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**Investigation of adaptive response in human tumour
and normal cell lines with varying radiosensitivity.**

by

Sheri Nadine Boyden, BSc

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

Master of Science

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March 26, 1998
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the Faculty of Graduate Studies and Research
acceptance of the thesis,

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Date May 4, 1998

ABSTRACT

The existence of cellular radioprotective mechanisms that can be up-regulated in response to exposure to small doses of ionizing radiation has developed into an important area of investigation. There appear to be two ways these 'induced' mechanisms operate. They either influence the response of cells to single doses so that small acute radiation exposures are more effective per unit dose at causing cell lethality than larger exposures above a threshold where the induced radioprotection is triggered, or they protect the cells against a subsequent exposure to radiation that may be substantially larger than the initial priming dose given several hours earlier. This study investigates the latter mechanism which is termed '*adaptive response*'. 6 cell lines were studied to determine if the adaptive response is dependent on the type of cell or on the radiosensitivity of the cell. Cells were primed with a small *priming* dose of X-radiation 6 h before the challenge dose of X-radiation. A range of priming doses, from 0.2-4Gy, was investigated as well as various challenge doses (1-8 Gy). Of the 6 cell lines studied, only the T47D breast carcinoma cells showed an adaptive response when primed prior to the challenge dose. The results from this study give no indication that radioadaptability is related to the type of cell, or to the cell's intrinsic radiosensitivity. The adaptive response was also not found to be related to the cell's ability to repair sub-lethal or potentially lethal radiation damage.

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LIST OF ABBREVIATIONS

AR	adaptive response
ATCC	American Type Culture Collection
BSF	backscatter factor
DNA	deoxyribonucleic acid
DP	delayed plating
dsb	double strand break
FBS	fetal bovine serum
HDR	high dose rate
HVL	half value layer
IP	immediate plating
IR	induced resistance
LET	linear energy transfer
LQ	linear quadratic
NCS	newborn calf serum
PDD	percent depth dose
PE	plating efficiency
PLD	potentially lethal damage
PLDR	potentially lethal damage repair
RR _{DP}	recovery ratio due to delayed plating
RR _{SD}	recovery ratio due to splitting a dose in two fractions separated by time
SEM	standard error of the mean
SF	surviving fraction
SF ₂	surviving fraction after 2 Gy
SF ₄	surviving fraction after 4 Gy
SLD	sub-lethal damage
SLDR	sub-lethal damage repair
ssb	single strand break
SSD	source to surface distance
TAR	tissue-air ratio
T-25	25cm ² flask

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1.0 GENERAL INTRODUCTION

1.1 Background

Over the last 30 years, there has been considerable interest in the idea that cellular radioprotective mechanisms exist that are up-regulated in response to exposure to small doses of ionizing radiation. It is generally accepted that mammalian cells can carry out some "passive" response to radiation in which the cells repair and control deoxyribonucleic acid (DNA) injury by constitutive processes. This system remains available whether the cell is damaged or not. Despite accumulating evidence that cells may trigger or increase the activity of processes specifically to facilitate the repair or removal of DNA injury following exposure to ionizing radiation (1-24), this idea has not yet been universally accepted.

The consequences of such a finding could affect how we treat cancer patients with radiotherapy and could also be important as far as radiation protection is concerned. If a radioresistant population of cells is induced by an initial radiation treatment, there may be serious problems treating patients who are presenting with a recurrent tumour or patients who are treated with a fractionated radiation protocol. The chance for tumour control or cure may be lost if such a radioresistant population is induced. The existence of these inducible radioprotective processes could also have an impact in the field of radiation protection. If cells can be made resistant to radiation, then radiation workers who are about to enter an area where they will be exposed to high levels of radiation, could be primed with a low dose of radiation, or by other means, in order to trigger their cells to become resistant to the harmful effects of the ionizing radiation that they will

subsequently receive. Furthermore, small repeated radiation exposures, permitted under current radiation safety programs, are delivered to large numbers of people (i.e patients through medical applications of radiation as well as radiation workers) so a knowledge of the response of humans to low levels of radiation exposure is basic to the safe and efficient use of radiation.

There appear to be two distinct ways in which these induced radioprotective mechanisms operate; they either influence the response of cells to single doses so that small acute radiation exposures (or exposures at very low dose-rates) are more effective per unit dose than larger exposures above a threshold where the induced radioprotection is triggered (1-7,12-14,23), or they protect against a subsequent exposure to radiation that may be substantially larger than the initial 'conditioning' or 'priming' dose given several hours earlier (2-11,14-20,22-25). The former mechanism is generally termed '*induced resistance*' while the latter is often referred to as '*adaptive response*'.

The idea of hypersensitivity to very low single radiation doses, as well as the radioadaptive response, is not a new one. In his "overview" papers on induced radioresistance, Joiner (2,3) pointed out that as early as 1963 experiments were performed by Eriksson on irradiated maize plants that demonstrated dose-response relationships for both mutation induction and pollen survival that showed hypersensitivity to doses of less than 1 Gy. In 1967, Calkins suggested the possibility of a threshold dose above which cells would acquire radioresistance. This idea was proposed to explain the real increase in cell survival that was observed when the radiation dose was raised above a hypothetical threshold level in the protozoan *Tetrahymena pyriformis* (26).

Induced resistance was also proposed to explain the increase in cellular survival observed in lower cell systems when the cells were primed with a much lower radiation dose (which itself produced little or no reduction in cell viability) several hours prior to delivery of the second, larger dose. This phenomena is now generally termed adaptive response. Boreham and Mitchel illustrated this effect in the yeast *Saccharomyces cerevisiae* (16). They demonstrated that prior irradiation increased the radioresistance by a factor of 1.5-3 to subsequent exposures given 2 hours later.

The adaptive response has also been observed in human systems. In a series of studies performed by Wolff et al. (17,19,27), human lymphocytes irradiated with very small X-ray doses (typically a few cGy) or by exposure to tritiated thymidine, became less susceptible to the effects of a subsequent acute γ -radiation or X-radiation dose given some time later. This was assessed by measuring chromosome aberrations. They also found that if the doses were administered acutely, the effect could not be induced by priming doses less than 0.5cGy or higher than 20cGy. In other words, there was a narrow window of doses below which and above which the phenomena did not occur. The response was not found to be the result of cell cycle related phenomena, such as differential cell stage sensitivity to radiation, or to the selective killing of a radiosensitive sub-population of lymphocytes.

Various single dose studies in mammalian cell lines have found multiphasic survival curves demonstrating low-dose hypersensitivity (1,4,5,14,19,28). It has been demonstrated that the linear quadratic model substantially underestimated the killing effect of single X-ray doses less than 1Gy in hamster cells (26) and various human

tumour cells (1,4,7,21,23). At very low doses, the survival curve has been found to be several times steeper than the initial slope of a linear quadratic model fitted to the data at doses above 1Gy and extrapolated into the low-dose region. As was the case in the adaptive response experiments, the low-dose hypersensitivity was not attributed to a sub-population of cells with different radiosensitivity.

There is some evidence that increased mutation is also detectable at very low radiation doses and that small radiation doses are able to protect against the mutagenic effects of subsequent exposures. At the *hprt* locus, Kelsey et al. (29) have found that conditioning doses of just 0.01Gy can protect human lymphocytes against mutation produced by a dose of 3 Gy given 16 h later. Work by Hall and Miller (30) measured radiation-induced transformation in C3H10T1/2 cells in single dose measurements which suggested a multiphasic response at low doses, with an apparent flat transition between regions of sensitivity at doses less than 0.3Gy and a more resistant response above about 1Gy.

Although the mechanism behind adaptive response and induced resistance is not known with certainty, several studies have suggested that high-linear energy transfer (LET) radiations are less effective than X-rays, at similar levels of cell killing, at initiating both of these responses (2,16). Small doses of hydrogen peroxide have also been shown to induce protection against subsequent doses of ionizing radiation. Both of these findings support the notion of oxidative species or single-strand breaks as inducers (8,14).

The majority of adaptive response studies published prior to commencement of this research project were conducted on rodent cell lines or on human lymphocytes. The range of priming and challenging doses reported in the literature was also very limited. We therefore decided to study a wide range of priming doses, from 0.2 Gy to 4 Gy and a wide range of challenge doses from 0 Gy to 8 Gy (depending on the radiosensitivity of the cells). The small priming doses of 0.2 and 0.5 Gy were chosen because the literature generally indicated that only priming doses under a gray were able to trigger adaptive response. Priming doses larger than 1 Gy were also selected to determine if clinical sized doses could initiate the adaptive response.

To determine if the adaptive response is dependent on the type of cell, we decided to study three different tissue types. As well, we selected two different cell lines from different individuals for each tissue type to determine if there is variability within a given type of tissue for the presence of an adaptive response.

Because the adaptive response is evidently related to the cell's ability to cope with radiation damage, either through increased repair mechanisms or through some other form of radioprotective process, it is possible that the ability of cells to undergo an adaptive response is related to the cell's ability to repair other forms of radiation damage. It is well known that many cells are able to repair radiation damage if left in plateau phase following irradiation. Plateau phase is the term used to describe the state of the cells in the flask when all the available growth surface is occupied and all the cells are in contact with surrounding cells. Cell proliferation nearly ceases at this time and the growth fraction falls to between zero and ten percent. Cell survival increases if the cells

are left in this density inhibited state following irradiation because they can repair radiation damage before undergoing mitosis. This form of repair is termed '*potentially lethal damage repair*' (PLDR) and is operationally defined as damage which, if unrepaired, is lethal (31-47).

Cell survival also increases due to repair of DNA damage if a radiation dose is split into two or more fractions separated by a time interval. This is termed '*sub-lethal damage repair*' (SLDR) (31,33,46-51). We set out to determine if the adaptive response is related to the cell's ability to repair potentially lethal damage (PLD) or sub-lethal damage (SLD).

1.2 Hypothesis and Specific Objectives

The hypothesis of this thesis is that small priming doses of X-radiation can trigger cells to become more resistant to a subsequent dose of X-radiation given some time later. This response is probably variable among cell lines and may be related to the cell's ability to repair potentially lethal damage and/or sub-lethal damage.

The purpose of this research was to achieve the following objectives:

- 1) To commission an X-ray unit to be used for high dose rate irradiation in this research project. Part of this task involved designing and installing a flattening filter.
- 2) To characterize the survival response of two established melanoma cell lines, two breast carcinoma cell lines and two human fibroblast cell lines subjected to X-radiation. This is required to determine if the presence of an adaptive response is related to the intrinsic radiosensitivity of the cells.

- 3) To determine whether an increase in cell survival results if cells are primed with ionizing X-radiation hours before being given a large second dose of X-radiation, i.e. check for an adaptive response in the cells.
- 4) To measure the recovery capacity of various cell lines maintained in a density inhibited state for 6 hours following irradiation before being assayed, compared to the recovery capacity if they are assayed immediately following irradiation, and to determine if this is related to the ability of the cells to initiate an adaptive response.
- 5) To measure the amount of cellular recovery that occurs in two breast carcinoma cell lines if the radiation dose is given as two fractions separated by a time interval, and to see if this is related to any observed adaptive response.

Each of these five objectives is addressed in a separate chapter in this thesis. Each chapter consists of four sections: introduction, methods, results and discussion.

2.0 MATERIALS AND METHODS

2.1 Cell Lines

Six cell lines, representing three different tissue/tumour types, were used in this study to determine whether the adaptive response is related to the type of cell. The tissues studied were human melanoma, breast carcinoma and human fibroblast. Two cell lines of different radiosensitivity from different patients were studied for each cell type to determine whether the adaptive response is dependent on the radiosensitivity of the cells.

2.1.1 Fibroblast Growth

The fibroblasts used in these experiments were grown from punch biopsies taken from the forearm (unless otherwise stated) of volunteers who gave informed consent. The 4mm diameter tissue biopsies were transported in saline solution and immediately soaked two times, for a period of 10 minutes each, in 5mL of a mixture of DMEM/F-12 (Gibco) containing 0.1 mM non-essential amino acids (Gibco), 20 mM HEPES (Boehringer Mannheim), 10 mM sodium bicarbonate (Sigma) and 30% v/v antibiotic-antimycotic (Sigma). The sample was then transferred to a petri dish where it was cut into small pieces (less than 1mm diameter) using two sterile scalpels. The pieces were pressed to the bottom of 25cm² (T-25) flasks with 6-8 pieces per flask. 1mL of the above medium containing 20% v/v fetal bovine serum (FBS) (Gibco BRL) and 1% antibiotic-antimycotic were carefully added to each flask. The flasks were observed daily until cell growth could be seen at which time the volume of medium was increased to 4ml per flask. Once the cell culture was established, the serum levels were changed to 7.5%

FBS and 7.5% newborn calf serum (NCS) (Gibco BRL). Experiments were performed on cell passage numbers less than 18. Extra cells were frozen for future use at -115°C at a concentration of 5×10^5 cells/ml in 1 ml aliquots in complete medium with 10% v/v Dimethyl Sulphoxide.

The fibroblast cell lines used throughout this study were the OMB 18 (Ottawa Medical Biophysics #18) and S11358.

The OMB18 cell line was derived from a skin biopsy received from a 68 year old male patient who had been treated for non-Hodgkins lymphoma of the stomach prior to donating the skin biopsy. This patient did not show any abnormal side effects to the radiation, and remains free of his lymphoma.

The S11358 cell line was grown from a biopsy from a 33 year old female patient with cervical cancer who had severe chronic normal tissue injury following conventional irradiation.

2.1.2 Melanoma Cell Lines

Two human malignant melanoma cell lines were used. The Sk-Mel3 cell line is a pigmented, radioresistant melanoma cell line established from a 42 year old female (American Type Culture Collection (ATCC)). The HT144 cell line is a non-pigmented, radiosensitive melanoma cell line established from a 29 year old male (ATCC).

2.1.3 Breast Carcinoma Cell Lines

Two human breast carcinoma cell lines were used. The T47D cell line is an epithelial-like, ductal carcinoma of the breast obtained from a 54 year old female

(ATCC). The HTB30 cell line is an epithelial-like, adenocarcinoma of the breast obtained from a 43 year old female (ATCC).

2.2 Clonogenic Methods

2.2.1 General tissue culture methods

Due to sterility requirements, all cell manipulations were carried out in a laminar flow hood. The melanoma and breast carcinoma cell lines were grown in the same medium as previously described with 7.5% FBS and 7.5% NCS, without antibiotics. The OMB18 and S11358 cell lines were grown in HEPES free medium. All cells were incubated at 37°C with 97.5% air, 2.5% CO₂ and 100% humidity.

All cell lines were grown in 80 cm² flasks and subcultured while still in exponential phase. Subculturing involved aspirating the medium, rinsing the cells with isotonic citrate saline (134mM KCl (BDH), 17.6 mM citric acid (Sigma)) to remove cellular debris and trypsin inhibiting substances found in the medium, and adding 0.9mL of trypsin (0.2% w/v in citrate saline)(Gibco BRL). Due to difficulties in obtaining a single cell suspension with the breast carcinoma cell lines, 0.9mL of trypsin-EDTA (0.25% w/v trypsin, 0.03% w/v EDTA in phosphate buffered saline)(Sigma) was used instead of trypsin. With the trypsin still on the cells, the flasks were incubated for 5 minutes at 37°C to allow the cells to loosen from the growing surface. The cells were then resuspended in 10mL of medium. The cells were counted by means of a Bright-Line haemocytometer (Reichert) or an automatic cell counter (Particle Data Elzone 80, calibrated by haemocytometer). Both methods gave comparable counts.

Growth curves were defined for the T47D and HTB30 cell lines by seeding 5×10^5 cells into T-25's containing 4 ml of medium. Cells were incubated at 37°C and each day one flask was trypsinized and counted. After 8 days, the remaining flasks were divided into three groups: one was given fresh medium, another was given fresh medium on day 8 and again on day 11, and the last group was left in the original medium. Daily counts were performed until the cells in each group remained confluent for several days. The growth curves for the T47D and HTB30 cell lines (Figures 2.2.1 and 2.2.2), were used to determine the optimal feeding schedule and initial cell concentration required to reach confluence.

The growth curves for the melanoma and fibroblast cell lines had been previously measured (52,53). All experiments were performed on plateau phase cultures of cells to minimize cell cycle redistribution effects following X-radiation. The melanoma cell lines were seeded at 5×10^5 cells/T-25, fed on day 5 and experiments were performed on day 8. The fibroblasts were seeded at 1×10^5 cells/T-25, fed on day 5 and experiments were carried out on day 10. The breast carcinoma cell lines were seeded at 5×10^5 cells/T-25, fed on days 8 and 11 and experiments were performed on day 13. All cell lines were seeded with 4 mL of complete medium (with or without HEPES depending on the cell line).

2.2.2 Colony forming assay

The colony forming assay, a measure of clonogenic cell survival originally described by Puck et al. (47,54,55), was used as the endpoint for all experiments.

Figure 2.2.1 Growth curve for T47D cells with various feeding schedules.

Figure 2.2.2. Growth curve for HTB30 following various feeding schedules.

This assay measures the proportion of cells which remain reproductively viable following an experimental treatment. During the course of an experiment, once the cells were subjected to the treatment of X-radiation, the medium on the plateau phase cells was aspirated, the cells were rinsed with citrate saline to remove any remaining medium, and trypsin (or trypsin-EDTA) was added. The cells were incubated for 5 minutes at 37°C. with the trypsin still on the cells, in order to loosen the cells from the growing surface. The cells were then resuspended in 4 mL complete medium with repeated pipetting to achieve a single cell suspension. A sample of this cell suspension was then counted as above. Once the cell concentration was determined, the number of cells required to ensure 50 surviving cells was plated in 60mm petri dishes with 4 mL of complete medium. In the case of fibroblasts, which are large cells that form large colonies, or when the survival of the cells was low, requiring large numbers of cells to be seeded (greater than 5×10^5 cells), cells were plated in 100 mm petri dishes with 10 mL of complete medium per dish. The dishes were then incubated undisturbed at 37°C to allow surviving cells to replicate and form colonies consisting of at least 50 cells/colony. The incubation period varied depending on the growth rate of the cell line: the Sk-Mel3 and HT144 cells were incubated for 11 days, OMB18 and S11358 cell lines for 14 days, the T47D cells for 18 days and the HTB30 cells for 23 days. At the end of the incubation period, the dishes were emptied of medium and methylene blue (0.2% w/v in 70% ethanol) was added for approximately 20 minutes. After gently rinsing away excess stain, colonies consisting of a minimum of 50 cells were counted under a Fisher Stereomaster II 10X stereoscopic microscope. In the colony forming assay, each colony is assumed to

have arisen from a single surviving cell which attached itself to the growing surface and entered the cell cycle, dividing itself and forming a colony.

For any given experiment, three to six dishes of cells were plated for each experimental dose of X-radiation, as well as an additional three to six plates for the control point (untreated), which was used to calculate the plating efficiency (PE) of the cells. The survival was then assessed by calculating the surviving fraction (SF) for each plate given by the following equations:

$$PE = \frac{\text{number of colonies per control plate}}{\text{number of cells seeded per control plate}} \quad (2.2.2.1)$$

$$SF = \frac{\text{number of colonies per plate}}{(\text{number of cells seeded per experimental plate}) \times (PE)} \quad (2.2.2.2)$$

The data from several independent experiments were pooled and the mean survival, $\overline{SF}_i$, and the standard error of the mean (SEM), $\overline{\sigma}_i$, were computed for each individual dose, i .

$$\overline{SF}_i = \sum_{j=1}^n \frac{SF_{ij}}{n} \quad (2.2.2.3)$$

$$\overline{\sigma}_i = \sqrt{\frac{\sum_{j=1}^n (SF_i - SF_{ij})^2}{n(n-1)}} \quad (2.2.2.4)$$

Here n is the number of experiments performed. Each experiment was performed a minimum of two times and up to as many as twelve times. The average SF between experiments was calculated for each cell line and was plotted versus the dose, with the error bars representing the standard error of the mean (SEM) between experiments.

2.2.3 Curve Fitting

The linear quadratic model was used to fit the survival curves. This model assumes there are 2 components of cell kill by radiation, one that is proportional to dose and one that is proportional to the square of the dose (31,56). This is discussed further in section 4.1. By taking the natural logarithm of the linear quadratic equation,

$$SF = e^{(-\alpha D - \beta D^2)} \quad (2.2.3.1)$$

and plotting it versus dose, a second order polynomial could be fit using the fitting routine in the Grapher version 1.24 (Golden Software Inc) plotting program. The data were then normalized to the zeroth order coefficient in order to set the point receiving zero dose to a surviving fraction of 1. The first and second order polynomial coefficients were taken to be α and β respectively.

2.3 Irradiations

All cell cultures were irradiated using high dose rate (HDR) X-rays. HDR radiation typically refers to dose rates greater than 100 cGy/min. Low dose rate generally refers to dose rates of less than 30 cGy/min. and medium dose rate X-rays fall between these two dose rate regions. The Sk-Mel3 experiments, as well as various experiments on the S11358, OMB18 and HT144 cell lines, were conducted using an X-ray machine with

a dose rate of 112 cGy/min (150kVp, 1mm Al filter). Due to relocation of the lab, experiments performed on the T47D, HTB30 and various S11358, HT144 and OMB18 cell lines were conducted using a different X-ray unit with a dose rate of 142cGy/min (250 kVp, 1.07 mm Al and a HVL of 1.75mm Cu). With this new X-ray unit, doses under 1 Gy were given at a lower dose rate of 56cGy/min. in order to increase the radiation exposure time to a reasonable time period. The cells were grown to confluence in T-25's and were irradiated in the flasks, on ice, in order to reduce the amount of repair being carried out while the cells were irradiated.

3.0 Commissioning of X-ray Machine

In December 1996, the Cancer Research Group at the Ottawa General Hospital received a Pantak Bipolar Model HF320 Industrial X-ray unit. This unit was to be used to irradiate cells with a tube voltage of 250 kVp and a desired dose rate of 200 cGy/min.

Commissioning of the irradiator involved determining the half value layer (HVL), obtaining a flat radiation field (by designing and installing a flattening filter), measuring a percentage depth dose (PDD) curve and calculating the dose rate. The HVL is the thickness of some standard material (usually copper or aluminum) required to attenuate the radiation beam by 50 percent. A PDD curve gives the variation of absorbed dose with depth within a patient (or phantom) along the central axis of the beam. It is expressed as a percentage of absorbed dose at any depth d , to the absorbed dose at a fixed reference depth d_0 , along the central axis of the beam.

The dose variation across the field at a source-surface distance (SSD) of 52 cm was determined by irradiating an X-ray film (Kodak X-Omat V) for 5 seconds, with tube parameters set to 6mA and 250 kVp, and then performing a film density scan to determine the relative dose (Lumisys Laser Film Scanner). The non-linearity of the films was not a concern at this point because we simply wanted to determine if the profile was flat. We were not concerned with using the films to determine the magnitude of the dose variation throughout the field. The beam profile in the direction from cathode to anode (horizontal slice) showed a decrease in dose toward the anode that was due to the “heel effect” (Figure 3.1) (57). The beam profile in the direction perpendicular to the cathode-anode direction (vertical slice), showed a gradual decrease in dose toward the

Figure 3.1 Initial X-ray beam profile in the direction from cathode to anode. The decrease in percent dose toward the anode is due to the heel effect.

Figure 3.2 Initial X-ray beam profile in the direction perpendicular to the cathode-anode direction (vertical slice).

edges of the beam (Figure 3.2). In order to obtain a flat isodose across the beam, a flattening filter had to be designed and installed.

A preliminary HVL measurement, determined by measuring the air kerma at a point in a narrow beam as increasing thicknesses of attenuating material (Copper) were placed in the beam (58,59), showed a large initial drop in beam intensity with very thin sheets of copper. This initial drop off, which was also observed with the use of an aluminum step wedge on an X-ray film, led us to the conclusion that the X-ray unit had no inherent filtration. As a result, a thin copper sheet had to be added to the flattening filter to attenuate the low energy photons in the X-ray beam.

To measure the dose rate, the distance from the X-ray tube to the level of the cells in the flask was set to 66cm. The distance from the X-ray tube to the surface of the medium was therefore 65.7cm (if we assumed 3 mm of medium overlaid the cells). The standard protocol used to determine the dose rate for medium energy X-rays is to measure the dose rate with the ion chamber placed at a depth of 2cm in a full scatter water phantom (59,60). The absorbed dose at this reference depth for a given field size is then given by

$$D_{w,z=2} = MN_k k_{ch} \left[\left(\frac{\mu_{en}}{\rho} \right)_{w:air} \right]_{z=2,\phi} \quad (3.1)$$

where $D_{w,z=2}$ is the dose to water in grays at the position of the chamber center at a depth $z=2$ cm when the chamber is replaced by water. M is the electrometer reading obtained

with a chamber corrected to standard pressure and temperature, N_k is the chamber calibration factor in grays per scale reading to convert the instrument reading at the beam quality (HVL) concerned to air kerma free in air at the reference point of the chamber with the chamber assembly replaced by air, $[(\mu_{en}/\rho)_{w/air}]_{z=2,\phi}$ is the mass energy absorption coefficient ratio, water to air, averaged over the photon spectrum at 2 cm depth of water and field diameter ϕ , and k_{ch} is a factor which accounts for the change in the response of the ionization chamber between calibration in air and measurement in a phantom (61,62). The Farmer dosimeter 2570/1 was used for the dose rate measurement and a HVL of 14mm Al was assumed (based on the use of an aluminum step wedge and X-ray film). With $N_k=8.9$, $k_{ch}=1.022$, $[(\mu_{en}/\rho)_{w/air}]_{z=2,\phi}=1.05$ and the tube voltage set to 250kVp, a tube current of 12.1mA was required to achieve a dose rate of about 200cGy/min at the level of the cells.

Before proceeding with the design of the flattening filter, the timer delay (time between when the machine is turned on and when the radiation is generated) was measured. To do so, the electrometer reading for various irradiation times was recorded and plotted (Figure3.3). The X-intercept on the graph is the timer delay and was calculated to be 3.35 seconds. This delay had to be accounted for in subsequent dose rate calculations.

The flattening filter had to consist of 2 parts; a copper sheet 0.5 mm thick to absorb the low energy component of the X-ray beam, and a lucite filter to differentially absorb the photon beam so as to obtain a flat beam profile. Lucite was chosen because it

Figure 3.3. Electrometer reading as a function of irradiation time at SSD 13cm and tube parameters 12.1 mA, 250 kVp. The equation of the line is $y=0.636161x-2.13166$ and the timer delay (x-intercept) is 3.35 seconds.

is water equivalent, i.e. has similar effective atomic number and number of electrons per gram (58).

To design the flattening filter, a percent depth dose (PDD) curve in water was measured using an NACP parallel plate ionization chamber and a water phantom. A sheet of copper 0.54mm thick was attached to the base of the X-ray tube to act as the absorber. Due to size constraints in the X-ray unit, the PDD curve was calculated from tissue-air ratio (TAR) measurements using the following equation (58)

$$P(d, W_m, F) = 100 \frac{T_a(d, W_d)}{B(W_m)} \left(\frac{F + d_m}{F + d} \right)^2 \quad (3.2)$$

where $P(d, W_m, F)$ is the PDD at a depth in water d , field size W_m defined at the depth of maximum dose (which was located on the surface of the phantom for our beam energy), and an SSD of F . $T_a(d, W_d)$ is the TAR, or dose in water at depth d to dose free in air, for the field size at that depth. $B(W_m)$ is the backscatter factor (BSF), i.e. the dose on the water surface to the dose free in air for field size W_m . The PDD curve illustrated in Figure 3.4 was verified by irradiation of an X-ray film on edge wedged between two sheets of “solid water” (MS11).

With the 0.54mm Cu absorber still attached to the base of the X-ray tube, the beam profile was determined, as before, using an X-ray film. This film, and all subsequent films, was irradiated for 20 seconds at an SSD of 65.7cm and the tube parameters set to 12mA and 250kVp. A sheet of plexiglass 1mm thick was placed on the

Figure 3.4. Percentage depth dose plotted against depth in water for X-ray unit with 0.54mm Copper absorber and SSD 65.7cm.

film to flatten it and mimic the medium over the cells. The heel effect disappeared due to absorption of the low energy photons in the copper absorber (Figure 3.5), but the roll off toward the edges of the field was still present (Figure 3.6). To design the flattening filter, we looked at the horizontal and vertical cross sections of the beam profile, together with the PDD curve, and calculated the thickness of lucite that would be required, at 2 cm intervals along each cross section, to obtain a flat profile. The dimensions were then scaled down from the size of the film (42X34cm²) to the size of the opening at the base of the X-ray tube, (6.6cm in diameter). The design of the flattening filter is shown in Figure 3.7. Once installed, slight modifications to the filter were required to obtain a flat beam profile.

The dose rate, measured as before but with the addition of the filter and a 0.81mm Copper absorber, was calculated to be 51cGy/min. This was far below the desired dose rate of 200 cGy/min. Table 3.1 illustrates the effect of varying several parameters in an attempt to increase the dose rate. Reducing the SSD was not an option because the large field size obtained at an SSD of 65.7cm was required. Removing the absorber all together was also not feasible due to the low energy photon contamination of the beam that would result in a poor PDD distribution. In the end, a compromise was made between beam quality and dose rate. A 1.07 mm Al absorber was installed in place of the 0.81 mm of copper, the SSD remained at 65.7 cm and the tube voltage at 250kVp, while the current was increased to 12.5 mA, which was the maximum allowed current for that tube voltage. Replacing the 0.81mm Cu absorber with 1.07 mm Al altered the beam so the dose profile was no longer even throughout the field. The flattening filter therefore

Figure 3.5. X-ray beam profile in the direction from cathode to anode with the addition of 0.54mm Cu absorber to the base of the tube. The heel effect is no longer present.

Figure 3.6. X-ray beam profile in direction perpendicular to the cathode anode direction with the addition of 0.54mm Cu absorber to the base of the tube.

Figure 3.7. Sketch illustrating the design of the lucite flattening filter in 2 dimensions. Filter was constructed symmetrically in 3 dimensions.

Table 3.1 Electrometer reading obtained by varying various parameters in an attempt to increase the dose rate.

Voltage (kV)	Current (mA)	SSD (cm)	Absorber	Flattening Filter	Electrometer Reading
250	12	65.7	0.81 mm Cu	yes	53.7
250	12	65.7	none	no	215
250	12	47	0.81 mm Cu	yes	98.5
300	10	47	0.81 mm Cu	yes	123.5
250	12	65.7	0.27 mm Cu	yes	78
250	12	65.7	none	yes	134.5
250	12	65.7	1 mm Al	yes	111.7

had to be readjusted with the use of epoxy and a file to obtain an even beam profile once again. Figures 3.8 and 3.9 show the final profiles. The beam profile obtained by film was verified by measuring the dose at various positions in the plane of interest using a PTW markus ion chamber (Frieburg) with 2 mm of solid water overlying the chamber to mimic the medium over the cells. It was feared that the photoelectric effect due to the high Z of the film, and the low energy component of the beam on the sides of the flattening filter where the thickness of lucite was reduced, might have caused the film to be a poor representation of the actual flatness of the field. Table 3.2 verifies that the X-ray film did accurately depict the field flatness.

To accurately measure the dose rate, the HVL had to be determined precisely. Using the narrow beam geometry previously described, and a PTW ion chamber, the HVL was measured to be 18.2 mm Al or 1.75 mm Cu. The PDD curve was

Figure 3.8 Final X-ray beam profile in the direction from cathode to anode (horizontal direction) obtained with the flattening filter and a 1.07mm thick Aluminum absorber in place.

Figure 3.9 Final X-ray beam profile in the direction perpendicular to the cathode-anode direction (vertical slice) obtained with the flattening filter and a 1.07mm thick Aluminum absorber in place

Table 3.2. Electrometer reading for various positions of the ion chamber in the plane of interest. The zero position represents the center of the plane.

Front-Back Position	Electrometer Reading	Left-Right Position	Electrometer Reading
15	1.044	15	0.995
12	1.000	12	1.000
9	1.022	9	1.010
6	1.000	6	0.995
3	1.000	3	1.000
0	1.000	0	1.000
-3	1.000	-3	1.000
-6	1.000	-6	0.995
-9	1.022	-9	0.990
-12	1.033	-12	0.985

remeasured because the addition of the flattening filter and absorber changed the quality of the radiation beam. The PDD curve was measured directly using sheets of solid water (MS11) and the PTW ion chamber, rather than by the TAR method previously described. To measure the PDD curve, the ion chamber was set in a piece of solid water at a distance of 65.7 cm from the X-ray source. Sheets of solid water of varying thicknesses were then added on top of the chamber and the chamber was lowered by a distance equal to the thickness of solid water added so that the distance from the X-ray source to surface of the solid water remained constant at 65.7 cm. The PDD curve showed very little variation in dose at low depths (less than 5 mm), which was what was desired (Figure 3.10).

Figure 3.10. Percentage depth dose plotted against depth in water for X-ray unit with flattening filter, 1.07mm Aluminum absorber and SSD 65.7cm.

The absolute dose rate was measured using the same protocol as previously described for determining the dose at a 2 cm depth in water. This time the Farmer ion chamber and electrometer that were calibrated by NRC were used. Equation 3.1 was modified to correct for ion recombination (P_{ion}) as well as the temperature and pressure ($k_{P,T}$) (63,64). P_{ion} was measured using a Farmer ion chamber with a 2570-1 model electrometer that allowed one to vary the potential across the chamber. P_{ion} , the ratio of the charge collected with 100% bias voltage to the charge collected with 50% bias voltage, was measured to be 1.005.

The dose was calculated using the following equation

$$D_{w,z=2} = MN_k k_{ch} \left[\left(\frac{\mu_{en}}{\rho} \right)_{w/air} \right]_{z=2,0} p_{ion} k_{P,T} \quad (3.3)$$

To account for the timer correction factor, an exposure time of 1 min 3 sec was used and the resulting dose was 126.1 cGy. The dose rate was therefore 126.84 cGy/min at a depth of 2 cm in water. The dose rate at a 3 mm depth in water (corresponding to the depth of the cells under the medium) was measured by looking at the ratio of the PDD at 3 mm depth to the PDD at 2 cm depth and was calculated to be 142.4 cGy/min with an uncertainty of 4.1%.

For radiation doses under 1 Gy, a lower dose rate was desired in order to increase exposure times. This was achieved by reducing the current to 5 mA, while maintaining the tube voltage at 250 kVp. The resulting dose rate was 56.04 cGy/min.

Knowing the dose rate allowed one to calculate the exposure time required to deliver any dose of radiation.

4.0 High Dose-rate Irradiation and the Linear Quadratic Survival Model

4.1 Introduction

Exposure of a population of cells to ionizing radiation can have various effects on the individual cells. Some cells may not receive any damage to a critical target and are therefore unaffected by the radiation. Other cells may accumulate enough damage in critical sites to be lethal, causing the cells to die as they attempt the next or a subsequent mitosis, while still other cells may accumulate damage to critical targets that in itself is not lethal. Given time, these latter cells can repair the damage. On the other hand, if more damage is accumulated before the initial damage is repaired, these cells could die. Most cells die either by necrosis, which is characterized by the loss of membrane integrity of the cell before the DNA degrades, or by apoptosis (programmed cell death), in which the DNA degrades before the loss of membrane integrity. Cells can also be irreversibly arrested in the cell cycle without ever having reached mitosis and are therefore reproductively, but not metabolically, dead (65,66).

Radiation induced damage to mammalian cells can be classified into three categories: a) lethal damage which is irreversible, irreparable and leads to cell death; b) sub-lethal damage (SLD) which, under normal circumstances, can be repaired in a few hours unless additional damage is added to the cell that can accumulate to result in lethal damage; and c) potentially lethal damage (PLD), the component of radiation damage that can be modified by postirradiation environmental conditions. SLD and PLD are further discussed in chapters 6 and 7.

Cells can also misrepair their damage if two damaged chromosomes recombine in such a way that the cell is no longer viable and is therefore not able to progress through mitosis and dies in the process of dividing (67).

Although there is no widespread consensus on which of the numerous models of radiation cell survival is best, it is widely accepted that a two-component model is necessary to adequately describe cell survival *in vitro* (68). The linear quadratic model assumes there are two components of cell kill by radiation, one that is proportional to dose and one that is proportional to the square of the dose (47,69).

$$SF = e^{(-\alpha D - \beta D^2)} \quad (4.1)$$

where SF is the surviving fraction of cells after delivery of a radiation dose D, and α and β are constants which depend on the cell type and the irradiation conditions. In the past, the α component has been related to a double strand break (dsb) produced by a single charged particle track producing lethal damage, and the β component to a dsb arising from the combination of two independently produced single strand breaks (ssb) resulting from uncorrelated particle tracks (47). In recent years, these components have been described as being related to chromosome aberrations (31,47), i.e. the α component related to damage to 2 chromosomes and the β component to damage to a single chromosome. However, many authors agree that it is unlikely that a single electron can cause breaks in two different chromosomes so we will disregard this latter interpretation. The former mechanism assumes that at low doses, the two strand breaks are the consequence of a single electron set in motion by the absorption of x-or γ -rays, so the

probability of a dsb is proportional to the dose. The α component is believed to represent the intrinsic sensitivity of the cell to lethal radiation events. At higher doses, the two strand breaks may result from two separate electrons. The probability of interaction is therefore proportional to the square of the dose. The β component is believed to reflect the repair of radiation damage. The survival curve is linear in the low dose region where the α term dominates and bends at higher doses where the quadratic term dominates. The relative radiosensitivity of tumour cells can be quantified by the ratio of α/β , this ratio being more useful than absolute values of α or β . Radioresistant cells have small α/β ratios and radiosensitive cells have large α/β ratios.

Several studies have shown that differences in radiosensitivity can be measured *in vitro* using the clonogenic assay for fibroblasts (70-72) and tumour cells (39,72-75). The radiosensitivity that is measured *in vitro* represents the intrinsic sensitivity of the cell and some authors have speculated that it may be representative of radiosensitivity *in vivo* and, therefore, of the patient's response to radiotherapy (70,71,76-79).

In this chapter, the results of irradiating two human melanoma, two human breast carcinoma and two human fibroblast cell lines with high dose rate X-rays are reported. Two cell lines for each cell type were studied to determine if differences in radiosensitivity between cells of the same origin could be deduced from survival curves. Survival curves were generated and fit to the LQ model.

4.2 Method

Plateau phase cells were irradiated on ice with one of the two HDR radiation units described in section 2.5. Single doses of X-rays ranging from 0 to 12 Gy, depending on the sensitivity of the cells, were administered to the cells in the flasks. Control flasks (for plating efficiency) received sham radiation treatment. Cells were then trypsinized immediately and assayed according to the colony forming assay described in section 2.2.2.

4.3 Results

The radiation response of the 6 cell lines following HDR irradiation was determined and the curves were fit using the LQ survival model (Figure 4.3.1). The two melanoma cell lines differed greatly in radiosensitivity. The HT144 cell line showed virtually no shoulder region while the Sk-Mel3 cell line was characterized by a large survival curve shoulder. A large shoulder region is indicative of accumulation of sub-lethal radiation damage, and may reflect repair capacity. The variation in the surviving fraction at 2 Gy was greater than 30-fold; ranging from 0.938 ± 0.067 for Sk-Mel3 to 0.0296 ± 0.0043 for HT144. The surviving fractions of the cell lines following doses of 2Gy (SF_2) and 4Gy (SF_4) compared the radiosensitivity of the cell lines at isodose levels (Table 4.3.1). The lower the surviving fraction, the more radiosensitive the cell line. A large ratio between two cell lines indicated a large difference in their radiosensitivities. The P-value, calculated from a two-tailed student's t-test using the Instat biostatistics program (GraphPad Software Inc), indicated the significance of the differences in SF 's between the two cell lines. The large difference in radiosensitivity between the Sk-Mel3

Figure 4.3.1. Survival curves for Sk-Mel3, HT144, T47D, HTB30, OMB18 and S11358 cells after high dose rate irradiation on ice. The error bars represent the SEM between experiments.

Table 4.3.1. Survival curve parameters for the 6 cell lines studied. SF_2 and SF_4 are the surviving fractions at 2 and 4 Gy respectively. "Ratio" is the ratio of SF of the resistant to sensitive cell line in each tissue type following 2 Gy or 4 Gy irradiation. The P-value indicates the significance of the difference in survival between the resistant and sensitive cells of each tissue type, obtained from a 2 tailed student's T-test. n is the number of independent experiments performed.

	Melanoma		Breast Carcinoma		Human Fibroblast	
	Sk-Mel3 n=3	Ht144 n=7	T47D n=4	HTB30 n=3	OMB 18 n=2	S11358 n=2
SF_2	.938 $\pm$ .067	.030 $\pm$ .004	.59 $\pm$ .09	.36 $\pm$ .08	.28 $\pm$ .08	.18 $\pm$ .01
ratio	31.7	P<.0001	1.62	P = 0.21	1.6	P = 0.39
SF_4	.667 $\pm$ .006	(1.1 $\pm$ .6)E-3	.15 $\pm$ .04	.112 $\pm$ .014	.04 $\pm$ .02	.026 $\pm$ .004
ratio	609	P<.0001	1.48	P = 0.41	1.54	P = 0.65
α	.012	1.64	.262	.214	.588	.778
β	.024	0	.067	.073	.051	.033
α/β	.505	infinite	3.9	2.9	11	23

and HT144 cell lines was further quantified by the large difference in α/β ratios between these cell lines.

The survival curves of two breast carcinoma cell lines and the two fibroblast cell lines fell between the survival curves of the resistant and sensitive melanoma cell lines. The breast cell lines showed larger shoulder regions than the fibroblasts, as was quantified by the α/β ratios (Table 4.3.1). Contrary to what was expected, the two fibroblast cell lines did not show very large differences in their in vitro radiosensitivity. With a dose of 2Gy, there was a 1.6-fold increase in survival of the OMB18 cells over the S11358 cells, although this difference was not statistically significant. The S11358 cells

had a 2-fold larger α/β ratio than the OMB18 cells. The two breast carcinoma cell lines likewise did not show much difference in their surviving fractions following irradiation.

4.4 Discussion

Several studies from the 80's have shown that each cell line has its own characteristic radiosensitivity which is reflected in the in vitro survival curve (72,75). It has been suggested that differences in clinical response to radiotherapy between patients might be reflected in the differences in radiosensitivity between cell lines and that the intrinsic radiosensitivity, measured by the surviving fraction at 2Gy, might furnish part of the clinical prognosis of local tumour control and normal tissue complications.

The two melanoma cell lines studied varied greatly in their radiation sensitivity, with the Sk-Mel3 cells being extremely radioresistant and the HT144 cells being extremely radiosensitive. This range in radiosensitivity is not representative of the typical radiosensitivities found in tumour cells. However, these cell lines were used because they represented a wide range in radiosensitivity. The SF_2 values for Sk-Mel3 and HT144 were 0.938 ± 0.067 and 0.0296 ± 0.0043 respectively.

The separation between the survival curves in the fibroblast cell lines was much smaller than was observed in the melanoma cells. Various studies have reported that differences in sensitive and resistant cell lines of both tumour and normal cells can be measured at low doses (70-75). It has been reported that fibroblasts from patients who did not show abnormal side effects to radiation have an SF_2 from 0.26 to 0.37, and cells derived from patients who experienced moderate to severe early or late normal tissue reactions have an SF_2 from 0.05 to 0.21 (80). The OMB18 cells were obtained from a

patient who did not show any abnormal side effects while the S11358 cells came from a patient who had a severe normal tissue reaction to radiation. The SF_2 for these two cell lines were 0.284 and 0.177 respectively, which agrees with the above finding. However, because of the large error bars, the two survival curves were not found to be significantly different. Due to the low plating efficiencies and high passage numbers of both fibroblast cell lines, these experiments could not be repeated. In Brown's studies on the S11358 and OMB1 cell lines, differences in cell survival were indistinguishable at low doses and it was not until higher doses that the two survival curves separated (53). The OMB1 cell line was derived from a volunteer for whom the normal tissue response to radiation was unknown. Other studies have likewise shown that in vitro survival curves are not accurate predictors of clinical radioresponsiveness (81).

The in vitro radiosensitivity of the breast carcinoma cell lines was between the sensitivity of the 2 melanoma cell lines and showed a larger radioresistance than the two fibroblast cell lines. There was only a very small difference observed in the survival response of the T47D and HTB30 cell lines, with the T47D cells showing increased survival at low doses.

Although the intrinsic radiosensitivity (SF_2) is still recognized by some as an important factor in determining the cellular response to radiotherapy, its usefulness as a predictive assay of radiotherapy outcome is now under debate. Because radiotherapy typically consists of multiple fractions, the measurement of the effect of a single dose of 2Gy does not take into account many of the factors directly related to inherent radiosensitivity such as (i) cell cycle related heterogeneity, (ii) heterogeneity related to

the possible selection of more resistant sub-populations, (iii) cell repair capacity and (iv) the possibility of an adaptive response after a priming dose. Factor (iii) is investigated further in chapters 6 (study of potentially lethal damage) and 7 (study of sub-lethal damage repair). Factor (iv) is discussed in chapter 5.

5.0 Adaptive Response Experiments

5.1 Introduction

In recent years there has been considerable interest in the search for radioprotective processes that are up-regulated in response to exposure to small doses of ionizing radiation. It has been proposed that there are two ways these 'induced mechanisms' operate: they either protect cells against a subsequent exposure to radiation that may be substantially larger than the initial '*priming*' dose (referred to as "*adaptive response*" (AR)) (2-11,14-20,22-25,65,82,83), or they influence the response to single doses so that small acute radiation exposures, or exposures at very low dose-rates, are more effective per unit dose than larger exposures above a threshold where the induced radioprotection is triggered (referred to as "*induced resistance*" (IR)) (1-7,12-14,23,65) . The precise mechanism by which this induced resistance or adaptive response occurs, whether it be repair, cell-cycle control, or some other process, remains unknown.

The purpose of the experiments described in this section was to look for an adaptive response in six different cell lines subjected to a small priming dose of radiation prior to exposure to a larger, second dose of radiation. Two priming doses under 1 Gy were investigated because much of the literature suggests that only very small priming doses cause an adaptive response (2,8,84,85). Priming doses of 1, 2 and 4 Gy were also studied to determine if clinical sized priming doses also turn on the adaptive response mechanism. The time interval between the priming and challenging doses was set to 6 hours because it has been reported that the adaptive response is initiated within a couple of hours following delivery of the priming dose, and is maintained for up to 24 hours

(2,3,7,9). Two priming doses under 1Gy were investigated because the literature states that only small priming doses illicit an adaptive response. Ratios of surviving fractions between primed and unprimed cells were examined at different dose levels.

5.2 Method

Various human normal and tumourogenic cells were exposed, on ice, to low 'priming' doses of ionizing X-radiation. Primed and unprimed growth-arrested human cells were then incubated at 37°C for 6 hours before being exposed on ice to a subsequent high 'challenging' dose of ionizing radiation. Cells were then immediately rinsed, trypsinized and plated according to the colony-forming assays (described in section 2.2.2) to assess cell survival as previously described. The priming doses used were 0.2, 0.5, 1, 2 and 4 Gy. Five challenge doses, ranging from 0 to 8 Gy, depending on the sensitivity of the cells, were used. The control group in these experiments was given the second, challenging dose of radiation without having been previously primed. Survival curves were generated for each priming dose in all cell lines according to the method outlined in section 2.2.2. In order to compare survival curves resulting from different priming doses within one cell line, the survival following the challenge dose was normalized to the survival following the priming dose alone rather than to the plating efficiency. This was done to normalize out the effect of the priming dose on the surviving fraction.

5.3 Results

The results are grouped into three sections; the melanoma cell lines, the breast carcinoma cell lines and the fibroblast cell lines.

5.3.1 Melanoma Cell Lines

The survival curves for the radioresistant Sk-Mel3 cell line, subjected to various "priming" doses 6 hours prior to delivery of the challenge dose, showed no apparent protection compared to the unprimed population at all challenge doses (Figure 5.3.1.1). In fact, the data showed that the cell populations surviving the priming doses were, if anything, somewhat more sensitive than the original unprimed population. Although a few studies have observed this sensitization following a priming dose of radiation (5.9), this is contrary to the usual pattern of adaptive response seen in human cells (4,8-10,14,15,23-25,83). The difference in survival between the cells that were primed and those that were not is quantified in Table 5.3.1.1, which shows the surviving fractions of the primed and unprimed cells following the conditioning dose. Experiments with this cell line were repeated a minimum of 4 times for all challenge doses and up to as many as 12 times for challenge doses of 2 and 8 Gy. The errors in the table are equal to the error bars on the survival curve and were calculated according to section 2.2.2. The P-value, calculated from a two-tailed student's t-test using the Instat biostatistics program (GraphPad Software Inc.), indicates the significance of the difference in SF's between the unprimed and primed cells.

The LQ model was originally used to fit the survival data for each priming dose and the data were renormalized to allow the fit LQ curves to pass through a SF of one. Statistical tests on the data normalized in this manner often resulted in slightly different P-values from the P-values obtained for the data normalized strictly to the priming dose SF. For the Sk-Mel3 cell line, these differences were minor. However, for the T47D cell

Figure 5.3.1.1. Effect of preirradiation on clonogenic survival of Sk-Mel3 cells. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction between 4 to 12 experiments and the error bars are SEM. The curves are normalized to the survival following the priming dose.

Table 5.3.1.1. Comparison between the surviving fractions in Sk-Mel3 cells when they are given a priming dose 6 h before the challenge dose, and when the cells are not primed prior to receiving the challenge dose. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given priming dose x, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF at 2Gy	.944±.073	.894±.099	.920±.044	.809±.047	.873±.044	.649±.037
P _{value}		.68	.79	.16	.43	.0034
ratio		.934	.974	.857	.925	.687
SF at 4Gy	.576±.028	.560±.064	.583±.049	.502±.059	.572±.023	.452±.027
P _{value}		.80	.90	.24	.93	.009
ratio		.972	1.01	.872	.994	.785
SF at 6Gy	.391±.036	.368±.040	.313±.018	.344±.032	.396±.042	.259±.024
P _{value}		.70	.094	.41	.93	.010
ratio		.941	.800	.880	1.01	.663
SF at 8Gy	.259±.020	.246±.028	.198±.020	.172±.014	.194±.013	.125±.014
P _{value}		.70	.038	.0027	.013	<.0001
ratio		.95	.75	.66	.75	.48

line discussed in section 5.3.3, normalization of the data to allow the fit LQ curves to pass through a SF of 1, as opposed to simply normalizing the data to the survival following the priming dose alone, occasionally resulted in a difference between the adaptive response being significant and not significant. It was decided that statistical analysis would be calculated on the data normalized to the SF following the priming dose because there was no evidence that the linear quadratic model, from which our survival curves were fit, is valid in the case of adaptive response. It is possible that some parameter may have to be added to the LQ equation to account for any increase (or decrease) in survival observed when the cells are primed prior to the challenge dose. For instance, the α and/or β component in the LQ model may be dose dependent in the case of adaptive response curves.

The HT144 radiosensitive cell line showed considerable variability between individual experiments. The survival curves for the primed versus unprimed cells for one individual experiment are illustrated in Figure 5.3.1.2. Table 5.3.1.2 lists the mean SF and SEM for three replicate plates with challenge doses of 1, 2 and 3 Gy following each priming dose. Cells that received a priming dose of X-rays 6 hours prior to the challenge dose appeared to have an increased resistance. Significantly different ($P < .05$) survival values from those of the single treatments were obtained for many X-ray challenge doses and various priming doses. The maximum increase in survival (2.8-fold increase) occurred with a priming dose of 0.5 Gy 6 hours prior to a challenge dose of 2 Gy.

However, this effect was variable in this cell line for not all experiments showed an adaptive survival response. When the mean SF and SEM were calculated from the

Figure 5.3.1.2. Effect of preirradiation on clonogenic survival of HT144 cells obtained from one experiment. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 3 plates and the error bars are the SEM between plates. The curves are normalized to the survival following the priming dose.

Table 5.3.1.2. Comparison between the surviving fractions in a single HT144 experiment when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_1	.092 $\pm$ .006	.138 $\pm$ .009	.156 $\pm$ .020	.102 $\pm$ .007	.218 $\pm$ .004	.143 $\pm$ .015
P _{value}		.011	.039	.32	<.0001	.032
ratio		1.51	1.69	1.11	2.37	1.56
SF_2	.018 $\pm$ .002	.018 $\pm$ .002	.051 $\pm$ .006	.036 $\pm$ .001	.033 $\pm$ .001	.019 $\pm$ .005
P _{value}		.89	.0002	.0009	.002	.58
ratio		1.02	2.86	2.04	1.88	1.05
SF_3	(3.2 $\pm$ .6)E-3	(7.9 $\pm$ .9)E-3	(4.3 $\pm$ .5)E-3	(7 $\pm$ 1)E-3	(2.4 $\pm$ .7)E-3	(3 $\pm$ 1)E-3
P _{value}		.010	.26	.036	.041	.74
ratio		2.45	1.45	2.11	.73	.82
SF_4	(5.4 $\pm$ .6)E-4	(1.1 $\pm$ .1)E-3		(5.8 $\pm$ .5)E-4	(4 $\pm$ 2)E-4	
P _{value}		.0012		.70	.40	
ratio		2.03		1.06	.700	

Figure 5.3.1.3. Effect of preirradiation on clonogenic survival of the average of 3 to 6 HT144 experiments. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction and the error bars are the SEM between experiments. The curves are normalized to the survival following the priming dose.

Table 5.3.1.3. Comparison between the average surviving fractions in the HT144 experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_1	.103±.023	.219±.053	.129±.023	.178±.049	.207±.010	.097±.045
P_{value}		.12	.46	.24	.015	.91
ratio		2.13	1.26	1.73	2.01	.95
SF_2	.030±.004	.042±.006	.030±.006	.026±.003	.031±.009	.015±.002
P_{value}		.13	.96	.57	.86	.066
ratio		1.42	1.01	.88	1.06	.50
SF_3	(6±1)E-3	(6.1±.9)E-3	(3.9±.3)E-3	(5.8±.5)E-3	(3.2±.8)E-3	(3.2±.7)E-3
P_{value}		.88	.25	.79	.29	.28
ratio		.95	.61	.91	.50	.49
SF_4	(1.1±.6)E-3	(5±2)E-4	(3.1±.1)E-4	(4±.1)E-4	(4±2)E-4	
P_{value}		.28	.40	.34	.43	
ratio		.43	.29	.35	.35	

average of 3 to 6 experiments, there was no sign of an adaptive response (Figure 5.3.1.3 and Table 5.3.1.3). The number of experiments performed depended on the challenge dose given. Six experiments were performed using a challenge dose of 2 Gy following all 5 priming doses while only 3 experiments were conducted for all other challenge doses given after the various priming doses. For a challenge dose of 1 Gy and priming doses from 0.2 to 2 Gy, the primed cells still showed an increase in survival over the unprimed cells, but it was not considered significant. As the challenge dose was increased, cell sensitization was observed for most priming doses rather than an adaptive survival response.

5.3.2 Breast Carcinoma Cell Lines

Five separate experiments were performed on the T47D cell line and each individual experiment showed significant survival enhancements when primed with conditioning doses less than 4 Gy. Figure 5.3.2.1 and Table 5.3.2.1 show the increase in survival obtained in a single experiment. This experiment was selected because it showed the greatest degree of adaptation seen in this cell line. All five priming doses produced an increase in cell survival over the unprimed case. Although there was variation in the amount of induction observed between experiments for each priming dose, this experiment demonstrated the degree of adaptation that could be observed among experiments with this cell line.

When the data from all five experiments were pooled together, priming doses from 0.2 to 2 Gy still produced an increase in survival over what was observed when the

Figure 5.3.2.1. Effect of preirradiation on clonogenic survival of one T47D experiment. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 3 to 6 plates and the error bars are the SEM between plates. The curves are normalized to the survival following the priming dose.

Table 5.3.2.1. Comparison between the surviving fractions in one T47D experiment when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_1	.677 $\pm$ .062	.797 $\pm$ .031	.821 $\pm$ .046	1.08 $\pm$ .07	.683 $\pm$ .004	.729 $\pm$ .040
P_{value}		.16	.13	.012	.93	.52
ratio		1.18	1.21	1.59	1.01	1.07
SF_2	.451 $\pm$ .031	.462 $\pm$ .019	.544 $\pm$ .026	.773 $\pm$ .002	.469 $\pm$ .015	.432 $\pm$ .003
P_{value}		.77	.084	.0005	.63	.57
ratio		1.02	1.21	1.71	1.04	.96
SF_4	.093 $\pm$ .011	.125 $\pm$ .005	.189 $\pm$ .006	.242 $\pm$ .006	.137 $\pm$ .006	.115 $\pm$ .005
P_{value}		.054	.0014	.0003	.022	.12
ratio		1.35	2.03	2.60	1.48	1.24
SF_6	.020 $\pm$ .002	.033 $\pm$ .003	.051 $\pm$ .003	.061 $\pm$ .003	.031 $\pm$ .003	.032 $\pm$ .004
P_{value}		.015	.0014	.0003	.030	.045
ratio		1.71	2.61	3.1	1.58	1.65

Figure 5.3.2.2. Effect of preirradiation on clonogenic survival of the average of five T47D experiments. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 5 experiments and the error bars are the SEM between experiments. The curves are normalized to the survival following the priming dose.

Table 5.3.2.2. Comparison between the surviving fractions in the average of the T47D experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_1	.627 $\pm$ .025	.757 $\pm$ .035	.798 $\pm$ .046	.76 $\pm$ .16	.795 $\pm$ .049	.74 $\pm$ .11
P_{value}		.016	.012	.31	.016	.31
ratio		1.21	1.27	1.22	1.27	1.17
SF_2	.409 $\pm$ .019	.472 $\pm$ .027	.599 $\pm$ .099	.580 $\pm$ .083	.506 $\pm$ .083	.414 $\pm$ .029
P_{value}		.092	.097	.059	.29	.87
ratio		1.16	1.46	1.42	1.24	1.01
SF_4	.131 $\pm$ .014	.142 $\pm$ .006	.139 $\pm$ .016	.168 $\pm$ .031	.157 $\pm$ .020	.122 $\pm$ .016
P_{value}		.49	.70	.28	.31	.69
ratio		1.08	1.06	1.28	1.20	.94
SF_6	.025 $\pm$ .002	.036 $\pm$ .006	.038 $\pm$ .008	.039 $\pm$ .009	.032 $\pm$ .007	.019 $\pm$ .006
P_{value}		.15	.13	.14	.37	.37
ratio		1.38	1.52	1.59	1.31	.78

cells were not primed (Figure 5.3.2.2). However, this increase was only significant with priming doses from 0.2 to 2Gy and a challenge dose of 1Gy (Table 5.3.2.2).

To test if a larger sample size would improve the statistical significance, the data were reanalyzed with the assumption that each individual petri dish was an independent result. Recall that when the experiments were performed, three to six petri dishes were plated for each flask given an inducing and challenging dose of radiation. Assuming that each petri dish was an independent sample, the surviving fraction was calculated for each dish and then the average surviving fraction of all the dishes from all experiments was calculated. The resulting survival curves showed a statistically significant increase in survival when the cells were primed (Figure 5.3.2.3 and Table 5.3.2.3). This was due to the larger sample size which reduced the error bars on the data points. The problem with this method is that the T-test requires independent samples. The replicate petri dishes that originated from a given flask were only partially independent. It was only after trypsinization that the cells destined for each of the three petri dish were manipulated independently. Although it would have been ideal to repeat these experiments many times in order to obtain a large sample size appropriate for statistical analysis, this was impractical.

In three separate experiments, T47D cells were primed with 0.5 Gy and challenged with a dose of 4 Gy at various times following the priming dose to determine if there was an optimum time for the initiation of an adaptive response. In hind sight, a challenge dose of 2 Gy would have been more appropriate due to the large increase in survival observed at this dose with a priming dose of 0.5 Gy. The survival data,

Figure 5.3.2.3. Effect of preirradiation on clonogenic survival of T47D cells when each petri dish is treated as an independent experiment. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 12 to 18 plates and the error bars are the SEM between plates from all experiments. The curves are normalized to the survival following the priming dose.

Table 5.3.2.3. Comparison between the surviving fractions in T47D experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. Each petri dish is treated as an independent experiment. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_1	.627 $\pm$ .026	.756 $\pm$ .036	.798 $\pm$ .028	.764 $\pm$ .085	.795 $\pm$ .031	.735 $\pm$ .058
P_{value}		.0067	.0001	.075	.0003	.082
ratio		1.21	1.27	1.22	1.27	1.17
SF_2	.409 $\pm$ .015	.599 $\pm$ .054	.579 $\pm$ .048	.516 $\pm$ .047	.472 $\pm$ .024	.414 $\pm$ .021
P_{value}		.0022	.001	.033	.033	.82
ratio		1.16	1.46	1.42	1.26	1.01
SF_4	.131 $\pm$ .008	.142 $\pm$ .005	.132 $\pm$ .013	.168 $\pm$ .017	.157 $\pm$ .012	.122 $\pm$ .009
P_{value}		.26	.95	.046	.075	.49
ratio		1.08	1.01	1.28	1.20	.94
SF_6	.025 $\pm$ .001	.034 $\pm$ .003	.038 $\pm$ .004	.039 $\pm$ .005	.032 $\pm$ .004	.019 $\pm$ .003
P_{value}		.0072	.0021	.0023	.06	.11
ratio		1.38	1.52	1.59	1.31	.78

Figure 5.3.2.4. Surviving fraction of T47D cells given a challenge dose of 4 Gy various times after being given a priming dose of 50 cGy.

normalized to the inducing dose, were plotted as a function of time (Figure 5.3.2.4). The survival increased over the first 1-2 hours by a factor of 1.6 relative to the case where the inducing dose and challenge dose were given simultaneously, and then leveled off. When the two doses were separated by 8 hours, the survival increased slightly, and then reached plateau again. The adaptive response was evidently initiated within the first 2 hours following the priming dose and did not fade away within 24 hours.

The HTB30 cell line, when primed with various conditioning doses, did not show an adaptive response in the average of 3 independent experiments (Figure 5.3.2.5 and Table 5.4.2.4). More often than not, the inducing dose caused a reduction in survival rather than the expected increase characteristic of an adaptive response.

5.3.3 Human Fibroblast Cell Lines

The OMB18 cell line showed the same sort of variability between experiments that the HT144 melanoma cell line exhibited. In a single experiment, priming doses of 0.5, 1 and 2 Gy caused an increase in survival over the unprimed case at challenge doses of 2 and 4 Gy (Figure 5.3.3.1 and Table 5.3.3.1). A priming dose of 0.2 Gy was not large enough to elicit the adaptive response and a priming dose of 4 Gy was too large to condition the cells. A priming dose of 2 Gy resulted in the largest adaptive response. When the results from this experiment were averaged with the results from two other experiments that in themselves were not significant, the statistically significant increase in survival that was observed in the single experiment essentially disappeared (Figure 5.3.3.2 and Table 5.3.3.2). The 1 and 2 Gy inducing doses still tended to cause an enhancement in cell survival, although it was not shown to be statistically significant, but

Figure 5.3.2.5. Effect of preirradiation on clonogenic survival of three HTB30 experiments. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction between experiments and the error bars are SEM. The curves are normalized to the survival following the priming dose.

Table 5.3.2.4. Comparison between the surviving fractions in HTB30 experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose				
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy
SF_1	.831 $\pm$ .074	.95 $\pm$ .25	.548 $\pm$ .015	.854 $\pm$ .086	.890 $\pm$ .070
P_{value}		.66	.02	.84	.59
ratio		1.14	.66	1.03	1.07
SF_2	.600 $\pm$ .089	.465 $\pm$ .075	.473 $\pm$ .075	.409 $\pm$ .025	.478 $\pm$ .016
P_{value}		.31	.34	.11	.25
ratio		.78	.79	.68	.80
SF_4	.140 $\pm$ .009	.133 $\pm$ .030	.116 $\pm$ .026	.148 $\pm$ .035	.122 $\pm$ .025
P_{value}		.83	.43	.84	.53
ratio		.95	.83	1.05	.87
SF_6	.020 $\pm$ .003	.027 $\pm$ .011	.027 $\pm$ .010	.014 $\pm$ .002	.013 $\pm$ .004
P_{value}		.60	.59	.20	.22
ratio		1.32	1.30	.69	.65

Figure 5.3.3.1. Effect of preirradiation on clonogenic survival of OMB18 cells obtained from one experiment. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 3 plates and the error bars are the SEM between plates. The curves are normalized to the survival following the priming dose.

Table 5.3.3.1. Comparison between the surviving fraction in one OMB18 experiment when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed. '-' indicates no P-value could be calculated due to insufficient data.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_2	.131 $\pm$ .023	.179 $\pm$ .025	.249 $\pm$ .031	.319 $\pm$ .013	.461 $\pm$ .013	.086 $\pm$ .008
P _{value}		.23	.038	.002	.0002	.047
ratio		1.36	1.90	2.43	3.51	.64
SF_4	.014 $\pm$ .002	.021 $\pm$ .006	.036 $\pm$ .007	.041 $\pm$ .008	.069 $\pm$ .014	.015 $\pm$ .003
P _{value}		.28	.035	.028	.019	.70
ratio		1.53	2.65	3.01	5.08	1.10
SF_6	(1.5 $\pm$ .4)E-3	(1.2 $\pm$ .6)E-3	4.0E-3	1.2E-3	(1.7 $\pm$ .6)E-3	8.1E-4
P _{value}		.73	--	--	.86	--
ratio		.82	2.61	.79	1.10	.53

Figure 5.3.3.2. Effect of preirradiation on clonogenic survival of the average of 2 to 3 OMB18 experiments. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction of 2 to 3 experiments and the error bars are the SEM between plates. The curves are normalized to the survival following the priming dose.

Table 5.3.3.2. Comparison between the surviving fractions in the average of the OMB18 experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose					
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy	4Gy
SF_2	.25 $\pm$ .11	.161 $\pm$ .017	.239 $\pm$ .010	.351 $\pm$ .028	.416 $\pm$ .030	.203 $\pm$ .072
P _{value}		.54	.96	.34	.17	.76
ratio		.66	.97	1.43	1.69	.83
SF_4	.039 $\pm$ .026	.024 $\pm$ .004	.046 $\pm$ .009	.063 $\pm$ .011	.084 $\pm$ .019	.031 $\pm$ .009
P _{value}		.62	.84	.40	.24	.73
ratio		.62	1.16	1.6	2.14	.78
SF_6	(5 $\pm$ 3)E-3	(2 $\pm$ 1)E-3	(4.7 $\pm$ .7)E-3	(1.0 $\pm$ .6)E-3	(1 $\pm$ 1)E-2	(3 $\pm$ 2)E-3
P _{value}		.54	.96	.52	.60	.67
ratio		.47	.96	2.09	2.69	.67

the 0.5 Gy priming dose no longer elicited an adaptive response in the cells. The survival curve for the 4Gy priming dose showed a deviant point at the 3 Gy challenge dose. This was observed in 2 out of 3 experiments and was probably due to the very low colony counts in these plates that resulted from not plating enough cells.

The S11358 cell line was similar to the Sk-Mel3 and HTB30 cell lines in that cells given a priming dose did not show an increase in survival over unprimed cells (Figure 5.3.3.3 and Table 5.3.3.3). Due to low plating efficiencies and very high passage numbers for this cell line, these experiments were only performed two times.

5.4 Discussion

The present study was undertaken to investigate whether a small, initial dose of ionizing X-radiation could increase the radioresistance of cells to a second, challenge dose of X-radiation given some time later.

Although our findings in the SK-Mel3, HTB30 and S11358 cell lines contradicted the observed increase in cellular survival observed by many others (2-4,8-10,14,15,23-25,83), the literature did suggest that not all cells could be adapted. (5,9,10,25,86). The HT144 and OMB18 cell lines showed that in a couple of independent experiments, prior exposure to a low dose of radiation reduced cell killing by a subsequent dose of X-rays. However, this phenomenon was not observed in all experiments. One can speculate that this observed variability was due to some unknown parameter that was not being properly controlled. Others have also noticed variability in results between experiments without any definitive reason (49). Perhaps some stress on the cells brought on by sub-optimal growth conditions, such as slight variability in the

Figure 5.3.3.3. Effect of preirradiation on clonogenic survival of two S11358 experiments. Cells were either non-irradiated or irradiated with various priming X-ray doses 6 h before the graded challenge doses. Each point represents the average surviving fraction and the error bars are the SEM. The curves are normalized to the survival following the priming dose.

Table 5.3.3.3. Comparison between the surviving fractions in S11358 experiments when the cells are given a priming dose 6 h before the challenge dose 'x', and when the cells are not primed prior to receiving the challenge dose. SF_x is the surviving fraction following challenge dose x. The effect of the priming dose has been normalized out. 'Ratio' is the ratio of SF between cells given a priming dose, and cells that were not primed.

	Inducing Dose				
	0Gy	0.2Gy	0.5Gy	1Gy	2Gy
SF_2	.177 $\pm$ .010	.247 $\pm$ .038	.226 $\pm$ .004	.235 $\pm$ .011	.158 $\pm$ .032
P_{value}		.22	.049	.064	.63
ratio		1.39	1.28	1.32	.89
SF_3	.075 $\pm$ .016	.187 $\pm$ .090	.093 $\pm$ .016	.071 $\pm$ .00008	.058 $\pm$ .011
P_{value}		.34	.46	.83	.49
ratio		2.51	1.25	.95	.78
SF_4	.025 $\pm$ .004	.019 $\pm$ .003	.023 $\pm$ .009	.017 $\pm$ .003	.015 $\pm$ .007
P_{value}		.35	.84	.22	.30
ratio		.75	.90	.65	.58

medium used to feed the cells, or temperature fluctuations or changes in CO₂ levels in the incubators masked the adaptive response in certain experiments. Perhaps it was some other unknown factor. Barquinero, who observed that occupational exposure to radiation induced an adaptive response in human lymphocytes, found the presence or absence of an AR varied in the same donor with time. He concluded that AR was not linked to the genetic constitution of the individual but depended on some unknown, transient, physiological parameter (87).

The T47D cell line demonstrated more consistently that cultured human cells respond to low doses of X-rays and induce some form of protective mechanism so that they are less susceptible to, or better able to deal with, the harmful effects of the subsequent higher dose. Priming doses from 0.2 Gy to 2 Gy resulted in an increased survival to the second dose of radiation. Several studies have reported that X-ray doses (2,18,23,83) or β ray doses (20,21) larger than a few centigray are incapable of inducing an adaptive response in human lymphocytes and Chinese hamster cells. On the other hand, other studies have found that larger priming doses (e.g. 0.1-0.5Gy for mS5 cells (8), 1.39 Gy with hypersensitive human fibroblasts GM498 cells (88) and 2 Gy in bone marrow cells (89)) are required to show a significantly enhanced survival to the challenge dose. One can speculate that the inducing dose needed to elicit the adaptive response is variable and dependent on the cell line. Various studies have suggested that both the dose and the dose rate must be manipulated to give rise to an adaptive survival response (9,22,25). Shadley, who worked with proliferating lymphocytes stimulated by PHA, found that the AR occurred with both high and low X-ray priming doses as long as the

high dose was given at a low dose-rate and the low dose was given at a high dose-rate (22).

In the work reported here for the T47D cell line, the largest level of protection using X-rays as a priming treatment was seen using conditioning doses of 0.5 Gy and 1 Gy at dose rates of 56.04cGy/min and 142.4cGy/min respectively. Perhaps the reason the 4 Gy dose was ineffective at initiating an AR was because it produced too many lesions in the DNA, leading to a saturation of the adapting effect that prevented the response from occurring. An alternative explanation is that the lesions may have interacted with each other to remove the inducing signal and then formed other types of damage that would not induce the response. A further possibility is that the high dose damaged the adaptive mechanism.

If the adaptive survival response is in fact critically dependent on dose and dose rate, as several studies claim (10,13,25), then one may speculate that the reason an adaptive response was not observed in the Sk-Mel3, S11358 and HTB30 cell lines was due to not hitting upon the proper dose and dose rate for these cell lines. However, due to the wide range of priming doses studied, as well as the 2 different dose rates used on these three cell lines, it seems fair to say that these cell lines are not inducible. The variability in the ability of our 6 different cell lines, from 6 different individuals, to exhibit an adaptive survival response is not unique. Indeed, the variable response among cell lines and among donors reported in the literature did not appear to be related to intrinsic radiosensitivity (18,24). For example, there was reported variability in lymphocytes from different individuals to exhibit an adaptive response (90,91).

The radioprotection afforded by the conditioning dose in the T47D cell line increased with the time interval between the first and second dose, and reached a plateau within a couple of hours without any signs of decaying away within 24 hours. Other studies have also shown that the radioprotection lasts up to 24 hours, but found that it decays away beyond such a time frame (2,3,8,14). Shadley found the AR in cycling lymphocytes persisted for about 40 hours (18). Boothman found that the adaptive survival response could be sustained for 2 to 3 days in HEP-2 or U1-Mel growth arrested cells, primed by low doses of ionizing radiation over a 4 day period before receiving the challenge dose, but this adaptive survival response was completely abolished within 5 days (9).

In the T47D cell line, and possibly the OMB18 and HT144 cell lines, it appears like the priming treatments produced an increase in radioresistance of the whole cell population, maybe by inducing a radioprotective mechanism. This type of induced resistance is not unprecedented. For example, small X-ray doses have been shown to induce transient protection against a second, higher dose in Chinese hamster cells (2,3) and human lymphocytes (2,3,18). Recent reports have shown that small doses of low-dose rate Co60 gamma rays (20,92), as well as low-energy beta rays from ^3H (19-21,27) protect human lymphocytes and Chinese hamster cells against subsequent doses of X-rays. The plating efficiency of unirradiated and neutron irradiated primate cells has been improved by previous exposure of the cells to small doses of gamma radiation (82,93). As well, low doses of radiation have had a stimulating effect on the growth of cultured cells in a limited dose range (0.02-0.2 Gy). Low doses have also

shown a stimulatory effect in the vital activity of an organism, on the mean life duration, fertility, body weight etc. and in stimulating cell division (25,94).

The triggering mechanism involved in this adaptive survival response has not yet been clarified. It has been speculated that DNA damage by free-radicals and low LET radiations might be particularly good at inducing radioprotection, and that single-strand breaks (ssb) might be candidates for such lesions. This has been supported by the fact that small neutron conditioning doses in yeast are less efficient than X-rays, at similar levels of cell kill, at producing an adaptive protection against subsequent large X-ray exposures (1,16). The number of ssb per unit of dose produced after high LET radiation, such as neutrons, has been reported to be lower than for low-LET radiations such as X-rays. Furthermore, at the very small conditioning doses (a few cGy) needed to produce the adaptive response seen in lymphocytes, only single-strand breaks or actual ionizations would have been abundant enough to be candidates for the inducing event. Further support of the notion that oxidative species or single-strand breaks are the inducers came from experiments that showed that small doses of hydrogen peroxide could induce protection against subsequent doses of ionizing radiation (8,14,16,23). While damage to DNA appears to be a strong candidate for triggering the adaptive survival response, this has yet to be proven. Radiation damage to other cellular structures, such as membranes, could also be important in initiating radioprotective signals.

It has been speculated that the adaptive response represents a modulation of repair processes, leading, perhaps, to increased DNA dsb rejoining or increased repair fidelity. This has been substantiated by evidence that small doses of radiation protect cells against

the mutagenic as well as the cell-killing effects of a subsequent exposure. The adaptive response is most likely mediated through the transient induction of a protein(s) involved in DNA repair because studies on human lymphocytes (14,95) have successfully used cycloheximide to block an adaptive response. The adaptive response has also been blocked by 3-aminobenzamide, indicating that repair pathways involving poly(ADP-ribose) polymerase (an enzyme involved in DNA strand-break rejoining) might be involved (17,21,24,27).

As Joiner pointed out in his "overview" papers on induced resistance, there are numerous examples of both protein upregulation and gene expression that occur within minutes to hours after small radiation exposures, and this would match the time scale needed to influence repair of radiation-produced DNA injury (2,3).

Radiation induced cell cycle delay has also been invoked to explain the observed adaptive survival response seen in our cell lines as well as in other studies. It has been proposed that gene transcripts which build up slowly in response to priming doses of radiation produce proteins that regulate or control cell division to "poise" cells in a certain state. The delay in cycle progression would then allow more time to complete high-fidelity repair of DNA damage by constitutive repair processes before replication or division fixed the unrepaired lesions. The clonogenic assay could then be used to detect an enhancement in cell survival.

In summary, the results from the 6 cell lines studied showed that the phenomenon known as "adaptive survival response" was only observed in some cell lines and could be dependent on the dose and dose rate used for the conditioning dose. It is known that

molecular processes can be up-regulated on a time scale of minutes to hours after small radiation exposures and this would match the time scale needed to influence repair of radiation-produced DNA injury. However, it still remains to be determined what, exactly, is the link between these molecular mechanisms and the ultimate fate of the cell following irradiation.

6.0 Potentially lethal damage repair experiments

6.1 Introduction

The way in which cells are treated following irradiation can affect the fraction of cells that survive after a given X-ray dose due to the expression or repair of potentially lethal damage (PLD). PLD is damage which leads to cell death if left unrepaired, but which is not lethal if post-irradiation conditions are such that they allow for the repair of PLD (47). Allowing cells to cycle generally fixes PLD, reducing the proportion of cells surviving the radiation dose. The word 'fix' is used in the sense of making something permanent, not in the sense of repairing damage. If post-irradiation conditions are suboptimal for growth, so that cells are non-cycling and therefore do not undergo mitosis while their chromosomes are damaged, repair of PLD occurs and the proportion of surviving cells increases. Little and others, who studied PLD repair (PLDR) in density-inhibited plateau phase cell cultures, showed that cell survival was enhanced considerably when the cells were allowed to remain in a density inhibited state for 6 to 12 hours post-irradiation before being subcultured and assayed for colony forming ability (32,33,38-41,47). The magnitude and kinetics of recovery from PLDR correlated well with the rejoining of DNA strand breaks, leading to the interpretation of PLD recovery as resulting from repair of this DNA lesion (31,42,43,45,46).

The experiments described in this section measured the capacity of the six cell lines (2 human melanoma, 2 human breast carcinoma and 2 human fibroblast) to recover from radiation damage when the cells were held in plateau phase for a post-irradiation incubation period of 6 hours. 6 hours was chosen as the time interval following

irradiation before trypsinization because the repair of PLD typically reaches a plateau by 6 hours. These experiments were conducted to determine if there is a relationship between the presence, or absence, of an adaptive survival response and the ability of cells to repair of PLD. The relationship between intrinsic radiosensitivity and PLDR was also studied.

6.2 Method

For delayed-plating experiments, five T-25 flasks of confluent cells were irradiated on ice with a single dose of X-radiation (0.2Gy, 0.5Gy, 1 Gy, 2 Gy and 4Gy). Immediately following irradiation, the flasks were placed in the incubator for 6 hours before being trypsinized and assayed according to the colony forming assay described in section 2.2.2. Another 4 flasks were irradiated on ice with single graded doses of X-radiation ranging from 1 to 8 Gy (depending on the sensitivity of the cells), and were then immediately trypsinized and assayed in the same manner as the delayed plating flasks. 2 control flasks were sham irradiated and one was plated immediately while the other was incubated for 6 hours before being assayed. Survival after immediate plating (IP) could then be compared with survival after plating delayed (DP) for 6 h, which allowed time for PLD repair.

6.3 Results

The results are divided into three sections according to the tissue type. The survival data from the average of all experiments within a given cell line was plotted as a function of dose with the error bars representing the standard error of the mean between experiments.

6.3.1 Human Melanoma Cell Lines

Due to the small doses used in the delayed plating experiments in the Sk-Mel3 cell line, it was impossible to determine whether PLDR occurred (Figure 6.3.1.1). However, because the PLD response in the Sk-Mel3 cell line had been previously reported to show PLDR at large doses and a response similar to what we observed at low doses (52,53,84), these experiments were not repeated.

In the HT144 cell line, PLDR was observed and the amount of repair increased with increasing dose (Figure 6.3.1.2). The recovery ratio (RR_{DP}), defined as the ratio of surviving cell fractions following delayed plating after irradiation vs. immediate plating, increased with increasing dose. The RR_{DP} with an acute 2 Gy dose was $1.69 \pm .27$ and the RR increased to 4.2 ± 1.2 for an acute dose of 4 Gy.

6.3.2 Human Breast Carcinoma Cell Lines

The T47D cell line also demonstrated the ability to repair PLD (Figure 6.3.2.1). Repair capacity clearly increased with increasing dose. The RR_{DP} for this cell line at doses of 1, 2 and 4 Gy were $1.345 \pm .089$, $1.64 \pm .11$ and $2.40 \pm .18$ respectively. The HTB30 cell line likewise repaired PLD if incubated for 6 h following irradiation before being subcultured (Figure 6.3.2.2). The RR_{DP} for this cell line at doses of 1, 2 and 4 Gy were $1.10 \pm .11$, $1.28 \pm .19$ and $2.23 \pm .45$ respectively.

Figure 6.3.1.1. Repair of PLD in Sk-Mel3 cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

Figure 6.3.1.2. Repair of PLD in HT144 cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

Figure 6.3.2.1. Repair of PLD in T47D cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

Figure 6.3.2.2. Repair of PLD in HTB30 cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

6.3.3 Human Fibroblast Cell Lines

The OMB18 cell line clearly demonstrated a large capacity for repair of PLD (Figure 6.3.3.1). Survival enhancement ratios of $2.69 \pm .58$ and 8.8 ± 3.7 were seen for the OMB18 cell line using doses of 2 and 4 Gy respectively with 6 hour delayed subculture. The S11358 cell line, on the other hand, showed no PLDR (Figure 6.3.3.2). In the case of the fibroblasts, the radiosensitive cell line lacked the ability to repair PLD while the normal cell line had a large capacity to repair such damage.

6.4 Discussion

PLD is damage that is normally lethal to the cell unless post-irradiation conditions are such that repair is facilitated. As was described by Iliakis, the exposure of cultured mammalian cells to a variety of post-irradiation conditions such as cycloheximide, low temperatures, incubation in a balanced salt solution and incubation with certain metabolic inhibitors, can enhance survival in the post-irradiation period (42). Maintaining cells in a density inhibited state following irradiation was also found to increase survival following irradiation. All of these treatments are associated with a slowing down of the progression of the cells through the cell cycle. Because the fraction of quiescent cells in a solid tumour is non-negligible, it was suggested that plateau-phase cultures, which contain a considerable proportion of non-dividing cells, could be a more relevant model for tumours in vivo, and that the radiosensitivity of a tumour could not be accurately predicted on the basis of the single cell survival curve alone without taking into consideration the amount of PLDR which occurred after each exposure. Wiechselbaum suggested that PLDR proficient tumours might be the cause of radiotherapeutic failure

Figure 6.3.3.1. Repair of PLD in OMB18 cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

Figure 6.3.3.2. Repair of PLD in S11358 cells after various doses of X-radiation. Cells were treated in plateau phase then were either harvested immediately following irradiation or were allowed to repair for 6 h prior to trypsinization.

(36). He based this on the observation that 7/10 of human tumour lines most proficient in PLDR were derived from tumours which either failed radiotherapy or were associated with a high probability of failure. He suggested that because the extent of PLDR may vary significantly between cell lines, the ability to repair PLD following treatment might serve as a possible predictor of tumour response to radiotherapy. However, future work by others suggested that this was not a good predictor.

There was considerable variability in the ability of each of the cell lines to repair PLD. After low doses, the HT144 cell line repaired more damage than the Sk-Mel3 cell line. At isosurvival levels, these two cell lines were previously shown to have similar recovery ratios of potentially lethal damage, although the HT144 cells repaired slightly more (84). The repair ratio in that study was defined to be the SF with 8 h delayed plating after irradiation divided by the SF with immediate plating when the cells were irradiated to isosurvival levels with doses of 10Gy and 2Gy for Sk-Mel3 and HT144 respectively. Under these conditions, the RR_{DP} 's were $2.1 \pm .3$ and $2.4 \pm .3$ for Sk-mel3 and Ht144 respectively. In the melanoma cell lines, there appeared to be no significant correlation between the in vitro radiosensitivity and the repair of PLD. Similar studies have also shown no correlation between PLDR and intrinsic radiosensitivity, or clinical radiosensitivity, in various tumour types (38,39). Although the HT144 cell line showed virtually no shoulder in the acute radiation survival curve, suggestive of very little SLDR, there was still considerable PLDR which suggests that there is little overlap between the mechanisms underlying the accumulation of SLDR and PLDR (33,52). There likewise appeared to be no correlation between PLDR and AR. Both melanoma cell lines repaired

PLD to the same extent (at isosurvival levels) yet the Sk-Mel3 cells exhibited cell sensitization when primed 6 h prior to the challenge dose, and the HT144 cells showed a variable response when primed; AR was observed in some experiments, but not in others.

The breast carcinoma cell lines had similar in vitro radiosensitivity and also repaired PLD to the same extent. As the dose increased, the recovery ratio also increased. It has been shown that RR_{DP} increases with dose according to a LQ exponential function in a variety of human tumour and fibroblast cell lines (96). Once again, PLDR was unrelated to AR because whereas the amount of PLDR was the same for these two cell lines, the T47D cells showed an AR when primed while the HTB30 cells did not.

The human fibroblasts showed a correlation between in vivo radiosensitivity and the ability to repair PLD, but no strong correlation between the amount of PLDR and the presence of an AR. The radiosensitive S11358 cell line showed no capacity to repair PLD, and no adaptive response when primed, whereas the normal OMB18 cell line showed a large PLDR capacity but no consistent adaptive survival response when primed prior to irradiation.

As was the case in these experiments, Deschavanne also noticed that the relationship between intrinsic radiosensitivity and PLDR in human tumours was different from that of nontransformed fibroblasts (39). He found that the magnitude of repair capacity for tumour cells was independent of their intrinsic immediate plating (IP) radiosensitivity, whereas it was proportional to the radiosensitivity of IP cells in fibroblasts. He attributed this observed difference to the fact that the proportion of non-cycling cells is generally lower in plateau-phase tumour cell cultures than in

plateau-phase fibroblasts. Also it has been suggested that there may be more variability in the proportion of quiescent cells from one tumour cell line to another (37-39). PLDR has been shown to be maximum in true plateau phase, where most of the cells are in G_1 or G_0 . The fact that normal fibroblasts have been reported to have a lower portion of cycling cells compared with tumours (37,39), may help to explain the large RR_{DP} observed in the normal OMB18 cell line compared to the tumours.

One must be careful in making deductions about repair capacity of cells based on PLD experiments. It has been shown that the amount of PLDR depends on the fraction of dsb repaired in cells under growth versus non-growth conditions. One may speculate that cell lines exhibiting only a small amount of PLDR in survival studies have a correspondingly small capacity to repair PLD. However, this may not be the case. The low levels of PLDR observed in some cell lines may not be due to a reduced capacity to repair PLD, but rather to an extensive repair of dsb under immediate plating conditions which reduces the difference between immediate and delayed plating survival curves.

Cox suggested that PLDR affected only the shoulder of the survival curve (44), Little and Hahn claimed PLDR was associated with a decrease in the slope of the survival curve, with no significant change in the shoulder (33,41). Deschavanne reported that both the alpha and beta parameters in the LQ model were decreased by PLDR, suggesting that both the direct damage effect (single hit damage) and the indirect damage effect (cumulative damage) undergo repair to a large extent (39). Our results agreed with this latter statement. The curves could not be fit to the LQ model due to the dip in the survival curves observed at low doses (Figures 6.3.1.1, 6.3.1.2, 6.3.2.1 and 6.3.3.2). The

α and/or β terms appeared to be dose dependent in these cell lines. This dip is similar to what has been observed in "induced resistance" experiments in which it appears as though low doses are more effective per unit dose than higher doses above some threshold level where a repair mechanism is triggered (1-7,13,14). Sections 1.1 and 5.1 elaborate more on this topic. Whether AR and induced resistance are manifestations of the same underlying mechanism is still under debate (2,3,5,7,23,82). The fact that the Sk-Mel3, S11358, HT144 and T47D cell lines all showed the induced resistance, while only the T47D (and some HT144 and OMB18) experiments showed an AR, leads one to speculate that these two phenomena are not manifestations of the same underlying mechanism.

7.0 Sub-Lethal Damage Repair Experiments

7.1 Introduction

Sub-lethal damage repair was initially observed by Elkind and Sutton who found that cell survival increased if a radiation dose was split into two or more fractions separated by a time interval.

Sub-lethal radiation damage (SLD) is damage that is not lethal to the cell on its own but which can become lethal if it is allowed to interact with similar damage from a further radiation treatment. Sub-lethal damage repair is typically referred to as the repair of radiation damage which occurs when a given dose of radiation is split into two fractions separated by a time interval. When a dose of radiation is delivered as two equal fractions separated by several hours, the fraction of cells surviving is greater than if the dose is given as a single exposure equal to the sum of the two doses. The fraction of cells killed by single-track damage (α component) is the same whether the dose is given in two fractions or as a single exposure. However, the multiple track damage may be repaired if the two chromosome breaks that must interact to form a lethal lesion occur hours apart so that the first break has time to be repaired before the second break occurs (47). In general, it has been found that there is a good correlation between the extent of repair of SLD and the size of the shoulder of the survival curve (31,33,46), although there are some exceptions.

SLDR results for in vitro systems are usually expressed as a recovery ratio (RR_{SD}), i.e. the ratio of survival observed when the doses are split by a time interval compared with that observed when the two doses are given simultaneously.

The experiments described in this section measured the capacity of the two breast carcinoma cell lines to recover from radiation damage when the dose was split into two equal fractions separated by varying time intervals. The experiments were performed to determine whether the presence or absence of an adaptive response is related to the repair of SLD. SLDR experiments were not performed on the melanoma cell lines because these results had been previously published (48). The published data is discussed in section 7.4. Due to high passage numbers and low plating efficiencies in the S11358 and OMB18 cell lines, SLDR experiments could not be performed on these cell lines.

7.2 Method

Confluent cultures of cells in flasks were irradiated on ice with two equal doses of 3 Gy separated by various times during which the cells were incubated at 37°C. The cells were trypsinized immediately following the second irradiation and assayed according to the colony forming assay described in section 2.2.2. A separate flask of confluent cells was sham irradiated to allow the plating efficiency of the cells to be determined, and another flask was given the two doses of 3 Gy simultaneously. Recovery ratios were calculated by dividing the survival after incubation at 37°C for the indicated times by the survival for no incubation.

7.3 Results

The SLDR results for both breast carcinoma cell lines were measured by determining the fraction of cells that survived 2 equal doses of 3 Gy, separated by various time intervals. The results were expressed as the recovery ratio versus time (Figure 7.3.1). Doses were chosen to reflect isosurvival between the two cell lines and because

Figure 7.3.1. The split dose response for T47D and HTB30 cells for doses of 3Gy+3Gy separated by incubation for various times. For the HTB30 curve, the data points represent the average of 4 experiments, and for the T47D curve, the data points represent the average of 3 experiments.

the two cell lines had similar HDR survival curves, the dose required to produce the same level of kill in the two cell lines was the same.

The SLDR capacity of the T47D cell line was larger than that of the HTB30 cell line. Recovery ratios after 8 h incubation were $3.96 \pm .41$ for T47D and $2.23 \pm .21$ for HTB30. The two cell lines had similar survival curves, with the T47D cells showing slightly increased survival over the HTB30 cells. The shoulder on the HTB30 survival curve was slightly larger (though not significantly so), yet the amount of SLDR in the T47D cell line was 1.77-fold higher than in the HTB30 cell line. If the shoulder of the survival curve reflects repair of SLD, then the HTB30 cells should have, if anything, shown more repair. RR_{SD} increased with time over the first 8 h and then reached plateau in the T47D cells. The HTB30 cells, on the other hand, showed an increase in RR over the first 2 h followed by a slight gradual decrease in RR over the next 14 h. The reason for this observed decrease in RR_{SD} with time is unknown. One hypothesis is that the HTB30 cells did not survive well in plateau phase and become senescent and unable to repair the radiation damage as effectively.

7.4 Discussion

The relative increase in survival that resulted from splitting a dose into 2 or more fractions separated by a variable time interval, was first observed in *Chlamydomonas* (a form of green algae) and was later investigated in mammalian cells. This increase in survival was due to repair of sub-lethal radiation damage in the time interval between irradiations before it had a chance to form a lethal lesion by interacting with subsequent SLD produced by the second dose.

After its discovery, SLDR was thought to be one of the most important factors influencing the curability of human cancers by radiation therapy. This was thought, in part, because fractionation protocols are often used in radiotherapy and this form of treatment allows SLDR between fractions. Several reports have suggested that the measurement of recovery from radiation damage could serve as a predictor of the response of tumours to radiation (50). It was hypothesized that cells from resistant tumours (such as glioblastoma multiforme) expressed a proficient recovery from radiation damage which would account for part of the radiation resistance observed in the clinic, and that extreme cellular radiosensitivity to ionizing radiation was the result of a deficiency in the repair of radiation-induced damage. However, some studies have shown no correlation between intrinsic radiosensitivity and recovery capacity (50).

It is generally thought that the SLDR capacity of cells is related to the radiation survival curve shoulder size. The survival curves for the two breast carcinoma cell lines were very similar, with the T47D cells showing only slightly increased survival over the HTB30 cells, and the HTB30 survival curves showing a slightly larger shoulder region (Table 4.3.1). If the shoulder on the survival curve does in fact reflect SLDR capacity, the T47D and HTB30 cells would be expected to repair SLD to the same extent, or perhaps slightly more in the HTB30 cells due to the slightly larger shoulder on the survival curve. However, the results indicate that the T47D cells repaired SLD to a greater extent than the HTB30 cells. There was a 1.77-fold increase in RR_{SD} of the T47D cells over the HTB30 cells. This suggests that it may not be possible to predict the capacity of a cell to repair SLD based on an acute, single dose survival curve.

The recovery ratios after 7 h incubation, previously published on the Sk-Mel3 and HT144 cell lines, were 95 for Sk-Mel3 (8Gy, 8Gy) and 3.4 for HT144 (1Gy, 1Gy) (48). The doses used reflected isosurvival between the two cell lines after the first dose. It was evident that the radioresistant Sk-Mel3 cells, characterized by a large shoulder in the survival curve, showed a large amount of SLDR. The HT144 cells, which had no shoulder region on the survival curve, had a much lower RR_{SD} , but were still able to repair sub-lethal radiation damage. This data supports the concept that the size of the survival curve shoulder may reflect SLDR capacity, but also shows that SLDR is still possible in the absence of a shoulder.

Although numerous studies have demonstrated a correlation between the presence of radiation-resistant and repair-proficient tumour cells with clinical failure, recent studies by Peacock et al. found that increased radiation sensitivity did not result from low recovery capacity (97,98). They found that cellular recovery was the greatest in cells of the most radiosensitive cell lines, i.e. cells with steep acute radiation survival curves. They claimed that a given radiation exposure induced greater numbers of irreparable and repairable lesions in sensitive cell lines compared to resistant lines. This might explain the SLD recovery observed in the HT144 experiments despite the steep acute radiation survival curve.

Direct comparison of the recovery capacity between the two melanoma and two carcinoma cells lines was difficult because the isosurvival level used between the melanoma experiments was different from that used between the breast carcinoma experiments. Because the repair that was assessed was that of the first dose interacting

with the second dose, SLDR was compared for isosurvival after the first dose only. Two fractions of 4 Gy, rather than 3Gy, would have to have been used in the breast carcinoma experiments to have the same isosurvival survival level as was used in the melanoma experiments.

Adaptive survival response did not appear to be related to the cell's ability to repair SLD. All four cell lines (2 breast carcinoma and 2 melanoma) showed repair of SLD, yet only the T47D cells showed an adaptive survival response when primed prior to receiving the challenge dose. The Sk-Mel3 cells, which showed the largest split dose survival enhancement, actually showed cell sensitization when primed prior to receiving the challenge dose, so the magnitude of the enhancement in survival in split dose experiments was not related to the existence of an adaptive response either.

8.0 Conclusions

There has been much discussion over the years about whether cells can induce radiation resistance. The adaptive response has been successfully observed in rodent cell lines and in human lymphocytes in the past, however, this response has been found to be variable between cell lines.

The ability of cells to express an adaptive survival response could affect the outcome of radiotherapy and could be important as far as radiation protection is concerned. If low doses of radiation can induce cells to become resistant to subsequent doses of radiation, then patients presenting with cancer who were previously exposed to radiation during an X-ray or mammogram could face treatment difficulties if the small radiation exposure induced an adaptive response in the cancer cells. Our studies have shown that clinical sized priming doses can also induce the radioprotective mechanism. This could affect the chance for tumour control or cure in patients presenting with a recurrent tumour, or patients treated with fractionated radiotherapy.

The existence of an adaptive survival response could also be beneficial as far as radiation protection is concerned. For example, if cells can be made resistant to radiation, then radiation workers, who are about to enter an area where they will be exposed to high levels of radiation, could be primed with a low dose of radiation in order to trigger their cells to become resistant to the harmful effects of the ionizing radiation they will subsequently receive. Other treatments such as drugs, hyperthermia etc. might also trigger this mechanism and could possibly be explored for their use in inducing radioresistance in radiation workers who are required to go into high radiation fields.

In this thesis, the effect of a priming dose of X-radiation given to cells prior to a challenge dose of X-radiation was studied. This type of experiment was designed to look for an adaptive survival response, initiated by the priming dose and manifested as an increase in survival following the challenge dose of radiation. Cell lines from three different types of tissue were studied to see if the presence of an adaptive survival response was dependent on the type of tissue. As well, biopsies from two different patients were studied for each tissue type to determine if the adaptability of the cells varied between patients. We further studied potentially lethal damage repair as well as sub-lethal damage repair to determine if there was any correlation between the presence of an adaptive survival response and these forms of repair.

In this study, only one cell line, the T47D breast carcinoma cell line, showed an adaptive survival response consistently among experiments. The HT144 melanoma and the OMB18 human fibroblast cell lines showed an enhancement in survival when the cells were primed in only a single experiment. When the results from this experiment were averaged with the results from other experiments, the adaptive survival response disappeared.

In the T47D cell line, priming doses of 0.2Gy to 2Gy were able to turn on the radioprotective mechanism that allowed the cells to better withstand the second dose of radiation. A priming dose of 4Gy, however, was too large to induce the adaptive survival response. Perhaps the large priming dose produced too many lesions in the DNA, leading to a saturation of the adapting effect that prevented the response from occurring. An alternative explanation could be that the lesions interacted with each other to remove the

inducing signal by forming other types of damage that would not induce the response. Another possibility is that the high dose damaged the adaptive mechanism. The adaptive response that was initiated with a priming dose of 0.5Gy, did not decay away within 24 hours.

The adaptability of the cells was not found to be related to the type of tissue nor to the intrinsic sensitivity of the cell line. In the melanoma cell lines, the only evidence of an adaptive response was in one HT144 experiment. These cells were shown to be intrinsically radiosensitive in the single dose survival curves. In the fibroblast cell lines, the OMB18 cells, which were slightly more radioresistant than the sensitive S11358 cells in vitro, showed some evidence of adaptive response, but this was not consistent among experiments. The T47D cells were shown to have only a slightly larger intrinsic radioresistance than the HTB30 cells and were the only cells to consistently show enhanced radiation survival when primed prior to delivery of the challenge dose. The presence of an AR therefore did not appear to be related to intrinsic radiosensitivity.

The existence of an adaptive survival response was also not found to be correlated with the ability to repair potentially lethal damage. Five out of 6 cell lines were able to repair PLD, yet only one cell line manifested an adaptive survival response. It was also observed that the ability to repair PLD was not necessarily correlated to intrinsic radiosensitivity in melanoma cell lines because the HT144 cells, which showed extreme radiosensitivity, showed the same amount of PLDR as the Sk-Mel3 cells at a given isosurvival level. The breast carcinoma cell lines and fibroblast cell lines showed a correlation between intrinsic radiosensitivity and the ability to repair PLD.

Finally, enhanced survival to a challenge dose of radiation by pre-treatment with a priming dose several hours earlier, was not correlated to the repair of SLD. In the breast carcinoma cell lines, the T47Ds, which showed increased repair of SLD over the HTB30 cells, did show an adaptive response. However, in the melanoma cell lines, the cells that showed the least SLDR, the HT144 cells, were the cells that were occasionally adaptable. Furthermore, the cell line that demonstrated the largest amount of sub-lethal damage repair, the Sk-Mel3 cells, showed cell sensitization when primed rather than an adaptive survival response.

The well-known variation in radiosensitivity of cells in the different stages of the cell cycle may be invoked to explain the adaptive response phenomena. The argument is that the first dose of radiation would produce partial synchrony by eliminating cells predominantly in sensitive phases of the cell cycle. The surviving cells would then be in a more resistant phase of the cycle when the second dose of radiation was given. However, several pieces of evidence have ruled out this possibility. The cells used in these experiments were in plateau phase, so only a very small portion of cells would have been cycling. The adaptive response observed was in excess of any change in sensitivity that could be explained on the basis of cell-cycle progression between the doses. Furthermore, the adaptive response was observed with very small priming doses that themselves caused no cell kill. Joiner cited various examples of previous studies performed on lower organisms like green algae that have observed this same increase in survival when cells were primed (23). Another example he referenced showed that in the desmid *Closterium*, the increased radioresistance that resulted from priming the cells

could not be explained by the known variation in radiosensitivity of these cells within the cell cycle because the adaptive response was also observed when the cells were kept in darkness, hence in cell cycle arrest. The adaptive response was also inhibited by the presence of cycloheximide during the time between the two doses.

If the adaptive response was attributed to cell cycle progression, then the experiments that varied the time interval between the inducing and challenge doses should not have increased then reached plateau (Figure 5.3.2.2). Were the cells cycling and partially synchronized by the first radiation dose, one would have observed fluctuations in the surviving fraction with time, with increases and decreases in survival corresponding to times when the second dose was given to the cells in resistant and sensitive phases of the cell cycle respectively.

Much has yet to be clarified with regards to the sometimes observed increase in cellular survival that results when cells are primed with a low dose of radiation prior to receiving the challenge dose. The expression of the adaptive response in the T47D cell line was consistent between experiments so future work should look at what is occurring on a molecular level in this cell line. Experiments should focus on determining what genes are being expressed that allow for the enhanced survival to the challenge dose. Experiments that attempt to induce the adaptive response by other means, say with the use of hydrogen peroxide, heat or X-radiation of different LETs, could give some insight into the triggering mechanism that initiates the adaptive response.

Another question which remains unanswered is what is the cause of the variability observed between experiments in the OMB18 and HT144 cell lines. Was some

parameter not being properly controlled in these experiments? This is difficult to test in a large lab where many parameters are hard to control. For example, it is difficult to maintain the incubators at 37°C when numerous people are in and out of the incubators dozens of times in the run of a day. In any regard, the source of this variability should be investigated.

It still remains unknown whether adaptive response and induced resistance are manifestations of the same underlying mechanism. Performing single dose experiments in the low dose range on the T47D cell line could be a step toward answering this question. If the T47D cells do not show the low-dose hypersensitivity, then it is unlikely that the two mechanisms are related. In order to examine the radiation response of cells at very low doses (less than 1 Gy), the microscopic assay or the cell sorting assay must be employed because the conventional colony forming assay can not accurately measure radiation-produced cell death in the low dose range. The microscopic assay recognizes and counts cells individually while the cell sorting assay allows one to count and plate an accurately known number of cells.

Although the ability of cells to adapt to radiation could have severe consequences in terms of tumour control or cure, it is impractical at this time to use predictive assays to determine the adaptability of the cells. This is due, in part, to the lack of consistency in the ability of certain cell lines to exhibit an adaptive survival response. Also, because the adaptive survival response is variable between cell lines, the application of adaptive survival response to the field of radiation protection is also not feasible at this time. Even if it were possible to predict the potential for an adaptive survival response, not all tissues

in an individual would necessarily be able to express an adaptive response. Whereas one tissue may show enhanced survival if primed, another tissue may show cell sensitization. Evidently, much still remains to be determined with regards to what causes the adaptive survival response before it can be used in any practical applications.

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