-13 (1994).
119. Mandelkow, E. and Mandelkow, E.M.: Microtubules and microtubule-associated proteins. *Curr. Opin. Cell Biol.*, **7**, 72-81 (1995).
120. Marder, B.A. and Morgan, W.F.: Delayed chromosomal instability induced by DNA damage. *Mol. Cell. Biol.*, **13**, 6667-6677 (1993).
121. Martin, D.W., Munoz, R.M., Subler, M.A. and Deb, S.: p53 binds to the TATA-binding protein-TATA complex. *J. Biol. Chem.*, **268**, 13062-13067 (1993).
122. Masui, Y. and Markert, C.L.: Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.*, **177**, 129-145 (1971).
123. McGuire, W.P., Rowinski, E.K., Grochow, L.B., Longnecker, S.M., Ettinger, D.S., Armstrong, D.K. and Donehower, R.C.: Taxol: a unique antineoplastic agent

with significant activity in advanced ovarian epithelial neoplasms. *Ann. Intern. Med.*, **111**, 273-279 (1989).

124. McKenna, W.G., Iliakis, G., Weiss, M.C., Bernhard, E.J. and Muschel, R.J.: Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and V-myc. *Radiat. Res.*, **125**, 283-287 (1991).
125. Mercer, W.E., Shields, M.T., Amin, M., Sauve, G.J., Appella, E., Romano, J.W. and Ullrich, S.J.: Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA*, **87**, 6166-6170 (1990).
126. Milner, J., Cook, A. and Mason, J.: p53 is associated with p34^{cdc2} in transformed cells. *EMBO J.*, **9**, 2885-2889 (1990).
127. Minotti, A.M., Barlow, S.B. and Cabral, F.: Resistance to anti-mitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. *J. Biol. Chem.*, **266**, 3987-3994 (1991).
128. Mitchison, T. and Kirschner, M.: Microtubule assembly nucleated by isolated centrosomes. *Nature*, **312**, 232-237 (1984).
129. Mitchison, T.J.: Compare and contrast actin filaments and microtubules. *Mol. Biol. Cell*, **3**, 1309-1315 (1992).
130. Momand, J., Zambetti, G.P., Olson, D.C., George, D. and Levine, A.J.: The Mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237-1245 (1992).
131. Morgan, D.O.: Principles of CDK regulation. *Nature*, **374**, 131-134 (1995).
132. Morgan, W.F., Yates, B.L., Rufer, J.T., Abella Columna, E., Valcarcel, E.R. and Phillips, J.W.: Chromosomal aberration induction in CHO cells by combined

exposure to restriction enzymes and X-rays. *Int. J. Radiat. Biol.*, **60**, 627-634 (1991).

133. Munro, T.R.: The relative radiosensitivity of the nucleus and cytoplasm of Chinese hamster fibroblasts. *Radiat. Res.*, **42**, 451-470 (1970).
134. Murnane, J.P.: Cell cycle regulation in response to DNA damage in mammalian cells: A historical perspective. *Cancer Metastasis Rev*, **14**, 17-29 (1995).
135. Murnane, J.P. and Kapp, L.N.: A critical look at the association of human genetic syndromes with sensitivity to ionizing radiation. *Sem. Cancer Biol.*, **4**, 93-104 (1993).
136. Murnane, J.P. and Schwartz, J.L.: Cell checkpoint and radiosensitivity. *Nature*, **365**, 22 (1993).
137. Murray, A.W.: Creative blocks: cell cycle checkpoints and feedback controls. *Nature*, **359**, 599-604 (1992).
138. Murray, A.W. and Hunt, T.: *The Cell Cycle*, W.H. Freeman, New York, 1993.
139. Muschel, R.J., Zhang, H.B., Iliakis, G. and McKenna, W.G.: Cyclin B expression in HeLa cells during the G2 block induced by ionizing radiation. *Cancer Res.*, **51**, 5113-5117 (1991).
140. Nagasawa, H., Latt, S.A., Lalande, M.E. and Little, J.B.: Effects of X-irradiation on cell cycle progression, induction of chromosomal aberrations and cell killing in ataxia telangiectasia (AT) fibroblasts. *Mutat. Res.*, **148**, 71-82 (1985).
141. Nasmyth, K.: Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.*, **5**, 166-179 (1993).
142. Natarajan, A.T., Balajee, A.S., Boei, J.J., Chatterjee, S., Darroudi, F., Grigorova, M., Noditi, M., Oh, H.J., Slijepcevic, P. and Vermeulen, S.: Recent developments

- in the assessment of chromosomal damage. *Int. J. Radiat. Biol.*, **66**, 615-623 (1994).
143. Nelson, W.G. and Kastan, M.B.: DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.*, **14**, 1815-1823 (1994).
144. Nigro, J.M., et al.: Mutations in the p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708 (1989).
145. Oberosler, P., Hloch, P., Ramsperger, U. and Stahl, H.: p53-catalyzed annealing of complementary single-stranded nucleic acids. *EMBO J.*, **12**, 2389-2396 (1993).
146. Okayasu, R., Bloecher, D. and Iliakis, G.: Variation through the cell cycle in the dose-response of DNA neutral filter elution in X-irradiated synchronous CHO-cells. *Int. J. Radiat. Biol.*, **53**, 729-747 (1988).
147. Oleinick, N.L., Chiu, S.M., Ramakrishnan, N. and Xue, L.Y.: The formation, identification, and significance of DNA-protein cross-links in mammalian cells. *Br. J. Cancer (Suppl)*, **8**, 135-140 (1987).
148. Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L. and Vogelstein, B.: Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80-83 (1992).
149. Painter, R.B.: The direct effect of X-irradiation on HeLa S3 deoxyribonucleic acid synthesis. *Radiat. Res.*, **16**, 846-859 (1962).
150. Painter, R.B., The role of DNA damage and repair in cell killing induced by ionizing radiation. In *Radiation Biology in Cancer Research* (R.E. Meyn and H.R. Withers, Eds.), Raven Press, New York, 1980.

151. Painter, R.B. and Robertson, J.S.: Effect of irradiation and theory of role of mitotic delay on the time course of labeling of HeLa S3 cells with tritiated thymidine. *Radiat. Res.*, **11**, 206-217 (1959).
152. Painter, R.B. and Young, B.R.: X-ray-induced inhibition of DNA synthesis in Chinese hamster ovary, human HeLa, and mouse L cells. *Radiat. Res.*, **64**, 648-656 (1975).
153. Papathanasiou, M.A., Kerr, N.C.K., Robbins, J.H., McBride, O.W., Alamo, I.J., Barrett, S.F., Hickson, I.D. and Fornace, A.J., Jr: Induction by ionizing radiation of the gadd45 gene in cultured human cells: Lack of mediation by protein kinase C. *Mol. Cell. Biol.*, **11**, 1009-1016 (1991).
154. Parness, J. and Horwitz, S.B.: Taxol binds to polymerized tubulin in vitro. *J. Cell Biol.*, **91**, 479-487 (1981).
155. Picksley, S.M. and Lane, D.P.: p53 and Rb: their cellular roles. *Curr. Opin. Cell Biol.*, **6**, 853-858 (1994).
156. Pietenpol, J.A., Tokino, T., Thiagalingam, S., el-Deiry, W.S., Kinzler, K.W. and Vogelstein, B.: Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci. USA*, **91**, 1998-2002 (1994).
157. Prelich, G., Kostura, M., Marshak, D.R., Mathews, M.B. and Stillman, B.: The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. *Nature*, **326**, 471-475 (1987).
158. Prelich, G., Tan, C.K., Kostura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B.: Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature*, **326**, 517-520 (1987).
159. Puck, T.T.: Action of radiation on mammalian cells. III. Relationship between reproductive death and induction of chromosome anomalies by X-irradiation of euploid human cells *in vitro*. *Proc. Natl. Acad. Sci. USA*, **44**, 772-780 (1958).

160. Quiet, C.A., Weichselbaum, R.R. and Grdina, D.J.: Variation in radiation sensitivity during the cell cycle of two human squamous cell carcinomas. *Int. J. Radiat. Oncol. Biol. Phys.*, **20**, 733-738 (1991).
161. Raff, M.C.: Social controls on cell survival and cell death. *Nature*, **356**, 397-400 (1992).
162. Ragimov, N., Krauskopf, A., Navot, N., Rotter, V., Oren, M. and Aloni, Y.: Wild-type but not mutant p53 can repress transcription initiation in vitro by interfering with the binding of basal transcription factors to the TATA motif. *Oncogene*, **8**, 1183-1193 (1993).
163. Revell, S.H., Relationship between chromosome damage and cell death. In *Radiation-Induced Chromosome Damage in Man* (T. Ishihara and M.S. Sasaki, Eds.), pp. 215-233. Alan R. Liss, New York, 1983.
164. Riley, P.A.: Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int. J. Radiat. Biol.*, **65**, 27-33 (1994).
165. Ritter, M.A., Cleaver, J.E. and Tobias, C.A.: High-LET radiations induce a large proportion of non-rejoining DNA breaks. *Nature*, **266**, 653-655 (1977).
166. Roberts, J.R., Allison, D.C., Donehower, R.C. and Rowinsky, E.K.: Development of polyploidization in taxol-resistant human leukemia cells in vitro. *Cancer Res.*, **50**, 710-716 (1990).
167. Roy, S.N. and Horwitz, S.B.: A phosphoglycoprotein associated with taxol-resistance in J774.2 cells. *Cancer Res.*, **45**, 3856-3863 (1985).
168. Rutka, J.T., Giblin, J.R., Dougherty, D.Y., Liu, H.C., McCulloch, J.R., Bell, C.W., Stern, R.S., Wilson, C.B. and Rosenblum, M.L.: Establishment and characterization of five cell lines derived from human malignant gliomas. *Acta Neuropathol. (Berl.)*, **75**, 92-103 (1987).

169. Sancar, A.: Mechanisms of DNA excision repair. *Science*, **266**, 1954-1956 (1994).
170. Sang, B.C., Chen, J.Y., Minna, J. and Barbosa, M.S.: Distinct regions of p53 have a differential role in transcriptional activation and repression functions. *Oncogene*, **9**, 853-859 (1994).
171. Savitsky, K., et al.: A single ataxia-telangiectasia gene with a product similar to PI-3 kinase. *Science*, **268**, 1749-1753 (1995).
172. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P. and Egly, J.M.: DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. *Science*, **260**, 58-63 (1993).
173. Schiff, P.B., Fant, J. and Horowitz, S.B.: Promotion of microtubule assembly in vitro by taxol. *Nature*, **277**, 665-667 (1979).
174. Schiff, P.B. and Horwitz, S.B.: Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA*, **77**, 1561-1565 (1980).
175. Schneiderman, M.H., Dewey, W.C., Leeper, D.B. and Nagasawa, H.: Use of the mitotic selection procedure for cell cycle analysis. Comparison between the X-ray and cycloheximide G2 markers. *Exp. Cell Res.*, **74**, 430-438 (1972).
176. Seewaldt, V., Greer, B., Cain, J., Figge, D., Tamimi, H., Brown, W. and Miller, S.: Paclitaxel (Taxol) treatment for refractory ovarian cancer: phase II clinical trial. *Am. J. Gynecol. Oncol.*, **170**, 1666-1670 (1994).
177. Seto, E., Usheva, A., Zambetti, G.P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A.J. and Shenk, T.: Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc. Natl. Acad. Sci. USA*, **89**, 12028-12032 (1992).

178. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B. and Costa, J.: Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA*, **89**, 4495-4499 (1992).
179. Shivji, M.K.K., Kenny, M.K. and Wood, R.D.: Proliferating cell nuclear antigen is required for DNA excision repair. *Cell*, **69**, 367-374 (1992).
180. Sinclair, W.K.: Cyclic X-ray responses in mammalian cells in vitro. *Radiat. Res.*, **33**, 620-643 (1968).
181. Singer, W.D., Jordan, M.A., Wilson, L. and Himes, R.: Binding of vinblastine to stabilized microtubules. *Mol. Pharmacol.*, **36**, 366-370 (1989).
182. Slichenmyer, W.J., Nelson, W.G., Slebos, R.J. and Kastan, M.B.: Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage. *Cancer Res.*, **53**, 4164-4168 (1993).
183. Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M. and Fornace, A.J., Jr.: Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science*, **266**, 1376-1380 (1994).
184. Snedecor, G.W. and Cochran, W.G.: *Statistical Methods*, 8th ed., Iowa State University Press, Ames, 1989.
185. Solomon, M.J.: Activation of the various cyclin/cdc2 protein kinases. *Curr Opin Cell Biol*, **5**, 180-186 (1993).
186. Steel, G.G.: Terminology in the description of drug-radiation interactions. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 1145-1150 (1979).
187. Steel, G.G. and Peckham, M.J.: Exploitable mechanisms in combined radiotherapy-chemotherapy: The concept of additivity. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 85-91 (1979).

188. Steren, A., Sevin, B., Perras, J., Angioli, R., Nguyen, H., Guerra, L., Koechli, O. and Averette, H.: Taxol sensitizes human ovarian cancer cells to radiation. *Gynecol. Oncol.*, **48**, 252-258 (1993).
189. Steren, A., Sevin, B.U., Perras, J., Ramos, R., Angioli, R., Nguyen, H., Koechli, O. and Averette, H.E.: Taxol as a radiation sensitizer: a flow cytometric study. *Gynecol. Oncol.*, **50**, 89-93 (1993).
190. Stewart, N., Hicks, G.G., Paraskevas, F. and Mowat, M.: Evidence for a second cell cycle block at G2/M by p53. *Oncogene*, **10**, 109-115 (1995).
191. Stromberg, J.S., Lee, Y.J., Armour, E.P., Martinez, A.A. and Corry, P.M.: Lack of radiosensitization after paclitaxel treatment of three human carcinoma cell lines. *Cancer*, **75**, 2262-2268 (1995).
192. Su, L.-N. and Little, J.B.: Transformation and radiosensitivity of human diploid skin fibroblasts transfected with SV40 T-antigen mutants defective in RB and p53 binding domains. *Int. J. Radiat. Biol.*, **62**, 461-468 (1992).
193. Su, L.N. and Little, J.B.: Prolonged cell cycle delay in radioresistant human cell lines transfected with activated *ras* oncogene and/or Simian Virus 40 T-antigen. *Radiat. Res.*, **133**, 73-79 (1993).
194. Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T. and Van Dyke, T.: p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell*, **78**, 703-711 (1994).
195. Szybalski, W.: X-ray sensitization by halopyrimidines. *Cancer Chemother. Rep.*, **58**, 539-557 (1974).
196. Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F. and Minna, J.D.: p53: a frequent target for genetic abnormalities in lung cancer. *Science*, **246**, 491-494 (1989).

197. Tang, H., Davis, M.A., Strickfaden, S.M., Maybaum, J. and Lawrence, T.S.: Influence of cell cycle phase on radiation-induced cytotoxicity and DNA damage in human colon cancer (HT29) and Chinese hamster ovary cells. *Radiat. Res.*, **138**, S109-112 (1994).
198. Terasima, T. and Tolmach, L.J.: Variations in several responses of HeLa cells to X-irradiation during the division cycle. *Biophys. J.*, **3**, 11-33 (1963).
199. Thacker, J.: The study of responses to 'model' DNA breaks induced by restriction endonucleases in cells and cell-free systems: achievements and difficulties. *Int. J. Radiat. Biol.*, **66**, 591-596 (1994).
200. Thigpen, T., Vance, R., Punecky, L. and Khansur, T.: Chemotherapy in advanced ovarian carcinoma: current standards of care based on randomized trials. *Gynecol. Oncol.*, **55**, S97-107 (1994).
201. Thut, C.J., Chen, J.L., Klemm, R. and Tjian, R.: p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science*, **267**, 100-104 (1995).
202. Tishler, R.B., Geard, C.R., Hall, E.J. and Schiff, P.B.: Taxol sensitizes human astrocytoma cells to radiation. *Cancer Res.*, **52**, 3495-3497 (1992).
203. Tishler, R.B., Schiff, P.B., Geard, C.R. and Hall, E.J.: Taxol: A novel radiation sensitizer. *Int. J. Radiat. Oncol. Biol. Phys.*, **22**, 613-617 (1992).
204. Truant, R., Xiao, H., Ingles, C.J. and Greenblatt, J.: Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. *J. Biol. Chem.*, **268**, 2284-2287 (1993).
205. Tucker, J.D., Morgan, W.F., Awa, A.A., Bauchinger, M., Blakey, D., Cornforth, M.N., Littlefield, L.G., Natarajan, A.T. and Shasserre, C.: A proposed system for scoring structural aberrations detected by chromosome painting. *Cytogenet. Cell Genet.*, **68**, 211-221 (1995).

206. Ullrich, S.J., Anderson, C.W., Mercer, W.E. and Appella, E.: The p53 tumor suppressor protein, a modulator of cell proliferation. *J. Biol. Chem.*, **267**, 15259-15262 (1992).
207. Vogelstein, B. and Kinzler, K.W.: p53 function and dysfunction. *Cell*, **70**, 523-526 (1992).
208. Vogelstein, B. and Kinzler, K.W.: The multistep nature of cancer. *Trends Genet.*, **9**, 138-141 (1993).
209. von Sonntag, C.: Radiation chemistry in the 1990s: pressing questions relating to the areas of radiation biology and environmental research. *Int. J. Radiat. Biol.*, **65**, 19-26 (1994).
210. Walters, R.A. and Petersen, D.F.: Radiosensitivity of mammalian cells. I. Timing and dose-dependency of radiation-induced division delay. *Biophys. J.*, **8**, 1475-1486 (1968).
211. Ward, J.F.: Biochemistry of DNA lesions. *Radiat. Res.*, **104**, S103-111 (1985).
212. Ward, J.F.: The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. *Int. J. Radiat. Biol.*, **57**, 1141-1150 (1990).
213. Ward, J.F.: Radiation mutagenesis: the initial DNA lesions responsible. *Radiat. Res.*, **142**, 362-368 (1995).
214. Warters, R.L., Hofer, K.G., Harris, C.R. and Smith, J.M.: Radionuclide toxicity in cultured mammalian cells: elucidation of the primary site of radiation damage. *Curr. Top. Radiat. Res. Q.*, **12**, 389-407 (1978).
215. Weinert, T. and Hartwell, L.: Control of G2 delay by the *RAD9* gene of *Saccharomyces cerevisiae*. *J. Cell. Sci. Suppl.*, **12**, 145-148 (1989).

216. Weinert, T.A.: Dual cell cycle checkpoints sensitive to chromosome replication and DNA damage in the budding yeast *Saccharomyces cerevisiae*. *Radiat. Res.*, **132**, 141-143 (1992).
217. Weinert, T.A. and Hartwell, L.H.: The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science*, **241**, 317-322 (1988).
218. Weinert, T.A. and Hartwell, L.H.: Characterization of *RAD9* of *Saccharomyces cerevisiae* and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. *Mol. Cell. Biol.*, **10**, 6554-6564 (1990).
219. West, C.M., Keng, P.C. and Sutherland, R.M.: Growth phase related variation in the radiation sensitivity of human colon adenocarcinoma cells. *Int. J. Radiat. Oncol. Biol. Phys.*, **14**, 1213-1219 (1988).
220. Williams, G.T. and Smith, C.A.: Molecular regulation of apoptosis: genetic controls on cell death. *Cell*, **74**, 777-779 (1993).
221. Wyllie, A.H., Kerr, J.F. and Currie, A.R.: Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251-306 (1980).
222. Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D.: p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701-704 (1993).
223. Xue, L.Y., Friedman, L.R., Oleinick, N.L. and Chiu, S.M.: Induction of DNA damage in gamma-irradiated nuclei stripped of nuclear protein classes: differential modulation of double-strand break and DNA-protein crosslink formation. *Int. J. Radiat. Biol.*, **66**, 11-21 (1994).
224. Yamada, M. and Puck, T.T.: Action of radiation on mammalian cells. IV. Reversible mitotic lag in the S3 HeLa cell produced by low doses of X-rays. *Proc. Natl. Acad. Sci. USA*, **47**, 1181-1191 (1961).

225. Yin, Y., Tainsky, M.A., Bischoff, F.Z., Strong, L.C. and Wahl, G.M.: Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell*, **70**, 937-948 (1992).
226. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A. and Oren, M.: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*, **352**, 345-347 (1991).
227. Yount, G.L., Haas-Kogan, D.A., Vidair, C., Levi, D., Haas, M., Dewey, W.C. and Israel, M.A.: Cell cycle synchrony unmasks influence of p53 function on radiation sensitivity of human glioblastoma cells. *Cancer Res.*, **56**, 500-506 (1996).
228. Yu, C.K. and Sinclair, W.K.: Mitotic delay and chromosomal aberrations induced by X-rays in synchronized Chinese hamster cells in vitro. *J. Natl. Cancer Inst.*, **39**, 619-632 (1967).
229. Zhan, Q., Carrier, F. and Fornace Jr., A.J.: Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol. Cell. Biol.*, **13**, 4242-4250 (1993).

Appendix A Comments on the Use of Paclitaxel as a Radiation Sensitizer

The discovery of paclitaxel (Taxol™) has generated considerable interest in its unique mechanism of action and therefore its applicability for clinical use. It is known that paclitaxel stabilizes microtubules by binding to tubulin heterodimers resulting in abnormal formation and function of organelles such as the mitotic spindle (37, 82). Recognition that cells treated with paclitaxel tend to arrest in mitosis led to the reasonable assumption that a second agent that could affect mitotic cells selectively might improve the therapeutic efficacy of paclitaxel. Radiation is, of course, such a agent since the cell age response of mammalian cells is well described (180). The cell cycle age response reflects differences in radiation sensitivity depending on which phase of the cell cycle radiation is delivered; typically cells in late G₁ and M are most sensitive.

After reading several recent reports which identified paclitaxel as a radiation sensitizer we became concerned by the imprecise use of nomenclature that might lead to improper assumptions by other readers (26, 45, 104, 188, 189, 202, 203). While some of these papers have used the terms *sensitizer* and *enhancer* appropriately, others have not been as careful. To avoid any confusion we would like to remind those interested in this field that a convention exists with respect to the terminology of drug/radiation interactions (186, 187). It is stated that for agents to be labeled as radiation sensitizers there must be little or no toxicity at the doses employed. The distinction is most important for those who might interpret the use of the term *sensitizer* to indicate an agent that has minimal cytotoxicity yet possesses a significant ability to augment the effect of radiation; thereby

leading to inaccurate assumptions concerning its potential use clinically. The rationale for carefully using the term *sensitization* vs. *enhancement* rests with the belief that it should be clear whether the agent being studied affects the biological system when used by itself.

It is evident that paclitaxel is a highly effective chemotherapeutic agent used by itself in vitro and in certain clinical situations. Indeed the cytotoxicity of paclitaxel in vitro is usually in the nanomolar range. It is clear from published reports with the drug concentrations used to study this agent in combination with radiation, anywhere from 50% to >99% of treated cells lose clonogenic potential prior to irradiation. This degree of cytotoxicity certainly does not allow paclitaxel to be classified as an "inactive" agent. While there is no reason to contest the fact that paclitaxel can act as a radiation enhancer in some systems, we believe that convincing proof of its use as a radiation sensitizer requires studying drug concentrations that carry a lesser degree of toxicity. Recent data from our group and others indicates that paclitaxel concentrations resulting in minimal cytotoxicity do not cause radiation sensitization (191).

The question of inherent drug toxicity raises leads us to question the assumption that enhanced radiation cell killing is a result of accumulation in G₂/M (the sensitive phase of the cell cycle). Since paclitaxel cytotoxicity is believed to result from aberrant mitosis, then loss of clonogenicity from paclitaxel alone of the magnitude reported above (>50%), is likely to preferentially eliminate viable cells at the G₂/M boundary. This would leave those cells that are in *other* phases of the cell cycle to account for the observed radiation enhancement. It may be that cells arrested at mitosis are merely held at this point free to re-enter the cell cycle once paclitaxel is removed or metabolized. This viewpoint leads to the question of which cells then, i.e. in which phase of the cell cycle if any, are dying as a result of paclitaxel treatment. Determining answers will require definition of the sensitivity of individual phases of the cell cycle in addition to careful cytokinetic studies. Exploration

of these and other interesting issues should lead to a clearer understanding of how paclitaxel and radiation interact at the cellular level.

Nalin Gupta, M.D.

Dennis F. Deen, Ph.D.

Brain Tumor Research Center

Department of Neurological Surgery

University of California San Francisco

San Francisco, CA 94143-0520

(This appendix was originally published as a letter to the editor in *The International Journal of Radiation Oncology Biology Physics*, vol. 33, p. 239, 1995.)

What they undertook to do
They brought to pass;
All things hang like a drop of dew
Upon a blade of grass.

Gratitude to the Unknown Instructor
W.B. Yeats

THE

For reference

Not to be taken
from the room.

