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A Study of the Role of p21/WAF1/CIP1 in Thermal Radiosensitization

by

Carey Feagan, B.Sc.

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of

Master of Science

Ottawa-Carleton Institute of Physics
Department of Physics
Carleton University
Ottawa, Ontario
November, 2001

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in Thermal Radiosensitization”

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Chair, Department of Physics

Thesis Supervisor

Carleton University
December 20, 2001

Abstract

The use of hyperthermia in combination with radiation therapy may be improved with a thorough understanding of the cellular mechanism of thermal radiosensitization. It is thought that the mechanism of thermal radiosensitization is related to the inhibition of repair of radiation-induced DNA damage by heat. Due to the apparent role of the gene p21/WAF1/CIP1 in the repair of radiation-induced DNA damage, its involvement in thermal radiosensitization was, therefore, investigated in this thesis. Two human colorectal cancer cell lines were used, both of which have wt p53 status but one was p21/WAF1/CIP1 deficient and the other was p21/WAF1/CIP1 proficient. I found no significant difference in the response of the two cell lines to exposure to 250 kV x-rays nor to hyperthermia treatments of two hours at 42°C or thirty minutes at 44°C. In addition, there was no difference in the clonogenic survival of both cell lines to hyperthermia before or after irradiation for both hyperthermia temperatures. Comparison of thermal enhancement ratios (TERs) showed that there was no difference in the amount of thermal radiosensitization induced in either cell line. The induction and subsequent repair of DNA double strand breaks, as measured by clamped homogenous gel electrophoresis (CHEF) was the same in both cell lines. This finding strongly suggests that the gene p21/WAF1/CIP1 does not have an effect on the degree of thermal radiosensitization expressed in these two cell lines.

Acknowledgements

I have been given the privilege of working with many amazing people throughout the completion of this Master's thesis. For his knowledge, encouragement, and ongoing support, I would like to thank my supervisor, Dr. Cheng Ng. His good advice and constructive criticism in the writing of this thesis were much appreciated. Thanks to Dr. Bog Jarosz for his helpful suggestions concerning the layout of this thesis.

I am also very grateful to my fellow researchers in the physics lab at the Ottawa Regional Cancer Center. Specifically, I would like to thank Gosia Niedbala for her knowledge and friendship. I would also like to thank Sami Qutob for sharing his knowledge, patience and many short cuts. Also, the experience of both Sheryl Cybulski and Julie-Maude Leblanc was much appreciated.

Finally, I would like to thank my family. The love, support and encouragement of my parents, has gotten me through to where I am today. Also, the strength and love of my fiancé, Stig, has helped me more than he could ever know.

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LIST OF ABBREVIATIONS

BER	Base Excision Repair
CHEF	Clamped Homogeneous Electric Field (electrophoresis)
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
FAR	Fraction of Activity Released
HDR	High Dose Rate
LQ	Linear Quadratic (model)
NER	Nucleotide Excision Repair
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PE	Plating Efficiency
PLD	Potentially Lethal Damage
PVDF	Polyvinylidene Difluoride (membrane)
SF	Surviving Fraction
SLD	Sub Lethal Damage
SSB	Single Strand Break
TBE	Tris/Boric Acid/EDTA (buffer)
TER	Thermal Enhancement Ratio
wt	Wild type

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Chapter 1

Introduction

Hyperthermia has been studied extensively over the past several years due to its potential use as an adjuvant to radiotherapy for the treatment of cancer. Many investigations have shown that hyperthermia can cause radiation sensitization (thermal radiosensitization) at the cellular (Dewey *et al.*, 1977), animal (Horsman and Overgaard, 1989; Marino and Civadalli, 1992), and human levels (Nishimura *et al.*, 1992; Overgaard *et al.*, 1996). This sensitizing effect is widely accepted to be due to the inhibition of repair of radiation-induced DNA damage by heat (Li *et al.*, 1976; Warters and Roti-Roti, 1979, Iliakis *et al.*, 1990; Raaphorst, 1992; Dahm-Daphi *et al.*, 1997;). Investigation of genes involved in DNA repair could help in identifying the means of heat-induced repair inhibition. The gene p21/WAF1/CIP1 has been shown in many studies to play a role in the repair of DNA damage (McDonald *et al.*, 1996; Jakob *et al.*, 2000; Groeger *et al.*, 2000). This study focuses on the role of p21/WAF1/CIP1 in thermal radiosensitization.

1.1 X-Radiation

The critical target for ionizing radiation in biological matter is widely accepted to be the DNA (deoxyribonucleic acid) of cells (Hall, 1994). When x-radiation is absorbed in biological material, there is a possibility that it will interact either directly with the critical targets in the cells (i.e. DNA atoms or molecules) or indirectly with other atoms or molecules in the cells (i.e. water) to produce free radicals that subsequently damage the critical target (Figure 1.1). The ionizations produced by x-radiation can cause a variety of lesions in the DNA resulting in altered molecular structure and function. Some of these lesions include single strand breaks (SSBs), double strand breaks (DSBs), base alterations and chromosome aberrations. Double strand breaks are generally thought to be the lethal lesion in cells (Ward, 1990).

Radiation damage itself is classified under three different categories. Lethal damage is irreversible, irreparable and leads to cell death. Sub-lethal damage (SLD) can, under normal circumstances, be repaired unless additional sub-lethal damage is added to form lethal damage. This type of repair is known as sub-lethal damage repair. Potentially lethal damage (PLD) is damage that can be influenced by post-irradiation environmental conditions. The repair of sub-lethal damage is an important aspect to consider in the treatment of cancer with external beam radiotherapy. Since HDR radiation is delivered in small fractions over a period of a few weeks, the potential for repair of sub-lethal damage is high. If this repair can

be inhibited in some way, the treatment will be made more effective. This is the basis of combined treatment therapies.

Figure 1.1 The mode of interaction of x-radiation with biological matter (Hall, 1994).

1.2 The Linear Quadratic (LQ) Model

There have been many models proposed to account for the response of cells to radiation. Early models based on the target theory of radiation action, such as the single-hit multitarget model (Lea, 1955) and the multi-hit multitarget model (Puck and Marcus, 1956), were replaced by models that better explained the cellular mechanisms involved. Such models include the Linear Quadratic (LQ) model (Chadwick and Leenhouts, 1973), the Saturable Repair model (Goodhead, 1985), the Repair-Misrepair model (Tobias, 1985), and the Lethal and Potentially Lethal (LPL) model (Curtis, 1986). Of these newer, more mechanistic models, there is no widespread agreement as to which one best describes cell survival. One study (Brenner *et al.*, 1998) went so far as to show that these models mentioned above result in similar predictions of time dose relationships. However, due to the small number of parameters involved in the LQ model, it has become the model of choice in radiotherapy.

The Linear Quadratic model was proposed by Chadwick and Leenhouts in 1973 (Chadwick and Leenhouts, 1973) to describe the effect of radiation on cell survival. The theory assumes that the primary action of radiation is breakage of a molecular bond and that a broken bond can be repaired. The theory also assumes that the critical molecule in a cell is the DNA, the critical damage leading to cell death is a DNA double strand break, and that a certain number of DSBs lead to cell death. The LQ model is expressed as follows:

$$S(D) = \exp (-\alpha D - \beta D^2), \quad (1)$$

where $S(D)$ is the surviving fraction, D is the dose, α , the linear component, accounts for DNA double strand breaks that occur from a single ionizing event and β , the quadratic component, accounts for DNA DSBs that occur from two independent ionizing events, i.e. two single strand breaks in close proximity. The quadratic component (β) is considered to be representative of the level of sub-lethal damage repair that occurs, because if the first SSB is repaired before the other occurs, then the DSB will not be produced. The LQ model was used to fit all survival curve data in this study with the exception of the heat response curves.

1.3 Hyperthermia and Thermal Radiosensitization

Hyperthermia is the addition of heat to cells above the normal body temperature of 37°C. Cell killing by hyperthermia is enhanced with increased temperature and duration of heating. Survival curves for cells exposed to heat for different periods of time at various temperatures are shown in Figure 1.2 (Hall, 1994). These curves are similar to those obtained for x-rays except that at lower temperatures the survival curve flattens out due to the development of thermotolerance or induced thermal resistance. The mechanism involved in cell killing by heat is however different to that of x-rays.

Figure 1.2 Hyperthermia survival curves for cells *in vitro* heated at different temperatures for varying lengths of time (Hall, 1994).

No general mechanism of heat-induced cell killing has been elucidated (Mackey, 1993). However, *in vitro* studies have suggested that membrane damage and protein inactivation play a major role in cellular heat damage (Hall, 1994). While hyperthermia has been used in the treatment of cancer for many years, it is generally agreed that local hyperthermia alone has no role in the curative treatment of cancer (Hall, 1994). It is widely accepted that exposure to heat sensitizes cells to radiation (Overgaard, 1989) and thus the combination of hyperthermia with radiation would be beneficial in the treatment of cancer.

Many studies have shown that cells that are resistant to radiation due to conditions such as hypoxia, reduced pH and nutrients, high repair abilities and cell cycle position are sensitive to hyperthermia (reviewed in Raaphorst, 1989). In fact, hyperthermia is probably the most potent cellular radiosensitizer known to date (Kampinga and Dikomey, 2001). The degree of thermal radiosensitization is dependent on the heating time and the temperature. The effect of moderate temperatures (around 40°C to 43°C) on the radiation survival curve is a reduction of the shoulder, and the largest potentiation effect is seen when heat and radiation are given concurrently. At higher temperatures, however, the survival curve is steepened and sequencing effects are minimal (Hall, 1994). These differences between higher and lower temperatures may be due to either different critical targets at the different temperatures or simply that thermotolerance may develop during the treatment at lower temperatures causing less cell killing by heat.

The enhanced radiosensitization seen when heat and radiation treatments are combined is normally expressed in terms of a thermal enhancement ratio (TER). A TER is defined as the radiation dose without heat divided by the radiation dose with heat required to produce the same amount of cell killing. A TER greater than one is considered advantageous when hyperthermia is combined with radiation and TER's up to 4.9 have been described *in vitro* (Ben Hur *et al.*, 1974; Harisiadis *et al.*, 1978; Raaphorst *et al.*, 1994).

The combination of heat and radiation produces interactive killing in virtually all cell lines and no clear trend in effectiveness between normal and

tumor cells has been seen (Hahn, 1982; Konings, 1995). The mechanism involved in thermal radiosensitization is still unknown but is believed to be the inhibition of repair of radiation-induced DNA damage. Many studies have shown that hyperthermia can inhibit the repair of potentially lethal damage (PLD) (Li *et al.*, 1976), sublethal damage (SLD) (Raaphost, 1992), DNA single strand breaks (Iliakis *et al.*, 1990), double strand breaks (Dahm-Daphi *et al.*, 1997), and base damage (Warters and Roti Roti, 1979). It was thought that the inhibition of DNA double strand breaks was the important mechanism involved in thermal radiosensitization. The current view, however, is that heat inhibits the religation step of base excision repair (BER) (Kampinga and Dikomey, 2001). Specifically, DNA damage is recognized and incision of the strand will occur but the removal of the damaged base and religation of the lesion are inhibited by heat. A result of this inhibition is that clustered damage has a high risk of being converted into additional double strand breaks, resulting in additional cell killing (Kampinga and Dikomey, 2001). *The inhibition of repair of radiation induced DNA damage by hyperthermia is central to the formulation of the hypothesis for this study.*

One concept that is useful in hyperthermia treatments is that of thermal dose. The effects of hyperthermia depend on the temperature and duration of heating. When a hyperthermia treatment is given at a fixed and constant temperature, the duration of heating would be a reasonable way to describe thermal dose. In clinical practice, where it is difficult to maintain a uniform temperature, the concept of thermal dose becomes more complicated. Since both

the temperature and duration of heating are variables in a treatment, the treatment must be related to an equivalent time at a chosen reference temperature (Sapareto and Dewey, 1984). As mentioned previously, the effect of high heating temperatures (above ~43°C) is different from those of lower temperatures. Above this transition temperature, heating times and temperatures may be related as follows:

$$\frac{t_2}{t_1} = 2^{T_1 - T_2}, \quad (2)$$

where t_1 and t_2 are heating times at temperatures T_1 and T_2 , respectively, to produce equal biological effect. Below the transition temperature, an increase in temperature of 1°C requires that time be decreased by a factor of 4 to 6 as follows:

$$\frac{t_2}{t_1} = (4 \text{ to } 6)^{T_1 - T_2}, \quad (3)$$

where the symbols are the same as above. These equations may be used to determine the thermal dose equivalent (the equivalent heating time at 43°C).

For the purpose of this study, wherein the experiments were performed *in vitro* and a constant uniform heating temperature could be applied, the concept of thermal dose may be described in terms of duration of heating. Specifically, to determine the isoeffective thermal dose of two different temperatures, clonogenic survival levels were used to deduce the approximate required duration of heating at each temperature.

1.4 The Gene p21/WAF1/CIP1

The gene p21/WAF1/CIP1, also known as CDKN1A, is located on the chromosome 6p21.1. It is involved in many cellular functions, such as cell cycle arrest, control of apoptosis, and activation of repair. The gene's most well known role is in the inhibition of cell cycle progression through its interaction with the tumor suppressor gene product p53 (El-Deiry *et al.*, 1993). Following DNA damage, p53 is induced causing transcriptional activation of p21/WAF1/CIP1. p21/WAF1/CIP1 then complexes with cyclin/cyclin-dependent kinases to inhibit their kinase activity, which maintains the cell in the G1 phase of the cell cycle. The cell will then remain in G1 until the DNA is repaired, unless the damage is irreparable, in which case the cell dies by apoptosis (Jacks and Weinberg, 1996). This p53 to p21 pathway also inhibits DNA replication through the interaction of p21/WAF1/CIP1 with proliferating-cell nuclear antigen (PCNA) (Miura, 1999).

PCNA is an essential factor in DNA replication as well as in both nucleotide excision repair (NER) and base excision repair (BER) (Miura, 1999). NER is the repair pathway activated following UV-induced DNA damage while BER is that activated following radiation-induced DNA damage. Results of a very recent study (Tusher *et al.*, 2001) suggest that NER might also play a role in the repair of DNA damaged by ionizing radiation. The interaction between p21/WAF1/CIP1 and PCNA in response to DNA damage suggests that these proteins may function in concert during a certain step of the DNA repair process

(Waga and Stillman, 1998). *In vitro*, p21/WAF1/CIP1 has been shown to prevent PCNA-dependent DNA replication but to stimulate PCNA-dependent repair (Li *et al.*, 1994). The specifics of this involvement have not been made clear thus far.

In addition, a study focusing on the role of p21/WAF1/CIP1 in nucleotide excision repair (NER) found that a deficiency in p21 was associated with a defect in DNA repair (McDonald *et al.*, 1996). The study examined the *in vivo* repair of *in vitro* UV-damaged or cis-platinum damaged CMV-driven β -galactosidase reporter plasmids transfected into p21 $+/+$ or p21 $-/-$ human colon cancer cells. They found that p21 $-/-$ cells were three to five fold less efficient at repair compared to p21 wild-type cells. They also found that cells deficient in p21/WAF1/CIP1 were more sensitive to UV radiation, which reinforces other findings that p21 $-/-$ cells were more sensitive to a variety of cytotoxic agents (Waldman *et al.*, 1996; Wouters *et al.*, 1997).

Another study examining the response of p21/WAF1/CIP1 after irradiation with heavy ions found that p21/WAF1/CIP1 accumulated at sites of primary DNA damage (Jakob *et al.*, 2000). Since heavy ions produce clusters of DSBs, this study suggests that the basis for an interaction of p21/WAF1/CIP1 with sites of DSB repair may be due to a radiation-induced binding of p21/WAF1/CIP1 to the KU70 antigen, an antigen known to bind to DSBs (Kumaravel *et al.*, 1998). All of these studies, therefore, strongly suggest a role for p21/WAF1/CIP1 in the repair of DNA damage.

1.5 Hypothesis and Specific Aims

Heat sensitizes cells to radiation by inhibiting the repair of radiation-induced DNA damage, a process known as thermal radiosensitization. The gene p21/WAF1/CIP1 seems to play a major role in the repair of DNA damage. The hypothesis of this thesis is therefore that cells deficient in p21/WAF1/CIP1 may exhibit reduced expression of thermal radiosensitization.

The specific aims of this study were:

- Determine that the cell lines to be used in this study express p53 and p21/WAF1/CIP1 proteins as expected.
- Establish the high dose rate (HDR) x-radiation response of the two cell lines and fit this response with the linear quadratic model.
- Establish the response of both cell lines to two hyperthermia temperatures (42°C and 44°C) to investigate differences between a mild hyperthermia treatment (below 43°C) and a higher temperature treatment (above 43°C). Use this response to determine the equivalent heat dose required to deliver equal cell killing by both temperatures.
- Establish the response of the two cell lines to radiation in combination with hyperthermia at both temperatures. Fit the responses with the linear quadratic model and determine thermal enhancement ratios for both cell lines at both temperatures.

- Determine the effect of sequencing the hyperthermia and radiation treatments. Fit the responses with the linear quadratic model and determine thermal enhancement ratios for both cell lines at both temperatures.
- Compare the initial and residual response of both cell lines to irradiation in the presence and absence of heat by examining the amount of DNA damage, in the form of double strand breaks, that occurs immediately and 5 hours after treatment using the CHEF assay.

The experimental procedures used to achieve these aims are described in the materials and methods section of this thesis. All data from these experiments are shown in the results section and are subsequently explained in the discussion section. A summary of this work, as well as comments on future work, is discussed in the conclusion section.

Chapter 2

Materials and Methods

2.1 Cell Lines

The kinetics of thermal radiosensitization are different in human cells compared to animal cells, therefore human cells were used in this study. Two human colorectal carcinoma cell lines were used, both of which were obtained from Dr. B. Vogelstein (Johns Hopkins Oncology Center in Baltimore, MD). The parental cell line, HCT116, contains a wild-type p53 gene and responds normally to DNA damaging agents with respect to the induction of p53. The other cell line is a derivative of the parental, identified as 80S4, in which both chromosomal p21/WAF1/CIP1 alleles have been deleted through homologous recombination (Waldman *et al.*, 1995). This cell line also has wt p53 status. Since these cell lines were isogenic except for their p21/WAF1/CIP1 status, they provided a model for the investigation into the role of p21/WAF1/CIP1 in thermal radiosensitization. This is because any differences in response can be ascribed to the presence or absence of p21/WAF1/CIP1 in these cells. Note that the use of colorectal cancer cells in this study plays no significance to the overall hypothesis.

The cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 0.1 mM MEM non-essential amino acids (Gibco), 20 mM HEPES (Boehringer Mannheim), and 10 mM sodium bicarbonate (Sigma). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Both cell lines required 10⁵ cells to be seeded into 25 cm² tissue culture flasks in a solution of 4mL of medium. The medium was changed on day 4, and the experiments were performed on day 7 when the cells had reached confluence (i.e. plateau phase). Cells are considered confluent when they completely fill their growing area (i.e. the surface of the flask). All experiments were performed at plateau phase to minimize cell cycle effects (Ng *et al.*, 1994).

2.2 HDR Irradiation and Hyperthermia Treatments

Confluent cells were treated with high dose rate (HDR) irradiation, hyperthermia, or a combination of the two treatments. The cells were irradiated in 25 cm² tissue culture flasks at room temperature with a 250-kVp x-ray unit at a dose rate of 168 cGy per minute. The x-ray unit was a Pantek Bipolar Series, model HF320 with a 1.87 mm-base aluminum filter and a customized aluminum flattening filter. The unit is calibrated using a Farmer ion chamber.

Cells were exposed to hyperthermia by sealing the flasks with parafilm and immersing them into thermally controlled ($\pm 0.05^\circ\text{C}$) water baths for the desired

heating time. Half time for temperature equilibration under these conditions was approximately 20 seconds for the temperatures used in this study (Ng *et al.*, 2002). For combined treatments, the effect of the sequence of the treatments was investigated, and the secondary treatment was given within 5 minutes of the first treatment.

2.3 Clonogenic Assay

The clonogenic assay is an *in vitro* assay that gives an indication of the survival level of treated cells. According to this assay, treated cells that have survived or retained their ability to proliferate indefinitely to produce a colony are said to be clonogenic (Hall, 1994). A loss of reproductive integrity is a relevant endpoint in cancer treatment because for a tumor to be eradicated, it is only necessary to render cells unable to proliferate and repopulate the tumor following treatment.

Treated cells to be assessed with the clonogenic assay were released from their flask with the proteolytic enzyme trypsin (0.2% w/v trypsin in citrate saline (Gibco)) as follows. The medium was aspirated and the cells were rinsed with 1 mL isotonic citrate saline (134 mM KCl (BDH), 17.6 mM citric acid trisodium salt (Sigma)) to remove any traces of medium that can inhibit the enzymatic action of trypsin. The citrate saline was aspirated and the cells were then washed with 500 μ L trypsin to release the cells from the monolayer. The flasks were placed in an

incubator at 37°C for 5 minutes to allow cells to detach from the flask. Fresh medium was added to suspend the cells in a single cell suspension and to stop the action of the trypsin. The cells in this suspension were counted using an electronic cell counter (Particle Data Elzone 80), which had been calibrated with a Bright Line haemocytometer. The desired number of cells was then plated with fresh medium so that approximately 50 colonies would form per plate. This number of cells per plate has been found to be optimum for the growth of the cells.

When fewer than 5×10^4 cells were plated, 60 mm tissue culture dishes were used with 4 mL of medium. When plating 5×10^4 cells or more, 100 mm dishes were used with 12 mL of medium. After plating, the dishes were incubated for 10 days at 37°C (5% CO₂, 95% air) to allow time for viable cells to form colonies. The medium was then removed, the colonies were stained with Methyl Blue (0.2% w/v Methylene Blue (Sigma) in 70% ethanol (BDH)) and colonies consisting of 50 or more cells were scored with a colony counter.

For each treatment point, the average count from three plates was used for subsequent calculations. Three control plates (no treatment) were scored, averaged, and used to determine the plating efficiency (PE). The plating efficiency accounts for cell death due to factors other than the treatment itself and was calculated as follows:

$$PE = \frac{\text{colonies counted}}{\text{cells plated}} \quad (4)$$

Once the plating efficiency was calculated, the surviving fraction of cells that were treated was calculated as follows:

$$SF = \frac{\text{colonies counted}}{\text{cells plated} \times PE} \quad (5)$$

All clonogenic experiments were repeated at least three times. The mean of the surviving fractions and the standard error in the mean were calculated for each experimental point. The surviving fraction was plotted on a semi logarithmic scale as a function of dose.

2.4 Western Blot Hybridizations

Electrophoresis is the process of moving charged particles through a solution by applying an electric field across the mixture (Hoefer, 1994). This procedure has been extensively developed for molecular separations (i.e. DNA, RNA, protein) using the fact that molecules in an electric field move with a speed dependent on their charge, shape and size. When a voltage is applied to a sample, different species of molecules in the sample will move through a porous matrix at different velocities. Once separated, the different molecules may be detected as bands at different positions in the matrix. A matrix is required to prevent the diffusion and convective mixing of the bands that would occur due to the generation of heat by the electric current through the solution. Polyacrylamide is the most common matrix for separating proteins.

Once the proteins have been separated, they must be transferred to a membrane to be detected, a process known as blotting. Western blotting is the blot transfer of proteins, while Southern blotting is the transfer of DNA and Northern blotting is that of RNA. Western blots were performed in this study to detect the presence of the p53 and p21 proteins.

Treated cells were incubated for 12 hours to allow for protein expression. Cells were then washed, trypsinized, and centrifuged for 10 minutes at 1500 rpm. Media was removed, cells were resuspended in 1 mL lysis buffer, transferred into 1.5 mL eppendorf tubes and put on ice. Protein concentration was quantified against protein standards using colorimetric determination (BioRad) on a Beckman spectrophotometer.

A 10% SDS-polyacrylamide separating gel was cast into a four well gel box and allowed to set for 1 hour. A 10% SDS-polyacrylamide stacking gel was then added to each well and ten-well combs were inserted into each gel. Five microliters of loading buffer was added to 30 μ L of each protein. This was centrifuged for 10 seconds at 1500 rpm, heated for 5 minutes at 100°C and centrifuged again for 10 seconds before being loaded into a well of the gel along with a pre-stained molecular size standard (Bio-Rad). The samples were then resolved by electrophoresis at 300 mA for 1¼ hours.

Following electrophoresis, the fractionated protein bands were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P) overnight at 4°C. The proteins are transferred from the gel to the PVDF membrane by placing the

membrane next to the gel in a cassette, which is then suspended in a tank of buffer between two electrodes. A voltage was applied to the electrodes overnight to allow the proteins to move out of the gel onto the membrane. The PVDF membranes were removed from the cassette, protein standard lines were marked and the membrane was washed for 1 hour with a blocking solution (5 g skim milk powder in 100 mL Tris buffered saline (TBS)) to block all the non-specific binding sites. The blocking solution was removed and the membranes were washed with the appropriate dilutions of primary antibodies (50 μ L p21/Waf1/Cip1 (EA10) in 10 mL milk solution; 20 μ L p53 (1801/Ab 2) in 10 mL milk solution) (Oncogene Research Products) for 1 hour at room temperature. These dilutions were within the manufacturers suggested ranges. The primary antibody was removed and the membranes were washed four times for ten minutes in TBS-T (1 mL 0.1% Tween-20 in 1 L Tris buffered saline (TBS)). Membranes were then washed with a secondary antibody (10 μ L goat anti-mouse (Boehringer Mannheim) in 20 mL milk solution) for 1 hour, and then rinsed with TBS-T four times for 10 minutes. Primary antibody-antigen complexes were then detected by Enhanced ChemiLuminescence (ECL) by covering membranes with chemiluminescence solution for five minutes. In a dark room, laboratory film was exposed to the membrane for 5 minutes and subsequently developed, yielding protein bands on the film.

The position of the bands was compared to the molecular size standard to determine the size of the protein expressed. From knowledge of the size of the

proteins that were probed for, the presence or absence of that protein in the cell was determined. The membranes can then be stripped of the primary antibody-antigen complex and re-probed with another antibody (for example the antibody α -actin). To do this, the membranes were placed in a strip buffer (700 μ L β -mercaptoethanol, 20 mL 10% SDS, 79.3 mL TBS) that was heated to 50°C for 30 minutes. The membrane was then washed twice with TBS-T for 15 minutes, and the new antibody was then added (20 μ L α -actin in 20 mL milk solution). The procedure following the addition of the primary antibody explained above was then repeated to obtain a film that exhibited bands of actin. These bands could be used to compare the actual amount of protein loaded into each well.

2.5 CHEF DNA Double Strand Break Assay

Gel Electrophoresis is a method used to separate different sized fragments of charged biological molecules such as DNA. Since DNA double strand breaks (DSBs) are considered the most important lesion in cells exposed to ionizing radiation, gel electrophoresis has been used to quantify the amount of DSBs that occur following radiation exposure. To specifically study the induction of DNA DSBs, Clamped Homogeneous Electric Field (CHEF) electrophoresis has been developed. In this method, damaged DNA is separated in agarose gels by alternately applying two homogeneous electric fields at an angle of 120° to each other. This causes the broken DNA to zigzag through the gel, resulting in DNA

migration along the net vector of the two electric fields, while intact DNA remains in place. CHEF electrophoresis can be used to measure damage equivalent to about 1 Gy of γ -rays (Blocher and Kunhi, 1990).

Cells used for the CHEF assay were grown in medium containing 0.02 $\mu\text{Ci/mL}$ ^{14}C -thymidine ($[2\text{-}^{14}\text{C}]$ Thymidine, 0.1mCi/mL (Mandel)). The cells were then treated, prepared into a suspension, and counted, as described above. A volume containing 10^6 cells was removed and centrifuged for 5 minutes at 1200 rpm. The supernatant was removed and the remaining pellet of cells was resuspended in 500 μL of phosphate buffered saline (PBS) (Sigma). Five hundred microliters of 2% low melting point (LMP) agarose (Gibco) was mixed with the cell suspension, creating a 1 % agarose solution with a 1×10^6 cells/mL concentration. This solution was cast into CHEF disposable plug molds (Bio-Rad) to form plugs, which were allowed to harden for 10 minutes on ice. Once hardened, the agarose plugs were pushed out of the mold into lysis buffer (0.5 mM EDTA (disodium dihydrate) (Sigma), 1.0% w/v Sarkosyl (Sigma), 1.0 $\mu\text{g/mL}$ Proteinase K (Sigma)) and left for 24 hours at 50°C. Lysis buffer was removed and the plugs were then washed four times in 1 mL TE buffer (20mM Tris HCl (Sigma), 50 mM EDTA (Sigma), pH 8.0) for 30 minutes to an hour each at room temperature. The plugs were then stored at 4°C until they were to be run in a gel. The plugs are stable at 4°C for three months to a year (Bio-Rad, 1992).

A 0.8% pulsed-field agarose gel (0.8 g pulsed-field certified agarose (Bio-Rad) in 100 mL tris-borate EDTA (TBE) buffer (Bio-Rad)) was cast into a level

14 cm x 13 cm casting stand, which was provided with the CHEF-DR II system. Two ten-well combs were placed in the gel to form wells and the gel was allowed to solidify at room temperature for approximately 30 minutes. Once the gel solidified, the combs were removed, a DNA size standard (*Schizosaccharomyces pombe* Strain 972 h (BioRad)) and the sample plugs were cut to size (5 mm x 3 mm), loaded into the wells and sealed with 1% low melting point agarose.

The CHEF electrophoresis unit (CHEF-DR II (Bio-Rad)) was filled with 0.5 x TBE buffer and the variable speed pump was turned on, allowing the buffer to circulate through the system at a flow rate of approximately 0.75 L/min. Part of this system consists of a chiller (Model 1000 Mini Chiller (Bio-Rad)), which cooled the buffer to 14°C. Once the temperature of the buffer had equilibrated, the gel was placed in the electrophoresis cell. The CHEF electrophoresis unit that was used consists of 24 electrodes positioned in a hexagonal orientation, which produces two electric fields at an angle of 120° to one another (Figure 2.1). The voltage was alternately applied to the two sets of electrodes for switch times of 75 minutes with an electric field strength of 1.5 V/cm for a total electrophoresis time of 18 hours. This protocol was determined from the manufacturers suggested values (Bio-Rad, 1992). The switch time refers to the length of time the electric field is in one direction before switching to the other. For an even number of switch intervals, the net migration direction for the DNA is from north to south.

Figure 2.1 The CHEF electrophoresis chamber with 24 electrodes in a hexagonal arrangement, with the electric field vectors shown for both pulse phases (Blocher and Kunhi, 1990).

Following electrophoresis, the gel was carefully removed from the unit and placed into a light protected container containing 50 mL TBE Buffer and 50 μ L SYBR Gold nucleic acid gel stain (Molecular Probes). The gel was agitated gently for 30 minutes at room temperature to allow the SYBR Gold to bind directly to the DNA. The gel was then viewed on a 312 nm variable intensity UV transilluminator (Fisher), which fluoresces the SYBR Gold so that the stained DNA can be viewed. Intact fluorescent labeled DNA remained in the well of the gel while broken DNA was removed from the well into lanes below.

The lanes were separated from the wells so that the agarose section containing the lane and the section containing the well could be cut apart and loaded into separate labeled scintillation vials. The agarose sections were heated to

95°C on a hot plate and fifty microliters of 10 N HCl was added to the vial. As the gel melted, acid hydrolysis of the agarose polymer prevented regelling. Ten milliliters of Ecolite scintillation liquid (ICN) was added to each vial and shaken vigorously to mix the fluid with the melted gel.

The vials were then placed in a liquid scintillation counter (1900 TR Liquid Scintillation Analyzer (Packard)), which determined the counts per minute (cpm) of ¹⁴C for each sample. The fraction of activity released (FAR) from the well was then calculated as follows:

$$FAR = \frac{cpm \text{ in lane}}{cpm \text{ in lane} + cpm \text{ in well}} \quad (6)$$

Each experiment was repeated three times and the mean FAR and the standard error of the mean were calculated for each set of three experiments. The FAR was then plotted as a function of dose, and all points were normalized to zero by subtracting the FAR of the control sample from them.

2.6 Curve Fitting

Survival curves were fitted to the linear quadratic formula, equation (1), using Sigma Plot version 4.00 (1997 SPSS Inc.). This software uses the Levenberg-Marquardt algorithm to find the coefficients of the independent variables that give the best fit between the linear quadratic equation and the

experimental data. The algorithm estimates the values of the parameters by the minimization of the following equation:

$$S = \sum_{i=1}^n (y_i - y)^2, \quad (7)$$

where y_i is the observed value of the dependent variable and y is the value of the dependent variable as predicted by the equation to which the data is being fit. It is an iterative process in which the algorithm continually makes better guesses at the values of the parameters until the difference between the residual sums of squares no longer decreases significantly. Once an acceptable minimum has been found, the parameter values (α and β in the case of a linear quadratic fit) are obtained with their uncertainties, which are based in part on the value of the residual sum of squares.

Chapter 3

Results

3.1 Expression of Proteins

Western blot hybridization experiments were performed to ensure that both cell lines were expressing proteins as expected. The cell line HCT116 is of wt p53 status and is expected to express the p21/WAF1/CIP1 protein. The 80S4 cell line is also of wt p53 status, however, the gene p21/WAF1/CIP1 has been knocked out (Waldman *et al.*, 1995). Alpha-actin was included as an internal control in all analyses to control for potential discrepancies in sample loading. Figure 3.1 shows Western blots for p53, p21, and α -actin for control cells and cells treated with 10 Gy of x-radiation. From Figure 3.1, it is clear that the HCT116 cell line expresses both p53 and p21 while the 80S4 cell line only expresses p53 as expected. There appeared to be an up regulation of p53 following irradiation in both cell lines. Up-regulation of p21 in the HCT116 cell line was also seen following irradiation. These up-regulations are due to the activation of p53 following DNA damage, and the subsequent induction of p21 to arrest the cells in the G1 phase of the cell cycle.

Figure 3.1 Western blots of p53, p21/WAF1/CIP1, and alpha-actin for HCT116 and 80S4 cell lines.

3.2 Thermal Radiosensitization

The purpose of these experiments was to compare the high dose rate (HDR) x-radiation response and the hyperthermia response of the HCT116 (p21+/+) and 80S4 (p21-/-) cell lines both individually and in combination. These data were produced from *in vitro* experiments utilizing the clonogenic assay. All data points are the average of at least three experiments with error bars representing the standard error of the mean.

3.2.1 High Dose Rate X-Radiation Response

The HDR response curves of the HCT116 p21 +/+ and 80S4 p21 -/- cell lines are shown in Figure 3.2. These curves were fit with the linear quadratic model (Chadwick and Leenhouts, 1973) and the estimated parameters α and β with their estimated standard errors are shown in Table 3.1. From Figure 3.2, both cell lines exhibit an almost equivalent HDR response. Both cell lines show similar shoulder regions, characterized by the β parameter (0.036 ± 0.004 for HCT116 cells compared to 0.035 ± 0.002 for 80S4 cells), as well as a similar linear region, described by the α parameter (0.267 ± 0.020 for HCT116 cells compared to 0.241 ± 0.012 for 80S4 cells). Point by point comparisons using a Student's t-test were performed, and it was determined that the differences in the HDR response of the HCT116 and 80S4 cell lines were not statistically significant ($P > 0.05$).

Figure 3.2 High dose rate x-radiation response of HCT116 and 80S4 cell lines.

Table 3.1 Comparison of the linear quadratic fits to the high dose rate x-radiation response curves of the HCT116 and 80S4 cell lines.

Cell Line	α (Gy ⁻¹)	β (Gy ⁻²)
HCT116	0.267 ± 0.020	0.036 ± 0.004
80S4	0.241 ± 0.012	0.035 ± 0.002

3.2.2 Heat Response

The hyperthermia response of the HCT116 and 80S4 cell lines was examined to determine the isoeffective heat dose of each cell line for the two heating temperatures (42°C and 44°C). These two heating temperatures were chosen since hyperthermia temperatures above and below 43°C exhibit different survival curve effects as mentioned above. The hyperthermia response curves of the HCT116 p21+/+ and 80S4 p21-/- cell lines are shown in Figure 3.3. The best-fit trend line was added to aid in the determination of the isoeffective heat dose. From Figure 3.3, the heat response of the two cell lines at each temperature shows that their trends lie almost directly on top of each other within the range of heating times used. It may thus be concluded that the heat response of the two cell lines is similar.

The equivalent or isoeffective heat dose for the cell lines was determined from the duration of heating at each temperature that yielded equivalent clonogenic survival. The duration of heating at 44°C required to yield the same surviving fraction for cells exposed to two hours of heating at 42°C was

Figure 3.3 Heat response of HCT116 and 80S4 cell lines.

determined to be approximately 30 minutes for both cell lines. The heating times used for all further hyperthermia experiments was therefore two hours at 42°C and 30 minutes at 44°C.

3.2.3 Hyperthermia and HDR Irradiation Response

Combined hyperthermia and HDR irradiation experiments were performed to determine if a reduced amount of thermal radiosensitization was exhibited in cells lacking p21/WAF1/CIP1 (the 80S4 cell line). Figure 3.4 shows the response of both cell lines to two hours of heating at 42°C followed by HDR irradiation and this response is compared to the previously determined response to HDR irradiation alone (Figure 3.2). The combined treatment response was normalized for cell killing by hyperthermia. This was done by dividing the surviving fraction of each combined treatment dose point by the surviving fraction of the cells exposed to heat alone. The linear quadratic model was used to fit the combined treatment data. The estimated parameters α and β along with their estimated standard errors are shown in Table 3.2. When compared to the response from HDR irradiation alone, cells treated with heat and x-radiation exhibit an increased amount of radiation sensitivity, evident from the steeper slope of the survival curve. This increase in sensitivity from combined heat and radiation treatments is an example of thermal radiosensitization.

Figure 3.4 Response of HCT116 and 80S4 cell lines to hyperthermia (42°C) and x-radiation.

Table 3.2 Comparison of the LQ fits to the combined hyperthermia (42°C) and HDR x-radiation response curves for HCT116 and 80S4.

Cell Line	α (Gy ⁻¹)	β (Gy ⁻²)
HCT116	0.36 ± 0.03	0.054 ± 0.006
80S4	0.41 ± 0.05	0.048 ± 0.010

From Figure 3.4, the two combined treatment curves appear to lie nearly on top of each other, although the α and β values are not as similar as for the HDR alone curves (Table 3.1). The error bars of both cell lines for each dose point in Figure 3.4 overlap. Also, comparison of the α values (0.36 ± 0.03 for HCT116 and 0.41 ± 0.05 for 80S4 cells) and β values (0.054 ± 0.006 for HCT116 and 0.048 ± 0.010 for 80S4 cells) shows that no significant difference exists between the two responses. To determine whether there is a decreased amount of thermal radiosensitization in the 80S4 p21 ^{-/-} cell line, thermal enhancement ratios (TERs) were calculated at the 10% and 1% survival levels and they are tabulated in Table 3.3. These values reveal that there is no difference in the amount of thermal radiosensitization induced in the two cell lines at either survival level.

Table 3.3 Comparison of thermal enhancement ratios at the 10% and 1% survival level for HCT116 and 80S4 when given hyperthermia (42°C) and HDR x-radiation.

Cell Line	TER _{10%}	TER _{1%}
HCT116	1.29 ± 0.14	1.27 ± 0.14
80S4	1.39 ± 0.21	1.33 ± 0.20

In order to determine the effect of sequencing on the amount of thermal radiosensitization induced, cells were treated with HDR irradiation followed by two hours of heating at 42°C. The response of both cell lines to this treatment is shown in Figure 3.5, along with the response to HDR irradiation alone. The data was fit with the linear quadratic model and the estimated α and β parameters and their estimated errors are shown in Table 3.4. Although there appears to be a slight difference between the two curves, many of the error bars of the data points overlap. The α value for the parental HCT116 cell line was 0.398 ± 0.014 compared to 0.38 ± 0.06 for the 80S4 cell line. The β value of the HCT116 cells was 0.057 ± 0.003 compared to 0.050 ± 0.011 for 80S4 cells. Neither the α , nor β parameters of the cell lines are significantly different, thus the two curves are similar.

Table 3.4 Comparison of the LQ fits to the combined HDR x-radiation and hyperthermia (42°C) response curves for HCT116 and 80S4.

Cell Lines	α (Gy ⁻¹)	β (Gy ⁻²)
HCT116	0.398 ± 0.014	0.057 ± 0.003
80S4	0.38 ± 0.06	0.050 ± 0.011

Thermal enhancement ratios (TERs) at the 10% and 1% survival levels were calculated to determine whether there was a reduced amount of thermal radiosensitization for the 80S4 cell line compared to the HCT116 cell line when given the reverse sequence of hyperthermia and radiation treatments. These values

Figure 3.5 Response of HCT116 and 80S4 cell lines to x-radiation and hyperthermia (42°C).

are tabulated in Table 3.5. At 10% survival, the TER for HCT116 cells is 1.36 ± 0.11 compared to 1.35 ± 0.22 for 80S4 cells. TER values at both survival levels for the two cell lines indicate that there is no reduction in the amount of thermal radiosensitization induced. It may therefore be concluded that sequencing the hyperthermia and HDR irradiation treatments had no effect on the amount of thermal radiosensitization exhibited.

Table 3.5 Comparison of TERs at the 10% and 1% survival level for the HCT116 and 80S4 when given HDR x-radiation and hyperthermia (42°C).

Cell Line	TER _{10%}	TER _{1%}
HCT116	1.36 ± 0.11	1.33 ± 0.11
80S4	1.35 ± 0.22	1.31 ± 0.21

Due to the different mechanisms involved in cell killing by hyperthermia above and below 43°C, it was necessary to examine the effect of combining hyperthermia at 44°C and HDR irradiation treatments on the levels of thermal radiosensitization between the two cell lines. The response of the HCT116 p21 +/+ and 80S4 p21 -/- cell lines to heating at 44°C for 30 minutes followed by HDR irradiation is shown in Figure 3.6, along with the response to HDR irradiation alone. The data was fit with the linear quadratic model and the estimated α and β parameters and their estimated errors are shown in Table 3.6. From Figure 3.6, the two combined treatment curves appear to lie nearly on top of each other. A Student's t-test was performed to compare the α and β values of the two cell lines

Figure 3.6 Response of HCT116 and 80S4 cell lines to hyperthermia (44°C) and x-radiation.

and no statistically significant difference was seen. Also, since the error bars of both cell lines for each dose point in Figure 3.6 overlap, there is no significant difference between the two responses.

Table 3.6 Comparison of the LQ fits to the combined hyperthermia (44°C) and HDR x-radiation response curves for HCT116 and 80S4.

Cell Line	α (Gy ⁻¹)	β (Gy ⁻²)
HCT116	0.927 ± 0.006	0.031 ± 0.002
80S4	0.997 ± 0.048	0.019 ± 0.010

The amount of thermal radiosensitization for each cell line was compared using TERs at the 10% and 1% survival levels. These values are tabulated in Table 3.7. Comparison of the TERs for the two cell lines indicate that there is no reduction in the amount of thermal radiosensitization induced in the 80S4 p21 -/- cell line. Therefore treatment with hyperthermia at 44°C followed by HDR irradiation had no effect on the amount of thermal radiosensitization exhibited between the two cell lines.

Table 3.7 Comparison of TERs at the 10% and 1% survival level for HCT116 and 80S4 when given hyperthermia (44°C) and HDR x-radiation.

Cell Lines	TER _{10%}	TER _{1%}
HCT116	2.21 ± 0.18	1.89 ± 0.15
80S4	2.4 ± 0.7	2.0 ± 0.6

To determine whether sequencing had an effect on the amount of thermal radiosensitization induced, cells were treated with HDR irradiation followed by thirty minutes of heating at 44°C. The response of both cell lines to this treatment is shown in Figure 3.7, along with the response to HDR irradiation alone. The data was fit with the linear quadratic model and the estimated α and β parameters and their estimated errors are shown in Table 3.8. From Figure 3.7, the two combined treatment curves appear to lie nearly on top of each other and the error bars of both cell lines for each dose point overlap. Comparison of the α and β parameters for both cell lines using a Student's t-test showed P-values greater than 0.05, therefore it was concluded that there is no significant difference between the two responses.

Table 3.8 Comparison of the LQ fits to the combined HDR x-radiation and hyperthermia (44°C) response curves for HCT116 and 80S4.

Cell Lines	α (Gy ⁻¹)	β (Gy ⁻²)
HCT116	0.90 ± 0.04	0.027 ± 0.008
80S4	0.875 ± 0.009	0.039 ± 0.003

Thermal enhancement ratios at the 10% and 1% survival levels were calculated to determine whether there was a reduced amount of thermal radiosensitization for the 80S4 cell line compared to the HCT116 cell line when given the reverse sequence of hyperthermia and radiation treatments. These values are tabulated in Table 3.9. Comparison of the TERs for the two cell lines indicate

Figure 3.7 Response of HCT116 and 80S4 cell lines to x-radiation and hyperthermia (44°C).

that there is no reduction in the amount of thermal radiosensitization induced. It may therefore be concluded that sequencing the hyperthermia and HDR irradiation treatments had no effect on the amount of thermal radiosensitization exhibited through clonogenic survival levels, nor did the temperature of the hyperthermia treatments.

Table 3.9 Comparison of TERs at the 10% and 1% survival level for HCT116 and 80S4 when given HDR x-radiation and hyperthermia (44°C).

Cell Line	TER_{10%}	TER_{1%}
HCT116	2.1 ± 0.4	1.8 ± 0.3
80S4	2.05 ± 0.12	1.78 ± 0.10

3.3 Comparison of DSB Induction and Repair

The clonogenic results determined that the amount of thermal radiosensitization expressed in cells treated with hyperthermia and radiation might not be dependent on the presence or absence of p21/WAF1/CIP1. The lack of difference in heat inhibition of radiation response between the p21^{+/+} and p21^{-/-} cell lines may, however, be interpreted in a couple of ways. If repair of radiation damage is different in the two cell lines, as hypothesized, but the amount of repair inhibition by heat is different, clonogenic survival levels could appear equivalent for both cell lines. For example, if the 80S4 cell line had a reduced repair ability compared to the HCT116 cells due to the removal of p21, overall heat would inhibit less repair compared to the HCT116 cells, and equivalent clonogenic

survival between the two cell lines would result. The other possibility is that there is no difference in repair of radiation damage between the two cell lines and heat inhibits this repair equally. To assess which of these situations is actually occurring, levels of DNA DSBs induced and subsequently repaired in both cell lines were compared using the CHEF assay.

A sample CHEF electrophoresis gel is shown in Figure 3.8 for cells treated with X-radiation of varying doses. As the dose of radiation increases, more DNA is seen to migrate out of the well into the lane due to the induction of DSBs in the DNA. Cells treated with x-radiation doses ranging from 0 Gy to 120 Gy were run in a CHEF gel immediately following treatment to examine the induction of DNA double strand breaks. CHEF electrophoresis was also performed on cells treated with the same radiation doses then incubated for 5 hours to examine the repair of DNA double strand breaks. To ensure that the addition of heat had the same effect on the two cell lines with respect to the induction and repair of DNA DSBs, the CHEF experiments were repeated for cells treated with x-radiation followed by either 2 hours of heating at 42°C or thirty minutes of heating at 44°C with and without 5 hours of incubation following treatment.

Figures 3.9 and 3.10 show the results for the HCT116 cells exposed to x-radiation alone and x-radiation followed by 2 hours of heating at 42°C or thirty minutes of heating at 44°C both immediately and 5 hours after treatment. A best-fit trend line was added to aid in the comparison of the curves. From the x-radiation alone curve, a large fraction of activity was released at the higher dose

Figure 3.8 Sample clamped homogeneous electric field (CHEF) electrophoresis gel. Three independent experiments are shown for HCT116 cells treated with radiation doses of 0 Gy to 120 Gy. The DNA standard was *schizosaccharomyces pombe*.

points (around 45% at 120 Gy), which relates to a large induction of DNA double strand breaks. When given 5 hours between treatment and CHEF electrophoresis, the FAR level is significantly reduced (approximately 7% at 120 Gy). This translates to repair of DNA damage occurring during the 5 hours.

Studies in our lab have shown that DNA double strand breaks are not induced in cells heated for the durations and temperatures used in these experiments (Niedbala, personal communication). Because of this, FAR curves for cells treated with radiation and hyperthermia were expected to be similar to those of cells treated with radiation alone. From Figure 3.9, the cells treated with radiation and 2 hours of heating at 42°C have a significantly lower FAR at higher doses (20% at 120 Gy) when compared to the radiation alone curve. Also, from Figure 3.10, the combined treatment of radiation and thirty minutes of heating at 44°C also yielded a consistently lower FAR (42% at 120 Gy) when compared to the radiation alone curve, although not as low as for the combined radiation and 42°C heat treatment. Since the FAR level was lower for cells given a longer heating time, the reduction in FAR for cells given the combined treatment was determined to be due to repair of radiation damage occurring during the heat treatment. Presumably, this could have been avoided if the hyperthermia treatment were given before irradiation. Nonetheless, cells given the combined treatment then incubated for 5 hours before electrophoresis showed even more of a reduction

in FAR levels (14% - 16% for both heat temperatures) compared to those given only the combined treatment,

Figure 3.9 Induction of DNA double strand breaks in HCT116 cells treated with x-radiation with or without hyperthermia (42°C).

Figure 3.10 Induction of DNA double strand breaks in HCT116 cells treated with x-radiation with or without hyperthermia (44°C).

since repair continued to occur during the five-hour incubation period. Both combined-treatment-plus-5-hour curves on Figure 3.9 and Figure 3.10 are however not as low as the radiation-plus-5-hour curves. This is due to the inhibition of repair of DNA damage by heat, which is thought to be the mechanism of thermal radiosensitization.

Figures 3.11 and 3.12 show similar curves for 80S4 cells. These curves also indicate that cells treated with radiation alone induced the highest level of FAR (44% at 120 Gy), cells given 5 hours of incubation following irradiation repaired the most damage (6% at 120 Gy), cells given the combined treatment showed FAR levels lower than the cells treated with radiation alone (18% at 120 Gy for 42°C; 41% at 120 Gy for 44°C) due to repair occurring during the heating time, and cells given the combined treatment then incubated for 5 hours showed even lower FAR levels (around 14% at 120 Gy for both temperatures), though not as low as for cells given 5 hours of incubation following irradiation due to the inhibition of repair of DNA damage by heat.

Although Figures 3.9 through 3.12 show similar trends for the HCT116 and 80S4 cell lines, a direct comparison is required. FAR values of HCT116 and 80S4 cells treated with radiation alone with and without 5 hours of incubation are shown in Figure 3.13. The error bars overlap one another at each dose point such that there is no significant difference between the two cell lines for these treatments. A Student's t test comparing the FAR of all dose points for both cell lines for each

Figure 3.11 Induction of DNA double strand breaks in 80S4 cells treated with x-radiation with or without hyperthermia (42°C).

Figure 3.12 Induction of DNA double strand breaks in 80S4 cells treated with x-radiation with or without hyperthermia (44°C).

treatment showed that there was in fact no significant difference. Figure 3.14 shows FAR curves for cells treated with x-radiation followed by 2 hours of heating at 42°C with and without 5 hours of incubation. Again, overlapping error bars and P-values from a Student's t-test comparing all dose points showed that there was no significant difference between the two cell lines for either treatment. FAR curves for cells treated with x-radiation followed by thirty minutes of heating at 44°C with and without 5 hours of incubation are shown in Figure 3.15. Again, no significant difference was seen between the two cell lines for either treatment. These findings allow for the conclusion that there was no significant difference in the induction and repair of DNA double strand breaks between the HCT116 and the 80S4 cell lines and therefore heat inhibited this repair equally in the two cell lines.

Figure 3.13 Comparison of DNA double strand breaks induced and repaired in HCT116 and 80S4 cells treated with x-radiation.

Figure 3.14 Comparison of DNA double strand breaks induced and repaired in HCT116 and 80S4 cells treated with x-radiation and 42°C hyperthermia.

Figure 3.15 Comparison of DNA double strand breaks induced and repaired in HCT116 and 80S4 cells treated with x-radiation and 44°C hyperthermia.

Chapter 4

Discussion

The use of hyperthermia in combination with radiation in the treatment of cancer results in an increased radiation sensitization via the inhibition of repair of radiation induced DNA damage by heat. This effect has been exploited clinically for some time, however, specific genes involved in this process have not been widely investigated. This study explored the gene p21/WAF1/CIP1's involvement in thermal radiosensitization due to the gene's putative role in the repair of radiation induced DNA damage. Using the parental colorectal cancer cell line HCT116 and its isogenic, p21/WAF1/CIP1-deficient clone, 80S4, the role of p21/WAF1/CIP1 in thermal radiosensitization was assessed. The results of this study are discussed below.

4.1 Expression of p53 and p21/WAF1/CIP1 Proteins

These experiments were performed to confirm that the p53 and p21/WAF1/CIP1 proteins were expressed in the cells as expected. The expression of these proteins was investigated for both cell lines using western blot hybridization experiments. The HCT116 cells expressed both the p53 and p21/WAF1/CIP1 proteins. The 80S4 cells expressed the p53 protein, but did not express p21/WAF1/CIP1. The cell lines therefore expressed p53 and p21/WAF1/CIP1 proteins as expected.

Following x-radiation induced DNA damage, the p53 protein became activated and up-regulated. The increase in the p53 protein transcriptionally activated p21/WAF1/CIP1, which in turn caused the inhibition of progression of the cell through the cell cycle. This cell cycle arrest allowed for repair of the DNA damage to occur (Waldman et al., 1995). Up-regulation of both p53 and p21/WAF1/CIP1 proteins was seen in HCT116 cells treated with 10 Gy of x-radiation compared to untreated cells. Up-regulation of p53 was also seen in the 80S4 cells treated with 10 Gy of x-radiation compared to untreated cells. The up-regulation of the p53 and p21/WAF1/CIP1 proteins following x-radiation indicated that the HCT116 and 80S4 cell lines responded normally to x-radiation with respect to p53 induction. These findings agree with published data (Waldman et al., 1995) that showed a similar induction of p53 and p21/WAF1/CIP1 in these cells following x-radiation.

4.2 Thermal Radiosensitization

4.2.1 High Dose Rate X-Radiation Response

The sensitivity of the HCT116 p21 $+/+$ and 80S4 p21 $-/-$ human colorectal cancer cells to HDR x-radiation was determined *in vitro*. Comparison of the two responses showed that there was no significant difference between the cell lines. This finding implies that total cell killing is equivalent in the two cell lines following x-radiation. The 80S4 cells, however, consistently showed a small, higher resistance to x-radiation compared to the HCT116 cells. This finding agrees with previously published results (Wouters et al, 1997), which showed a small increase in cell survival for the p21/WAF1/CIP1 knockout cell line (80S4). The amount of protection seen by Wouters et al. (1997) was, however, greater than that seen in this study. This difference may have been due to the cells having been irradiated in exponential phase (Wouters et al, 1997) as opposed to in plateau phase (this study), since exponentially growing cells exhibit a different radiation response due to cell cycle effects.

The use of human cells in this study was due to differences in radiation response (Goettsch et al., 1998; Haines et al., 1998) and heat response (Armour et al., 1993) between human cell lines compared to rodent cell lines. Rodent cells also behave differently in regard to transformation, spontaneous immortalization and perhaps genomic stability when compared to human cells (Tlsty, 1998). Human cells also provide a more clinically relevant model.

4.2.2 Heat Response

The response of both cell lines to two hyperthermia temperatures for different durations was investigated to examine the cytotoxic effect of the hyperthermia treatments. The lower temperature (42°C) induced thermotolerance in both cell lines after about 2 hours of heating. This phenomenon has been seen in many cell lines (Dewey et al., 1977; Raaphorst et al., 1979) for heating temperatures less than ~43°C, however, different cell lines exhibit different levels of thermotolerance. Our lab has recently shown that p21/WAF1/CIP1 does not play a role in the induction of thermotolerance (Ng, unpublished results), and the equivalent induction of thermotolerance in both HCT116 p21 +/+ and 80S4 p21 -/- cell lines supports this finding. At the higher temperature (44°C), cell killing was much more severe and no thermotolerance was induced.

Targets for heat-induced cell lethality are thought to be the cellular membrane and inhibition of protein synthesis (Hall, 1994). Comparison of the heat survival curves for HCT116 and 80S4 cells indicated that there was no statistically significant difference in heat response between the two cell lines. Thus, the removal of p21/WAF1/CIP1 had no apparent effect on the cytotoxicity of the heat treatments. Alternatively, heat might have inhibited the expression of p21/WAF1/CIP1 in the parental cell line so that it behaved similarly to the 80S4 cell line. A western blot experiment was performed to detect the expression of p21/WAF1/CIP1 following exposure to thirty minutes of heating at 44°C and a

small amount of inhibition of p21/WAF1/CIP1 was seen (data not shown). While these results suggest that hyperthermia may have caused a small down-regulation of all proteins (including p21/WAF1/CIP1), it is obvious that heat did not entirely inhibit p21/WAF1/CIP1 expression. Therefore, it seems that the removal of p21/WAF1/CIP1 had no effect on the cytotoxicity of the heat treatments.

The heat survival curves were also used to determine the isoeffective heat dose for both cell lines. Since this study focused on the clonogenic survival of cells exposed to radiation and/or heat, the isoeffective heat dose was determined from clonogenic survival levels of the heat response. It was found that both cell lines yielded almost equivalent clonogenic surviving fractions when heated for thirty minutes at 44°C as when heated for 2 hours at 42°C. This method for determining the isoeffective heat dose was chosen to ensure that equivalent cell killing by hyperthermia at both temperatures was achieved so that thermal enhancement levels induced by the two temperatures could be compared.

Previous studies (Sapareto and Dewey, 1984) determined the equivalent heating time at ~43°C using equations 2 and 3 (see chapter 1.3). One problem with using the Sapareto and Dewey equations is that there is a range of transition temperatures that may be used (between 42.5°C to 43°C). The choice of a transition temperature is therefore somewhat arbitrary, unless an analysis of the slope transition on an Arrhenius plot for each cell line is performed. The slope transition occurs at the temperature that no longer induces thermotolerance in cells. For the two cell lines used in this study, as the transition temperature is

lowered closer to 42°C, the treatment time determined from the Sapareto and Dewey equations at 44°C approaches thirty minutes. However, due to the onset of thermotolerance in these cells when heated at 42°C, it is unlikely that a transition temperature of 42°C is reasonable. In order to avoid these problems, clonogenic survival levels were used to determine the isoeffective heat dose.

4.2.3 Hyperthermia and HDR Irradiation Response

When exposed to a combination of hyperthermia and x-radiation treatments, both cell lines exhibited an increased radiosensitivity when compared to the response to a treatment of radiation alone. The cytotoxic effect of heat on its own was normalized out of the survival response in order to compare the degree of sensitization in the radiation response of both cell lines. A significant amount of thermal radiosensitization was seen in both cell lines when they were exposed to all the treatment combinations. When treated with the higher hyperthermia temperature, a greater degree of thermal radiosensitization was seen. Similar results have been exhibited in other cell lines (Raaphorst, 1989).

It was hypothesized in this study that a loss of p21/WAF1/CIP1 would result in a decreased amount of thermal radiosensitization. The degree of thermal radiosensitization was assessed using thermal enhancement ratios (TERs). If the hypothesis were indeed correct, the 80S4 cell line would be expected to exhibit a lower TER than the HCT116 cells. The results of this study disagreed with the

hypothesis. This is evident from the statistically equivalent TER values for both cell lines for each treatment combination. The similarity between the combined treatment response of the HCT116 cells and that of the 80S4 cells demonstrated that no reduction in thermal radiosensitization occurred in cells deficient in p21WAF1/CIP1. This suggests that p21WAF1/CIP1 does not play a role in thermal radiosensitization.

Both of the cell lines used in this study are mismatch repair (MMR) deficient (Wladman et al., 1995). Mismatch repair is a specialized form of base excision repair, which involves the removal of mismatched DNA bases occurring as errors of DNA replication or from miscoding properties of damaged bases (Moustacchi, 2000). Since BER plays a role in thermal radiosensitization (Kampinga and Dikomey, 2001) and since these cells are deficient in a kind of BER, both cell lines should be more sensitive to heat-induced radiosensitization. A study examining MMR in relation to ionizing radiation (Davis et al., 1998) found that HCT116 cells were more sensitive to ionizing radiation than MMR-corrected HCT116 cells. No analysis of thermal radiosensitization levels has yet been performed in these cells. Comparison of thermal radiosensitization levels for HCT116 and 80S4 cells to other cell lines was not conclusive since different cell lines can vary widely in their response to radiation alone or in combination with hyperthermia (Raaphorst, 1989). The only conclusive way to determine whether MMR has an effect on the level of thermal radiosensitization induced would be to compare the response of HCT116 cells and MMR-corrected HCT116 cells

exposed to radiation alone and in combination with hyperthermia. It is important to remember , however, that both HCT116 and 80S4 cells are MMR deficient and the similarity in thermal radiosensitization may not be attributed to a difference in this type of repair.

4.3 Comparison of DSB Induction and Repair

There are two possible ways to explain the lack of difference in thermal radiosensitization induction between the HCT116 and 80S4 cells. First, if the 80S4 p21^{-/-} cells did not repair as much radiation induced DNA damage as the HCT116 cells, then less repair inhibition by heat would need to occur in 80S4 cells in order to achieve equivalent thermal radiosensitization levels as HCT116 cells. The other possibility is that the repair of radiation induced DNA damage is equivalent in both cell lines, resulting in equal heat inhibition of repair. To examine which of these two possibilities was true, the induction and subsequent repair of DNA double strand breaks was examined using CHEF electrophoresis. It was concluded that the induction and repair of DNA double strand breaks was equivalent between the two cell lines.

Although the recent study by Kampinga and Dikomey (Kampinga and Dikomey, 2001) implicated the inhibition of repair of base damage as the important mechanism involved in thermal radiosensitization, the inhibition of DNA double strand break repair by heat was previously thought to be responsible

at the time that these studies were carried out. Also, our lab has previously shown that heat inhibition of DNA double strand breaks was important in some cell lines (Raaphorst et al., 1999). It was for these reasons that the induction and subsequent repair of x-radiation induced DNA double strand breaks was investigated in this study.

Chapter 5

Conclusion

Hyperthermia sensitizes cells to radiation. An understanding of the cellular mechanism of thermal radiosensitization may permit the optimization of combined radiation and heat treatments that will enhance cell killing of tumors but not of normal tissues. Many studies have shown that the mechanism of thermal radiosensitization is related to the inhibition of repair of radiation-induced DNA damage by heat. Due to the apparent role of the gene p21/WAF1/CIP1 in the repair of radiation-induced DNA damage, its involvement in thermal radiosensitization was, therefore, investigated.

In this thesis, the response of the two human colorectal cancer cell lines to radiation and heat treatments were observed to be similar and statistical analyses of the responses showed that they were not statistically different. Both cell lines showed a normal radiation response with respect to p53 induction but different responses in regard to p21/WAF1/CIP1 induction. As expected, p21/WAF1/CIP1 was not induced in the 80S4 cell line. In addition, p21/WAF1/CIP1 does not play a role in eliciting thermotolerance, as inducible thermotolerance was found to be similarly expressed in both the HCT116 (p21^{+/+}) and 80S4 (p21^{-/-}) cell lines.

The response of both cell lines treated with hyperthermia before or after irradiation for both hyperthermia temperatures revealed no difference between the cell lines. Comparison of thermal enhancement ratios showed that there was no difference in the amount of thermal radiosensitization induced in either cell line. The inactivation of the gene p21/WAF1/CIP1 from the 80S4 cells, therefore, does not seem to have an effect on the degree of thermal radiosensitization expressed in these cells. We may generalize these findings as suggesting that p21/WAF1/CIP1 does not play an essential role in thermal radiosensitization in cells that express wt p53.

The induction and subsequent repair of DNA double strand breaks was found to be the same in both cell lines. The lack of difference in thermal radiosensitization levels was, therefore, not due to a difference in the repair abilities of the two cell lines. Conversely, the similarity in thermal radiosensitization levels in these two cell lines was likely due to similar levels of repair of radiation damage and similar levels of heat inhibition of that repair. This finding strongly supports the idea that the knocking out of the gene p21/WAF1/CIP1 from the 80S4 cells does not have an effect on the degree of thermal radiosensitization expressed in these two cell lines. The hypothesis that a loss of p21/WAF1/CIP1 would result in a reduced amount of thermal radiosensitization seems to be incorrect.

5.1 Future Research

This study focused on the response of p21/WAF1/CIP1-deficient cells and their wild-type parental cells to combined hyperthermia and radiation treatments *in vitro*. The results suggest that p21/WAF1/CIP1 may not play a role in thermal radiosensitization. While further research may not seem entirely necessary due to the negative result obtained from this study, some further experiments may still prove interesting. For example, thermal radiosensitization experiments could also be performed *in vivo* to assess p21's role. The response of the 80S4 cells *in vivo* to radiation alone has been shown to be significantly more sensitive than that of the HCT116 cells (Wouters et al., 1997). It has also been suggested that cells grown *in vivo* have interactions with other molecules that may have important effects on repair proficiency. Thus p21/WAF1/CIP1 may interact very differently with these repair molecules *in vivo* compared to *in vitro*. Therefore, similar combined treatment experiments performed *in vivo* could provide a more sensitive method for assessing thermal radiosensitization levels.

Other molecules involved in the repair of radiation-induced DNA damage should also be investigated. A recent review of hyperthermic radiosensitization (Kampinga and Dikomey, 2001) stated that the determination of molecular effects underlying thermal radiosensitization is important. Since the most recently suggested mechanism of thermal radiosensitization is the inhibition of radiation-induced base excision repair, similar experiments should be performed using cell lines deficient in BER. To date, no naturally occurring mutants of BER have been

detected and cells in which BER has been modulated by either transfection or mutational inactivation are very susceptible to apoptosis (Kaina et al., 2001). If these mutants become available in the future, however, they would provide an explicit method for determining the role of BER in thermal radiosensitization.

In the meantime, it has been shown that cell lines that are deficient in either DNA ligase I (known as 46BR.1G1) or DNA ligase III (known as EM9) are defective in catalyzing BER *in vitro* (Tomkinson et al, 2001). Both of these DNA ligases interact with other BER proteins, such as PCNA and DNA polymerase beta. Since it is the religation step of BER that is thought to be inhibited by heat (Kampinga and Dikomey, 2001), an investigation of DNA ligases in thermal radiosensitization could prove interesting. These cell lines could therefore be used to examine the role of BER in thermal radiosensitization and could potentially implicate specific enzymes involved in this process.

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