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**Radiobiological aspects of cellular recovery following high and
low dose-rate irradiation with/without mild hyperthermia in a
human glioma cell model**

by

Dennis P. Heller

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

Doctor of Philosophy

Department of Physics
Carleton University
Ottawa-Carleton Institute of Physics
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**Radiobiological aspects of cellular recovery following high and low
dose-rate irradiation with/without mild hyperthermia in a human glioma
cell model**

submitted by

Dennis P. Heller, M.Sc.

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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Thesis Supervisor

External Examiner

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ABSTRACT

Despite current treatment modalities the prognosis for patients with malignant glioma is poor. The intrinsic radioresistance of these tumours is thought to play a major role in the failure of radiotherapy. A major component of intrinsic radioresistance is the cellular recovery from radiation damage.

One primary reason for the success of combined hyperthermia and radiation is thought to be the ability of hyperthermia to overcome radioresistance by inhibiting the cellular recovery of radiation damage. Recent clinical trials of simultaneous low dose-rate irradiation (LDRI) and mild ($<42^{\circ}\text{C}$) hyperthermia have shown great promise.

In this thesis, cell survival and recovery following radiation damage were measured under a variety of conditions in human glioma cells. Recovery during LDRI was examined in a non-proliferating cell population. Recovery was investigated following high dose-rate irradiation (HDRI) in proliferating and non-proliferating cell populations under various environmental and physiological conditions. The kinetic nature of this recovery was also measured. Lastly, the inhibition of recovery by mild hyperthermia was investigated.

A range in cell survival and recovery levels was observed in proliferating and non-proliferating cells by pre- and post-irradiation manipulation of the growth medium. The degree of metabolic and proliferative quiescence affected by a change in pH was

significant in determining the immediate and delayed plating survival levels, and the amount of recovery observed, with pre- and post-irradiation manipulation of the growth medium, respectively.

Recovery post-HDRI followed mono-exponential kinetics. The recovery half-time increased as a function of dose implying that the recovery processes were saturated ($T_{1/2}$ from 0.9 to 3.5 h for dose of 3.0 to 9.0 Gy). This increase was similar to the increase in the amount of recovery observed as a function of dose. At an iso-recovery level the rate of recovery was similar in proliferating and non-proliferating cells.

Cellular recovery from radiation damage was measured during LDRI and following HDRI. While the recovery in either case was maximal under the same conditions (non-proliferating cells, low pH, low metabolic activity), under alternate conditions the recovery was different.

A large degree of thermoradiosensitization was seen with concurrent 41°C heating and LDRI (TER of 1.5 to 2.1 at 1% survival level). Less sensitization was seen at 39 and 40°C. This study indicates that mild hyperthermia may be an effective inhibitor of cellular recovery which occurs during LDRI and following HDRI, only if applied while the recovery processes are taking place. This has implications in clinical trials in which hyperthermia is administered before or after irradiation, especially when only mild hyperthermic temperatures are achieved. It also supports the role of simultaneous mild hyperthermia as an effective way to improve brachytherapy.

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1.0 INTRODUCTION

1.1 BACKGROUND

Despite current treatment modalities (surgery, radiation therapy, chemotherapy) the prognosis for patients with malignant glioma is poor, with localized recurrence within a few years cited as the main cause for failure. While the lifetime probability of developing brain cancer is low ($\sim 1\%$), the lifetime probability of dying from the disease is virtually identical [1]. Glioblastoma multiforme (GM) and anaplastic astrocytoma (AA) are the two classifications of brain tumours that are the most difficult to eradicate, with the 5 year survival rate for GM being essentially zero [2,3,4].

Since brain tumours are typically unifocal, of limited size, and well circumscribed, radiation sources located in the tumour volume allow an increased total dose to the tumour with minimal dose escalation in the surrounding normal tissue. Interstitial radiotherapy or brachytherapy has yielded modest improvements in the time to recurrence especially for AA, but long term survival has not changed significantly [5,6,7]. A recent review has implicated the intrinsic radioresistance of these tumours as a major cause for failure with brachytherapy [2].

A major component of intrinsic radioresistance is the cellular recovery from sublethal and potentially lethal damage. Typical dose-rates employed in brachytherapy are 0.3 to 1 Gy/h. In this range of low dose-rates, increased cellular recovery occurs due to the repair of sublethal damage [8]. Recovery from sublethal damage may also occur

between dose fractions of a fractionated course of radiotherapy. It is thought that irradiating cell lines derived from human tumours at low dose-rates allows the assessment of recovery from sublethal damage and hence may reflect the intrinsic radiosensitivity of the original tumour and aid in the prediction of treatment outcome *in vivo* [9,10]. Low dose-rate studies of glioma cell lines indeed show that they are at the radioresistant end of the radiation sensitivity spectrum [11,12,13].

Many experiments that have measured the recovery from a portion of radiation damage termed potentially lethal damage have shown that cell cultures derived from human tumours express maximum recovery when in a stationary growth state (low cycling population) and surrounded by culture medium at low pH [14-17]. Many solid tumours are thought to include acidic (low pH) and nutrient deprived regions containing slowly proliferating cells [18]. However studies have shown a discrepancy between the amount of recovery from potentially lethal damage demonstrated *in vitro* and *in vivo* [15,19]. This may be related to a difference in the metabolic and/or proliferative status under which the two cell systems are studied, or due to low pH adapted cells *in vivo*. Despite these differences it has been argued that this type of recovery process also contributes to the resistance of human tumours to radiotherapy [10,14,20,21].

There are factors other than the recovery capacity of tumour cells that are of concern when considering the resistance of tumours to fractionation and low dose-rate protocols. Of prime importance are the rates of cellular recovery and

repopulation/proliferation in tumours compared to the surrounding normal tissue during the treatment period. Assessment of the kinetic nature of cellular recovery in both tumour and normal tissues is crucial in mathematical models which attempt to predict an optimal radiotherapy treatment protocol or the biological equivalence of different irradiation schedules [22-25].

Hyperthermia in combination with radiotherapy has been shown to be more effective than either modality alone, in its ability to successfully treat cancer. Both fractionated high dose-rate radiotherapy and brachytherapy treatment regimes have benefited from the addition of hyperthermia [26,27]. The effects of hyperthermia on cell survival *in vitro* have been extensively studied. It has been demonstrated that the cytotoxicity of hyperthermia is complementary to that of radiation [26,28]. One of the primary reasons for the potential efficacy of a combined hyperthermia/radiation treatment is thought to be the ability of hyperthermia to overcome radioresistance by inhibiting the cellular recovery of radiation damage. Many studies have shown that hyperthermia can inhibit the repair of DNA strand breaks [28-30], DNA polymerases [31,32] and the cellular recovery of sublethal and potentially lethal damage [33,34]. These studies have typically involved temperatures $> 42^{\circ}\text{C}$. Less is known about the mechanisms of interaction of heat and radiation at temperatures $< 42^{\circ}\text{C}$. Recently the mechanisms of combined mild hyperthermia and radiation has become a topic of great interest. The failure of many clinical trials that combined heat and radiation is thought to be due to the

failure to heat the tumour to high enough temperatures to fully realize the thermoradiosensitization of temperatures $> 42^{\circ}\text{C}$ [35,36]. In addition, recent clinical trials of simultaneous low dose-rate irradiation and mild ($\sim 42^{\circ}\text{C}$) hyperthermia have shown great promise [37,38]. The success in these recent trials may be related to the inhibition of sublethal damage during low dose-rate irradiation by mild hyperthermia

Obviously, cellular recovery plays a role in the resistance of human tumours to radiotherapy. Examining the cellular recovery of sublethal damage under low dose-rate irradiation conditions, the recovery of potentially lethal damage as a function of various physiological factors such as the rate of glycolysis, the rate of proliferation and pH, and examining the kinetics of sublethal and potentially lethal damage recovery, are major recommendations for ongoing research in radiotherapy [39].

1.2 SPECIFIC OBJECTIVES

The purpose of this research was to achieve the following objectives

- 1) Design and implement a low dose-rate cellular irradiator – Design considerations included the incubation of cell cultures at physiological and hyperthermic temperatures ($39\text{--}41^{\circ}\text{C}$), dose uniformity, and shielding for radiation protection.

- 2) Characterize the survival response of resistant tumour (glioma) cells in a stationary growth phase treated with high (acute), and low (chronic) dose-rate irradiation to establish a model of increased cellular recovery
- 3) Measure the kinetic nature of cellular recovery following high dose-rate irradiation of cells in different proliferative states to establish a relationship between radiation dose, recovery capacity, and the time to achieve this recovery.
- 4) Measure the recovery capacity of cells under different metabolic and proliferative states induced by several environmental stresses which may be present *in vivo* such as low glucose/high lactate levels, and high and low intercellular pH, to establish a range of recovery levels *in vitro* and investigate the dependence of recovery mechanisms on cell cycle distribution, pH, and metabolic activity.
- 5) Apply mild hyperthermia in conjunction with high and low dose-rate irradiation in various sequences and compare the levels of recovery to those achieved without hyperthermia to assess the effectiveness of this treatment as a recovery inhibitor with radioresistant tumour cells.

Investigation of these key aspects of cellular recovery in radiation resistant cell lines is vital to understanding the role of radioresistance in radiotherapy. In addition,

examining the ability of mild hyperthermia to inhibit cellular recovery is crucial to the advancement of the combined treatment modality of hyperthermia and radiotherapy.

1.3 THESIS OUTLINE

This thesis is comprised of nine chapters and two appendices. Each chapter is a comprehensive study which addresses a specific objective of the thesis.

Chapter 2 addresses objective two and introduces the Linear-quadratic model of cell survival and its usefulness as a measure of radiation sensitivity or resistance

Chapter 3 examines objective three. The kinetics of cellular recovery are quantified in plateau and exponential growth phase cells. The recovery half-time is resolved as a function of dose, and compared to the magnitude of recovery. The differences in the kinetics of sublethal and potentially lethal damage recovery are also addressed.

Objective four is investigated in chapters 4 and 5. Chapter 4 examines the range in recovery levels observed as cells progress from an exponential to a plateau growth phase. These recovery levels are correlated to pH and to the metabolic and proliferative states of the cell cultures. In addition recovery is measured following periods of metabolic stimulus prior to irradiation. Chapter 5 examines the effect on recovery of post-irradiation changes in proliferation and metabolic status.

Chapters 6 and 7 address the inhibition of recovery processes by mild hyperthermia (objective five). Chapter 6 examines the simultaneous combination of mild hyperthermia and low dose-rate irradiation. Various temperatures are investigated to establish a temperature threshold for thermoradiosensitization. In addition, the efficacy of pre-low dose-rate irradiation heating is examined. Chapter 7 tests the ability of mild hyperthermia to inhibit cellular recovery following high dose-rate irradiation.

Chapter 8 presents three complementary studies which relate to the studies in the previous chapters and enhance the significance of the findings.

Objective one is described in appendix I.

Due to the extensive scope of this thesis, each chapter excluding chapter 8 deals with a specific area of study with regard to cellular recovery, and is therefore comprised of 4 sections: introduction, materials and methods, results, and discussion. While the findings of each chapter are significant in their contribution to an aspect of cellular recovery, together they represent a comprehensive examination of the factors which govern cellular recovery in a radiation resistant cell line as well as address the ability of mild hyperthermia to inhibit this recovery. This is discussed in chapter 9 as are possible areas for future research.

2.0 LOW DOSE-RATE IRRADIATION AND THE LINEAR-QUADRATIC SURVIVAL MODEL

2.1 INTRODUCTION

When a population of cells is exposed to a dose of ionizing radiation, some individual cells may: A) not receive damage in a critical site or target and therefore are unaffected by the radiation, B) receive damage to all critical sites such that this damage becomes lethal as the cells attempt the next or a subsequent mitosis (lethal damage), or C) receive damage in some, but not all, of its critical target sites (sublethal damage). Given enough time, energy and nutrients, the cells may repair this damage and recover completely from its effects. However, misrepair or error-prone repair may also result which, in the majority of cases, leads to cell death after a subsequent mitosis [40]

In terms of repair there are two types of radiation damage which affect cell survival: 1) unreparable damage which is irreversible and leads to cell death, and 2) reparable damage which may or may not result in cell death. Repairable damage has been classified as both sublethal and potentially lethal. Sublethal and potentially lethal are terms which have been operationally defined by the type of experiment used to measure this damage [40,41]. Recently, Bedford has equated sublethal damage with non-lethal damage and potentially lethal damage with the interaction of several units of sublethal damage [41]. The relationship between sublethal and potentially lethal damage is discussed further in the next chapter.

Although there is no widespread agreement 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* [42]. The linear-quadratic (LQ) model assumes that there are two components of cell kill, one proportional to dose (D) and the other proportional to the square of the dose (D^2). This model stems from the work of Chadwick and Leenhouts [43,44] who claimed that the direct production of a double-strand break (dsb) leads to an irreversible lethal event (L). If the dsb is produced by a single charged particle track, the probability of L is related to dose. If a dsb arises from a combination of two independently induced single-strand breaks produced by uncorrelated particle tracks, then the probability of L is proportional to the dose squared. Thus L is given by

$$L = \alpha D + \beta D^2 \quad (1)$$

This LQ relationship has been correlated to the induction of chromosome aberrations following irradiation. In this analogy the yield of chromosome break-pairs is the measure of L. [41]. In either model, since the frequency of L per cell is an average number, a close approximation of the proportion of cells with an L of exactly 0,1,2 etc. is given by the Poisson formula. A cell will survive (S) if it contains no lethal events ($L=0$), and the probability of this according to the Poisson formula is,

$$P_0 = S = e^{-L} = e^{-(\alpha D + \beta D^2)} \quad (2)$$

The same LQ relationship to describe cell survival following irradiation was also derived by Kellerer and Rossi [45,46]. In their dual radiation action theory they correlated initial physical absorption events to biological actions. The dual radiation action theory predicts that α is independent of dose-rate while β varies with dose-rate. In all 3 derivations of the LQ survival model mentioned above, β is assumed to be dependent on a repair time factor.

The publication of a number of *in vitro* low dose-rate (LDR) survival response curves has stimulated radiobiologists and radiation oncologists to analyze the survival characteristics of these curves in order to predict the radioresponsiveness of the tumours from which they were derived. It has been shown that the differences in the relative radiosensitivity or resistance between various cell lines are better distinguished by the survival response measured at low dose-rates rather than at high dose-rates [47]. During low dose-rate irradiation (LDRI) increased cell survival occurs due to the recovery of sublethal damage, resulting in a survival response curve which is shallower than its high dose rate (HDR) counterpart [8,40]. Correlations have been made between clinically radioresistant tumour cell types and a shallow slope in the *in vitro* LDR survival curve [10,17,42,47,48].

In the LQ model α represents the inherent sensitivity of a cell to lethal events. A LQ fit of a mono-exponential survival curve results in β being equal to zero. Most human tumour cell lines irradiated at low dose-rates exhibit survival response curves which can be approximated by a mono-exponential function of dose. Under these conditions α corresponds to the limiting slope of the LDR survival response and represents the inherent sensitivity of the cell line to lethal events. For this reason α is thought to be an important parameter in assessing the radiosensitivity of tumours to fractionated and low dose rate therapy regimes [42,49].

If β is zero in the fit of a LDR survival curve, a value for α may be determined more accurately than from a HDR survival curve alone [50]. If β goes to zero as the dose-rate is lowered then the cells have expressed the maximum recovery of SLD. In this case, the β derived from the LQ fit of the HDR survival response of the same cell line is a measure of the recovery of radiation damage [42].

In this chapter the results of irradiating two human glioma cell lines at high and low dose-rates are reported. The cell lines were irradiated under stationary or plateau growth phase conditions. The resulting survival curves were fit to the LQ model. The significance of the results are discussed in terms of the recovery of radiation damage and the parameters of the LQ model. The importance of using plateau growth phase cells when measuring cellular recovery under low dose-rate conditions is also discussed.

2.2 MATERIALS AND METHODS

The cell lines U-87MG and U-373MG, were established from a human malignant glioblastoma and astrocytoma, respectively [51]. They were originally obtained from the American Type Culture Collection cell repository catalogue. The cells were grown in the medium combination DMEM:F-12 (1:1) (Sigma) supplemented with 7.5% Fetal Bovine serum (Gibco), 7.5% Newborn Calf serum (Gibco), 1mM MEM non-essential amino acids (Sigma), 10 mM sodium bicarbonate, and 20 mM Hepes. All cell cultures were incubated at 37°C in a humidified atmosphere of 2% CO₂ and 98% air.

For all experiments 4×10^3 cells per cm² were plated in 25 cm² flasks for HDRI and in 1.3 cm² glass vials for LDRI. The medium was changed (4 ml in flasks and 0.5 ml in vials) on days 4 and 7 post-plating with experiments commencing on day 10.

For HDRI, cells were irradiated on ice with a ⁶⁰Co therapy unit at a dose-rate of 150-200 cGy/min. The dose to the cells was determined by TLD dosimetry.

For LDRI, cells were irradiated by an array of ²²⁶Ra sources (total activity 800 mCi) contained in a temperature controlled incubator (See Appendix I). Two low dose-rates were used, LDR₁ at 0.88 cGy/min. and LDR₂ at 0.41 cGy/min. These dose-rates were determined by TLD dosimetry relative to a ⁶⁰Co reference protocol. For LDRI, accurate temperature control (± 0.1 °C) was achieved by monitoring thermocouples placed in the irradiation chamber.

Cell survival was assayed by the colony formation assay which is described in detail in appendix II. Briefly, after counting, the cell suspensions were appropriately diluted and plated in either 60 or 100 mm dishes, depending on the number of cells required, to achieve 30-80 colonies per dish. Fourteen days later, the cells were stained and surviving colonies containing > 50 cells were enumerated.

All data points for U-87MG results represent the average of a least 4 independent experiments while data points for U-373MG results represent the average of 2-3 independent experiments. Error bars on data points in figures 3 and 4 are shown only when they extend further than the data symbol. All uncertainty estimates ($\pm$ values) represent the inter-experimental Standard Error of the Mean (SEM).

2.3 RESULTS

Growth curves for U-87MG and U-373MG are shown in figures 1 and 2 respectively. Cell population doubling times calculated from the exponential portion of the growth curves were similar (28 ± 1 h) for both cell lines in either flasks or vials. This value is within the range of values reported for other glioma cell lines [52]. In vials the cells had a slightly lower plating efficiency upon seeding, and took a little longer to enter an exponential growth phase. It can be seen from figure 1 that changing the medium on days 4 and 7 resulted in a higher cell density as the cell population reached a plateau growth phase. This was desirable to insure a high percentage of non-cycling cells for experiments. Cell densities at plateau (day 10) were $1-1.5 \times 10^5$ and $2-2.5 \times 10^5$ per cm^2 for U-87MG and U-373MG, respectively.

Medium pH at the start of experiments for both cell lines was 6.7-6.8 in flasks and 6.9-7.0 in vials. The pH did not decrease by more than 0.1 unit during 2.5 days of HDR. In the colony formation assay, the plating efficiencies for these cells from either flasks or vials were approximately 25-35% and 18-25% for U-87MG and U-373MG, respectively.

Survival response curves for the two cell lines following HDR and LDR are shown in figures 3 and 4. They have been fitted to the linear quadratic (LQ) survival model expressed as:

$$\%S(D) = e^{-(\alpha D + \beta D^2)} \times 100 \quad (3)$$

Values for α and β were obtained by regression analysis of the linearized form of equation (3), namely:

$$\frac{-\ln S}{D} = \alpha + \beta D \quad (4)$$

The I.Q parameters are reported in table 1. The proportion of the variation in the dependent variable that is explained by the linear regression is given in table 1 by R^2 . R^2 compares the total sum of squares and the regression sum of squares. Note that while α is fairly constant, β tends toward zero as the dose-rate was lowered. The linearized form of the data is shown in figures 5 and 6. If β is assumed to be zero then the data may be fit to a simple linear exponential function of dose, namely:

$$\%S(D) = e^{-(\alpha D)} \times 100 \quad (5)$$

The survival data at low dose-rates expressed in the linearized form of this equation is shown in figure 7. The fits have been constrained through zero since survival at zero dose must be 100%. With this constraint the data at low doses does not coincide with the linear fit as well as the data at high doses. The value of α from these fits is also given in table 1. If β is assumed to be zero the values for α are greater than if β is not assumed to be zero.

2.4 DISCUSSION

It has been shown that the LQ parameters obtained from one set of HDR data, although they have reasonable uncertainty values, may be significantly different from values obtained at a later date from another set of HDR data [52]. This may be due to a variation in radiosensitivity of a cell line over time. A variation in radiosensitivity *in vitro* is most prevalent with newly established cell lines from patient tumour samples, questioning the usefulness of these *in vitro* survival parameters in predicting patient radiosensitivity. However, as mentioned in the introduction, *in vitro* survival parameters from established cell lines have shown good correlation to clinical radiosensitivity.

The data points in the survival curves have been averaged from many experiments and consequently the values obtained for α and β represent the response of the cell lines over a long period of time. The values shown in table 1 are similar to those from other glioma cell lines reported previously [49].

Qualitatively a HDR survival curve for mammalian cells exposed to sparsely ionizing radiation (e.g. x-rays, γ -rays) has the characteristic shape displayed by these glioma cell lines, with an initial 'shoulder' region followed by a steeper bending portion. This type of response implies that a certain amount of SLD must be accumulated, or is repaired quickly before a lethal event becomes evident [17]. Repair is thought to become saturated very quickly at a HDR [53]. Cells exposed to densely ionizing radiation (e.g. alpha particles, low energy neutrons) yield a dose response curve which has little or no

shoulder region, implying that there are few sublethal reparable events and that the majority of events are singularly lethal (non-reparable).

As the dose-rate is lowered, a point is reached when all SLD capable of being repaired is repaired, resulting in a mono-exponential survival curve with a limiting slope. This is the classic dose-rate effect. Typically human tumor cell lines show maximum LDR sparing at or below a dose-rate of approximately 1 cGy/min. For stationary cell cultures, if recovery of SLD is complete, then the LDR survival curve should be an extension of the initial slope of the HDR survival curve [17,49,54]. It is clear from their LDR survival responses (figures 3 and 4) that these glioma cell lines in a stationary growth phase reach maximum LDR sparing at a dose-rate ≤ 0.41 cGy/min. (LDR_2). The magnitude of the dose-rate effect varies enormously between different cell types as does the initial shoulder, however there is generally good correlation between the two. In this study, the results for the two glioma cell lines support these concepts since the values for α obtained from the HDR survival curves, which are dominated by the data in the shoulder region, are the same as the values for α from the LDR response curves, within experimental error.

Cell survival at 2Gy, a dose which usually reflects survival in the shoulder region, has been shown to discriminate between resistant and sensitive tumour cell types [50,55,56] with some exceptions [17]. Many researchers consider that α from the LQ fit of a LDR response curve is a better predictor of the sensitivity or resistance of a cell line

provided that the recovery of SLD in stationary growth phase cells is the only process that is reflected in the survival response [17,42,49,50,54]. In this study, the linearized representation of the data (equation 4, figures 5 and 6) reveals that LDR response curves do not always fit well to a linear function. Therefore a single LQ fit of an entire LDR curve may not always yield the most accurate value for α , especially if one is concerned primarily with characterizing the survival response in the 2 Gy region. Recently, Skarsgard et al. [102,103] have examined the substructure of HDR survival curves in the low dose region and have applied a two-component LQ fit of the entire survival curve. Perhaps a two-component LQ survival model may prove useful in the parameterization of LDR survival curves as well.

There are two other factors, in addition to the recovery of SLD, known to contribute to the net dose-rate effect. These factors are: 1) increased cell sensitivity due to cells being blocked in the G_2 phase of the cell cycle, and 2) increased cell resistance due to proliferation. For HDR exposures cells are frozen in the phase of the cell cycle that they happened to be in at the time of the exposure. In most cell lines a lowering of the dose-rate past the point at which all SLD is repaired allows cells to progress through the cell cycle and accumulate in G_2 [57]. G_2 is a relatively radiosensitive phase of the cell cycle [58] and so a survival curve for cells irradiated at this particular dose-rate becomes steeper. This is known as the inverse dose-rate effect. A further reduction in the dose rate will allow cells to pass through the G_2 block, redistribute within the cell

cycle, and divide. This will lead to an apparent increase in survival since the daughter cells will have received less dose than cells which have not divided. In addition as the cells move through S phase they are more resistant to irradiation [58], which would also tend to increase survival.

To avoid the effects of these factors and only examine the recovery of SLD, all LDR experiments were conducted with stationary or plateau growth phase cells with a very low fraction of cycling cells. The maximal recovery of SLD under LDRI with plateau phase glioma cells has been used as a model to examine recovery inhibition by mild hyperthermia. This is examined in a later chapter.

Table 1: Linear-quadratic parameters: High and low dose-rate irradiation

CELL LINE	DOSE-RATE	$\alpha(\text{Gy}^{-1})$ (+/-)	$\beta(\text{Gy}^{-2})$ (+/-)	R^2	$\alpha(\text{no } \beta)^*$
U-87MG	HDR	0.20 (0.03)	0.061 (0.004)	.973	--
	LDR ₁	0.21 (0.01)	0.011 (0.001)	.914	.33
	LDR ₂	0.18 (0.01)	0.004 (0.001)	.754	.23
U-373MG	HDR	0.19 (0.03)	0.047 (0.004)	.950	--
	LDR ₁	0.23 (0.02)	0.005 (0.001)	.695	.28
	LDR ₂	0.22 (0.01)	0.004 (0.001)	.719	.27

* - α from simple exponential fit: $\%S = \exp(-\alpha D) \times 100$, $\beta = 0$ assumed, $R^2 > .995$ in each case.

HDR = 150-200 cGy/min ^{60}Co

LDR₁ = 0.88 cGy/min ^{226}Ra , LDR₂ = 0.41 cGy/min ^{226}Ra

R^2 = Regression SS/Total SS

Total SS = Regression SS + Residual SS

SS = Sum of Squares

Figure 1: Growth curve: U-87MG

Figure 2: Growth curve: U-373MG

Figure 3: Cell survival: U-87MG in plateau growth phase. High dose-rate (HDR) - ^{60}Co , low dose-rate (LDR) - ^{226}Ra . LDR 1 - 0.88 cGy/min, LDR 2 - 0.41 cGy/min. Survival curves fit to $\%S = \exp(-\alpha D - \beta D^2) \times 100$.

Figure 4: Cell survival: U-373MG in plateau growth phase. High dose-rate (HDR) - ^{60}Co , low dose-rate (LDR) - ^{226}Ra . LDR 1 = 0.88 cGy/min, LDR 2 = 0.41 cGy/min. Survival curves fit to $\%S = \exp(-\alpha D - \beta D^2) \times 100$.

Figure 5: $-\ln(S)/D$ v. D : U-87MG. High dose-rate (HDR) - ^{60}Co , low dose-rate (LDR) - ^{226}Ra . LDR 1 - 0.88 cGy/min, LDR 2 - 0.41 cGy/min.

Figure 6: $-\ln(S)/D$ v. D : U-373MG. High dose-rate (HDR) - ^{60}Co , low dose-rate (LDR) - ^{226}Ra . LDR 1 ≈ 0.88 cGy/min, LDR 2 ≈ 0.41 cGy/min.

Figure 7: $-\ln(S)$ v. D : U-87MG and U-373MG. Low dose-rates (LDR) only. LDR 1 = 0.88 cGy/min, LDR 2 = 0.41 cGy/min.

3.0 THE KINETICS OF CELLULAR RECOVERY IN EXPONENTIAL AND PLATEAU GROWTH PHASE CELLS

3.1 INTRODUCTION

It is well established that cellular recovery from radiation damage, both sublethal and potentially lethal is a dominant factor governing the effectiveness of a multifraction or low dose-rate (LDR) course of radiotherapy [10]. Sublethal damage recovery (SLDR) is typically referred to as recovery which occurs between dose fractions of HDR or during protracted or LDR [59]. Potentially lethal damage recovery (PLDR) has been operationally defined as recovery which occurs post-irradiation [60]. Studies of post-irradiation recovery have shown that PLDR is maximal under environmental conditions which inhibit cellular proliferation such as when cells are in a plateau growth phase [61,62,63]. Under such conditions both SLDR and PLDR have been demonstrated in rodent [61-64] and in human tumour cell lines [10] and thought to be manifestations of the same underlying processes operating on the same basic lesions [59,65]. Current models which incorporate SLDR and PLDR to characterize cell survival during fractionated or LDR irradiation protocols have included a mono-exponential repair term to describe cellular recovery [59,66,67]. More recently, extensions of these models to predict the biological equivalence of different irradiation schemes (alternate dose-fraction size, dose-rates, time between doses, etc.) have called for the accurate determination of recovery kinetics in both normal and tumour tissues [22-25]. Outstanding questions that remain are whether recovery follows mono- or bi-exponential kinetics, does the recovery

half-time ($T_{1/2}$) vary with dose or dose-rate, and is $T_{1/2}$, determined by SLDR, PLDR or LDR studies, a measure of the same recovery process.

The recovery of PLD, expressed as a recovery ratio (RR), has been shown to increase with dose according to a linear-quadratic (LQ) exponential function in a wide variety of human tumour [21,68,69] and fibroblast [21,69,70] cell lines, as well as in rodent tumour cell lines [71,72]. Although the kinetics of PLDR has been studied in human tumour cell lines [19] and fibroblasts [70], accurate determination of recovery half-times have not been made. Such analyses have been made in rodent cell systems however, and have resulted in both mono-exponential [71,73] or bi-phasic [74,75] recovery kinetics. In the study by Reddy et al. [71] $T_{1/2}$ was found to increase linearly with dose for V79 cells in an exponential growth phase.

Evidence that recovery of PLD follows mono-exponential repair kinetics in plateau phase cells comes from the study of the induction and rejoining of interphase chromosome breaks. Repair half-times similar to those from PLDR studies has led to the suggestion that chromosomal repair is the underlying mechanism of PLDR in rodent tumour cells [76] and in human fibroblast cells [77]. Studies measuring the repair of DNA double strand breaks (dsbs) have revealed bi-phasic repair kinetics. The $T_{1/2}$ of the slow component has been shown to increase as a function of dose [78,79]. If repair of DNA dsbs is the underlying process leading to PLDR [63], then this is further evidence that recovery half-times should increase with dose. The difficulty in comparing $T_{1/2}$

values from the repair of dsbs and chromosome aberrations with those for PLDR, is that much higher doses are used in the latter two assays [73].

In this study we have examined the kinetics of PLDR in a human glioma cell line in an exponential and a plateau growth phase. We have also compared PLDR and SLDR in plateau growth phase cells. The specific objectives of this study are: to determine whether recovery is mono- or bi-exponential in nature; to determine if $T_{1/2}$ values are different in exponential and plateau growth phase cells; to determine if $T_{1/2}$ values increase as a function of dose, and if so does this increase compare to the increase in the amount of recovery; and finally, to determine if the kinetics of SLDR and PLDR are similar in plateau growth phase cells.

3.2 MATERIALS & METHODS

The human glioma cell line U-87MG was cultured as described in section 2.2. Cells were irradiated with a clinical ^{60}Co (120-200 cGy/min) therapy unit while suspended in an ice water bath.

For all experiments 10^5 cells were plated in 25 cm^2 flasks. For experiments on exponentially growing cultures, the cells were irradiated on day 4 post plating. At this time the cell density was $\sim 2.5 \times 10^4\text{ cm}^{-2}$ and the distribution of cells within the cell cycle was $\sim 60\%$ G_0/G_1 , 15% S , and 25% G_2/M . On day 4 the pH of the medium was 6.95 ± 0.05 . For experiments on plateau growth phase cultures the medium was changed on

days 4 and 7 post plating. The cells were irradiated on day 10 at which time the cell density was $\sim 10^5 \text{ cm}^{-2}$, and the distribution of cells within the cell cycle was $\sim 85\%$ G_0/G_1 , 5% S, and 10% G_2/M . On day 10 the pH of the medium was $6.75 (\pm 0.05)$.

For delayed plating (DP) experiments the flasks were immediately warmed post-irradiation for 2 minutes in a 37°C water bath and then placed, medium unaltered, in a 37°C tissue culture incubator for the required period of time 't'. This procedure was followed whether 't' followed a single dose fraction (PLDR) or t was between dose fractions (SLDR). For immediate plating (IP) or 't' = 0, the cells were trypsinized immediately post-irradiation, without being warmed in a 37°C water bath. Survival values were normalized to the plating efficiency of control flasks which were treated in the same manner. The plating efficiency under these conditions was approximately 35% with an observed range of 25-45%.

Cell survival was assayed by the colony formation assay (see appendix II).

All data points on all graphs represent the average of 3-4 independent experiments with uncertainty ($\pm$) values signifying the Standard Error of the Mean. Error bars are not shown when smaller than the datum symbol.

3.3 RESULTS

Complete immediate and delayed plating (IP and DP) survival curves for U-87MG in exponential and plateau growth phase are shown in figure 8. They have been

fitted to the linear quadratic (LQ) survival model (equation (3), section 2.3). Values for α and β were obtained by regression analysis of equation (4) and are given in table 2. The DP survival values were assayed 24 hours post-irradiation and therefore represent the maximum recovery of potentially lethal damage (PLD) under the experimental conditions. Recovery ratios are typically defined as:

$$RR = \frac{\%S_{DP}}{\%S_{IP}} \quad (6)$$

Using equation (3) for the IP and DP survival curves, the RR can be expressed as:

$$RR(D) = e^{(\alpha_{IP}-\alpha_{DP})D + (\beta_{IP}-\beta_{DP})D^2} \quad (7)$$

$$\text{or} \quad RR(D) = e^{(\xi D + \psi D^2)} \quad (8)$$

Values for the parameters in equation (8) are given in table 2. The corresponding RR response functions are shown in figure 3. The same values for ξ and ψ could have been obtained directly by fitting the individual RR values to equation (8).

Survival recovery as a function of the DP time 't' for plateau and exponential growth phase cells irradiated at several different doses is shown in figures 9 and 10, respectively. Within the first hour of post-irradiation incubation, survival was assayed

every 15 minutes, and then at 1.5, 2.0, 4.0 hours etc. such that the resulting recovery curves could be accurately fit to the following general equation:

$$\%S(t) = 100 - (\sum A_i e^{-k_i t} + B) \quad (9)$$

where $\sum A_i + B$ = total initial cell kill at $t=0$ ($1-\%S(0)$), and B = residual cell kill after A_i are repaired at the rates k_i ($1-\%S(\infty)$) [75].

This equation differs from a simple sum of exponentials used to describe the repair of DNA dsbs [80] in that it includes the B term which represents initial unreparable lethal damage and misrepaired damage. Equation (9) was fit to the data in its mono- and bi-exponential form using a non-linear regression program (SPSS/PC+ Advanced Statistics v. 5.0) in which partial derivatives were supplied and calculated analytically. In all cases the coefficient of determination (R^2) was very close to 1.0 ($> .9$). R^2 is a measure of the proportion of the total variation of the dependent variable about its mean that is explained by the fitted model, and was defined in table 1. The goodness of fit was monitored by calculating a chi squares statistic (χ^2). In each case a bi-exponential model resulted in large increases in the error values for the parameters estimated, with minimal decrease in the χ^2 value, as compared to a mono-exponential fit. With 100-200% error on the parameter estimates, a bi-exponential model was rejected in each case. With reasonable error values for the estimates of the model parameters (see tables 2,3,4), a

mono-exponential model was adopted in each case. The resulting values are shown in table 3, as are values for the recovery half-time ($T_{1/2}$). The mono-exponential form of equation (9) may be expressed in the form:

$$S(t) = S_0 + (S_\infty - S_0)(1 - e^{-kt}) \quad (10)$$

where $(1 - S_0) = (\Sigma A_i + B)$ and $(1 - S_\infty) = B$ [73].

Using S_{IP} for S_0 and $S_{IP(24)}$ for S_∞ , a value for 'k' may be estimated. Equation (9) was used however, since it allows a free form fit of all the data without specifying initial and final survival values, and therefore provides a more rigorous test of the model function.

The constant 'A' reflects the amount of survival recovered at the rate 'k' and therefore represents repairable type damage. The results in table 3 indicate that 'A' decreases as a function of increasing dose, while the recovery half-time increases. In addition, at iso-dose measurements exponential growth phase cells sustained less repairable type damage (A) than plateau growth phase cells. At 7.5 Gy, exponentially growing cells had a higher IP survival while recovering to the same final survival as plateau growth phase cells. At an iso-IP survival dose (9.75 Gy), exponentially growing cells did not recover to the same final survival level as plateau growth phase cells (figure 10).

One of the aims of this study was to investigate the relationship between the degree of recovery and the time to achieve it. Figure 11 compares the RR and the recovery half-time at the various doses for which the kinetics of recovery were measured. At an iso-recovery level, the rate of recovery, expressed by $T_{1/2}$, is not significantly different in exponential and plateau growth phase cells.

Values for $T_{1/2}$ have been plotted as a function of dose in figure 12. Within the dose range examined, $T_{1/2}$ increased with dose in an exponential manner for cells in either growth phase. The inset in figure 12 indicates that this limited set of $T_{1/2}$ values also fits reasonably well to a linear function of dose. In either case the $T_{1/2}$ value at 9.0 Gy for plateau growth phase cells was excluded from the fit. In figure 11, to approximate a value for $T_{1/2}$ at a RR of 1.0, corresponding to zero dose, a linear extrapolation of the limited data was employed. In this manner an approximate value for $T_{1/2}$ of 0.77 h for both exponential and plateau growth phase cells was estimated. With this value included in figure 12, the data was fit to a linear-quadratic equation similar to equation (8), namely,

$$T_{1/2}(D) = T_{1/2}(D=0)e^{(\mu D + \nu D^2)} \quad (11)$$

The results of fitting this data to equation (11) are shown in figure 12.

For cells in a plateau growth phase survival recovery was also investigated via split dose experiments with two equal dose fractions (X) of 4.5 Gy. Partial recovery was allowed between dose fractions with IP after the second dose (X-t-X-0, $t = 0-24$ h). Two other protocols were examined, allowing partial recovery after the first dose fraction and complete recovery after the second dose fraction (X-t-X-24) and vice versa (X-24-X-t). The results of these protocols are shown in figure 13. Time course recovery following single dose fractions of 4.5Gy (X-t) and 9.0 Gy (2X-t) have been drawn in from figure 9 for comparison. When $t = 0$ or $t = 24$ there are several survival points within these protocols which should be inherently the same. The results are consistent with this fact. Once again a mono-exponential kinetic model yielded the best of the data. The kinetic parameters are shown in table 4.

The X-t-X-0 response (SLDR) is similar to the 2X-t response (PLDR) from $t = 0$ to $t = 1$ h, after which there is reduced rate of survival recovery in the SLDR case. Although the final recovery values are similar ($t=24$ h), this reduced rate of recovery dominated the fit of the data, resulting in a $T_{1/2}$ for SLDR that is significantly longer than in the PLDR case. It is interesting to note that 'A' is the same in both these cases. For the two split-dose protocols in which full recovery occurred following one of the dose fractions, 'A' was less than that observed for the single dose fraction X-t, but greater than that for the double dose fraction protocols 2X-t and X-t-X-0. With partial recovery between dose fractions (X-t-X-24) the rate of recovery was reduced as compared to full

recovery between doses (X-24-X-t), resulting in a longer $T_{1/2}$. In both cases $T_{1/2}$ was longer than the $T_{1/2}$ for PLDR at iso-RRs. These split dose experiments suggest that in addition to PLDR following each dose fraction, there may be some interaction between dose fractions that results in damage which recovers at a slower rate. This is most evident when only partial recovery from the first dose fraction has occurred prior to the second dose fraction (X-t-X).

3.4 DISCUSSION

It is well known that there is increased IP-survival in proliferating cells as compared to quiescent cells [21,81]. In the LQ model this has been manifested as a decrease in both α and β [82]. My results with U-87MG cells in an exponential and plateau growth phase are consistent with these observations. The decrease in α and β observed in the 24 h DP survival response curves as compared to the IP response curves is also consistent with previous studies with human cell lines [21]. There was little change in the DP survival response between both growth phases however, resulting in a lower RR as a function of dose for exponentially growing cells as compared to plateau growth phase cells (figure 8). This was expressed with use of equation (8) by decreases in both ξ and ψ . This equation has been proposed to quantify the recovery of PLDR [72]. The decrease in RR observed in exponential growth phase cells may be related to several post-irradiation factors which during the DP period tend to decrease the level of PLDR.

These factors include increased glycolysis [chapter 5], a slightly higher pH [83], and increased proliferation [61,62,chapter 5]. In these experiments there was approximately 3x as much glucose utilized during the DP period in exponential growth phase cells (data not shown). It has been observed in rodent cells that the majority of PLDR occurs in G_1 cells [84]. This may also explain why there was less recovery in exponentially growing cells.

The analyses in this report have resulted in mono-exponential recovery kinetics for both exponential and plateau growth phase cells. This does not contradict the bi-phasic kinetics observed with DNA dsb repair. Lack of detection of a fast recovery component may be related to the lack of measurement points in the initial few minutes of recovery. Since the first measurement point was at 15 minutes post-irradiation, the underlying fast repair of dsbs may have already been complete. In addition, even though the cells were irradiated on ice, preventing enzymatic repair during irradiation, the $t = 0$ survival point may have included some repair occurring in the interval between irradiation and cell dispersal [75]. Treatment with a hypertonic salt solution prior to plating the $t = 0$ point has been used to prevent any of this repair [64,75]. This would result in a lower initial survival. Since several of the mono-exponential fits in figure 9 also point to a lower initial survival, it is doubtful that this factor would influence the mono-exponential fit of the data to a significant degree.

Figure 12 reveals that within the range of measurements, $T_{1/2}$ may be increasing exponentially with dose, although with this limited data set a linear increase cannot be ruled out (figure 12 inset). This implies that within this dose range, the repair processes are saturated, i.e. the velocity of repair is constant, resulting in a $T_{1/2}$ which increases with dose. It has been proposed that below a certain dose, repair is unsaturated and therefore the velocity of repair increases with dose and $T_{1/2}$ is constant [53]. This relationship has been demonstrated in plateau phase rat astrocytoma 9L cells with regard to the repair of DNA dsbs [79]. In that study the $T_{1/2}$ of the slow component was shown to increase in an exponential manner beyond a certain dose threshold. In this study, with an estimate for $T_{1/2}$ at zero dose from figure 11, a constant $T_{1/2}$ at lower doses followed by an exponential rise in $T_{1/2}$ within the measured dose range, has been drawn in figure 12. This estimation puts the threshold dose at approximately 3 Gy for exponential and plateau growth phase cells.

It has been shown in rodent cells that the $T_{1/2}$ for PLDR increased linearly with dose, while the RR increased linear-quadratically [71]. By using an estimate of the $T_{1/2}$ at zero dose from figure 11, we have shown that $T_{1/2}$ also fits reasonable well to a LQ exponential function of dose (equation (11)). This no threshold-dose model does not rule out the repair saturation model of Goodhead [53]. With this model the degree of repair saturation would increase as the amount of recovery increases, according to a LQ exponential relationship, with very little saturation at the lower doses. The fact that RR

and $T_{1/2}$ may be linearly related within a range of RRs (figure 11) also suggests that they follow the same relationship with respect to dose. The anomalous point in figures 11 and 12 corresponding to a 9 Gy dose to plateau phase cells suggests that there may be an upper limit to $T_{1/2}$ which in these cells is approximately 3.5 h. Alternatively this point indicates that the velocity of recovery may have increased to keep $T_{1/2}$ constant. At this point an induced repair system may play a role in increasing the recovery velocity

It has been observed with rodent cells that kinetics of the repair of DNA dsbs varies little throughout the cell cycle [80] and is similar in proliferating and quiescent cells [74,82]. However there have also been conflicting reports that the repair kinetics for DNA dsbs was slower in quiescent cells than in proliferating cells [85] and vice versa [86]. It appears from figure 10 and tables 3 and 4, when comparing iso-dose or iso-IP-survival points, that recovery is faster in exponential growth phase cells. However this is not a valid comparison since $T_{1/2}$ is also related to the level of recovery (figure 11), which is different in each case. At an iso-RR, $T_{1/2}$ is not statistically different in both growth phases.

It is thought by many that SLDR and PLDR may not differ in their molecular basis [59,65], and cellular recovery in either case corresponds to very complex mechanisms, such as the repair of DNA dsbs, which may have different rates of completion associated with them. Our data indicates that in plateau phase cells a large degree of recovery (PLDR or SLDR) occurred between or after a dose fraction. The split-dose experiments indicated that at an iso-recovery level, the rate of recovery between

dose fractions is less than the rate of recovery following a single dose fraction. This may be due to some residual damage from the first dose fraction interacting with the damage from the second dose fraction resulting in a decrease in the rate of recovery. It has been proposed that the first dose fraction alters the accessibility to lesions incurred following the second dose fraction [87]. An accessibility hypothesis has been proposed to explain the fast and slow kinetics observed for the repair of DNA dsbs [80,88]. Perhaps the decrease in the rate of recovery observed for SLDR reflects a change in the proportion of these two repair rates. It has also been suggested that DNA packaging and organization in the nucleus plays a role in DNA damage and repair [89]. Structural differences caused by the first dose may alter the amount and/or the repair rate of damage following the second dose.

This study has shown that the recovery of PLD in exponential and plateau growth phase human glioma cells is mono-exponential in nature for recovery times greater than 15 min. and occurs at a similar rate when iso-recovery values are compared. Recovery was saturated within the observed dose range in that $T_{1/2}$ increases as a function of dose. There was not enough data to rule out a linear relationship, however the data did suggest that the recovery half-time increased according to an exponential function of dose. Evidence was presented that this exponential function may be linear-quadratic in nature. This would imply that the recovery half-time and the recovery ratio increase with dose in a similar fashion. Lastly, this study indicates that significant recovery, whether it be termed PLDR or SLDR, occurs between dose fractions and that this recovery proceeds at a slower rate than recovery following a single dose of radiation.

Table 2: Linear-quadratic parameters: Exponential and plateau growth phase cells

U-87MG	R²	α (Gy⁻¹) (+/-)	β (Gy⁻²) (+/-)	α/β
Plateau				
IP	.973	0.20 (0.03)	0.061 (0.004)	3.2
DP	.919	0.14 (0.02)	0.025 (0.003)	5.8
$\xi(\alpha_{IP}-\alpha_{DP})$		0.06 (0.01)		
$\psi(\beta_{IP}-\beta_{DP})$			0.036 (0.005)	
Exponential				
IP	.950	0.16 (0.03)	0.035 (0.003)	4.5
DP	.965	0.13 (0.02)	0.022 (0.002)	6.0
$\xi(\alpha_{IP}-\alpha_{DP})$		0.03 (0.01)		
$\psi(\beta_{IP}-\beta_{DP})$			0.013 (0.002)	

D = dose (Gy) ⁶⁰Co

IP = Immediate Plating

DP = Delayed Plating (24 h)

Table 3: PLDR kinetic parameters

DOSE (Gy)	R²	A (%) (+/-)	k (+/-)	B (%) (+/-)	T_{1/2}(hrs)* (+/-)
Plateau					
3.0	.988	30.8 (1.3)	0.79 (0.08)	43.1 (0.9)	0.88 (0.09)
4.5	.981	14.3 (0.8)	0.55 (0.07)	76.9 (0.6)	1.3 (0.2)
7.5	.990	9.8 (0.4)	0.20 (0.02)	89.6 (0.4)	3.5 (0.4)
9.0	.990	3.5 (0.1)	0.20 (0.02)	96.4 (0.1)	3.5 (0.4)
Exponential					
4.5	.990	11.9 (0.5)	0.81 (0.07)	62.9 (0.3)	0.86 (0.08)
7.5	.993	6.2 (0.2)	0.49 (0.04)	90.6 (0.2)	1.4 (0.1)
9.75	.994	2.31 (0.08)	0.39 (0.04)	96.74 (0.07)	1.8 (0.2)

*T_{1/2} = ln (2) / k

(+/-) = Asymptotic Std. Error

(A + B) = total initial cell kill at t=0 (1-%S(0))

(B) = residual cell kill after (A) is repaired at the rate (k)
(1-%S(∞))

Table 4: SLDR kinetic parameters.

	R²	A (%) (+/-)	k (+/-)	B (%) (+/-)	T_{1/2}(hrs)* (+/-)
Protocol					
XtX0	.993	3.5 (0.2)	0.09 (0.01)	96.2 (0.3)	7.9 (1.3)
XtX24	.971	7.2 (0.6)	0.17 (0.04)	88.9 (0.6)	4.0 (1.0)
X24Xt	.987	8.5 (0.5)	0.32 (0.05)	87.7 (0.4)	2.2 (0.4)

$$*T_{1/2} = \ln(2) / k$$

(+/-) = Asymptotic Std. Error

(A + B) = total initial cell kill (t=0) (1-%S(0))

(B) = residual cell kill after (A) is repaired at the rate (k)
(1-%S(∞))

X = 4.5 Gy

t = incubation time at 37°C, 24 = 24 h at 37°C.

Figure 8: U-87MG cell survival following HDRI (^{60}Co) in an exponential (EXP) and plateau (PLA) growth phase, under immediate plating (IP) and delayed plating (DP) conditions. Curves fit to $\%S = \exp(-\alpha D - \beta D^2) \times 100$. Recovery ratio (RR) functions, $RR = \exp(\xi D + \psi D^2)$.

Figure 9: PLDR at various doses for cells in a plateau growth phase % survival following a delayed plating period of 't' hours at 37°C. Curves fit to $\%S = 100 - (A \exp(-kt) + B)$.

Figure 10: PLDR at various doses for cells in an exponential growth phase. % survival following a delayed plating period of 't' hours at 37°C. Curves fit to $\%S = 100 - (A \exp(-kt))$ (B). Iso-dose and iso-IP-survival responses have been compared to the 7.5 Gy response in plateau cells.

Figure 11: Recovery Ratio (RR) as a function of recovery half-time ($T_{1/2}$) for exponential and plateau growth phase cells. The lines drawn are linear fits of the data. The point at a RR of approx. 29 has been excluded from the fit in the plateau cell case.

Figure 12: Recovery half-time ($T_{1/2}$) as a function of dose for exponential and plateau growth phase cells. The thin lines drawn are exponential fits of the data. The point at 9.0 Gy has been excluded from the fit in the plateau cell case. The thick lines are a fit of the data to $T_{1/2} = \exp(\mu D + \nu D^2) \times 0.77$, with the inclusion of the $T_{1/2}$ value estimated from figure 4 of 0.77 at zero dose. Inset: Linear representation and fit of the same data.

Figure 13: SLDR for cells in a plateau growth phase. % survival after three split-dose protocols with 't' being the time at 37°C and X = 4.5 Gy HDR. Curves fit to %S = 100 - (Aexp(-kt) + B). PLDR curves for 4.5 and 9.0 Gy from figure 2 have been included

4.0 Changes in Survival and Potentially Lethal Damage Recovery following Periods of High and Low Metabolic Activity

4.1 INTRODUCTION

A large degree of potentially lethal damage recovery (PLDR) has been demonstrated in many human tumour cells lines and is thought to play a role in the resistance of these tumours to radiotherapy [10,20,21]. Glioblastoma are considered clinically to be very radioresistant [2] and *in vitro*, glioblastoma cell lines have shown a large degree of PLDR [33]. However studies have shown a discrepancy between the amount of PLDR demonstrated *in vitro* and *in vivo* [15,19]. This may be related to a difference in the proliferative and metabolic status under which the two cell systems are studied, or the fact that cells *in vivo* are adapted to low pH conditions. Many solid tumours are thought to include acidic and nutrient deprived regions containing slowly proliferating cells [18]. In such regions the functional vasculature is often inadequate to supply nutrients to the cells and to remove waste products generated by the cells. In addition, cellular proliferation, DNA synthesis and glycolysis may be inhibited due to acidic pH [90]. Such a quiescent state has been modeled *in vitro* by growing cells to a plateau growth phase and allowing them to remain in culture under depleted medium conditions at an acidic pH. PLDR has been shown to be maximal under these environmental conditions which favour the cellular processes necessary for damage repair and/or the inhibition of damage fixation [61-63]. Decreased PLDR is associated with

damage fixation and/or error prone repair that occurs under metabolic conditions which promote progression through the cell cycle [61-63].

Studies with rodent cells have indicated that cells in a plateau growth phase and in conditioned medium exhibit increased radiosensitivity and an increased amplitude of PLDR as the time in culture is increased [81]. In a study with human astrocytoma cells similar results were seen as a function of decreasing pH. In that study the cells were exposed to new medium adjusted to various acidic pH values [91].

It is a well known biological phenomenon that a decreasing pH causes a decrease in glycolysis in intact organisms and in a variety of systems *in vitro*, and that glycolysis is maximal under basic conditions [92,93]. Cellular proliferation also decreases with decreasing pH [94,95] although this decrease may be a result of a concomitant reduction in glycolysis or DNA synthesis. DNA synthesis is also inhibited by a decreasing pH [96]. In this study cell survival and capacity for PLDR were measured in a human glioma cell line as the cells progressed from an exponential to a plateau growth phase and as the cells aged in a plateau growth phase. During this period the interdependence of cellular proliferation, glycolysis, and pH were closely monitored. This study expands on previous studies by measuring cell survival and PLDR throughout a range of metabolic and proliferative states.

Increased metabolic activity has been associated with increased tumor radiation resistance *in vivo* [97]. Numerous studies have reported temporal increases in metabolic

activity post-irradiation in animal tumour models using ^{31}P NMR spectroscopy [98-101]. Maximum increases occurred between 24-48 hours post-irradiation and decayed within the next several days. A recent study using ^{18}F - fluorodeoxyglucose PET detected an increase in the regional cerebral metabolic rate for glucose in a human glioma following stereotatic radiotherapy [105]. The increase was maximal at 24 hours post-irradiation and then decayed. This may have implications in fractionated radiotherapy regimes employed in the clinic. Following a particular fraction, a transient period of increased metabolic activity may occur in regions of the tumour which were previously metabolically quiescent.

This study addresses the radiosensitivity of such a subpopulation to a subsequent fraction of radiotherapy. We examined the effect of a period of increased glycolytic activity in a previously metabolic quiescent cell population on cell survival and recovery from PLD. Glycolysis was increased by raising the pH of the existing medium to 7.4. Prior to irradiating, the rate of glycolysis was allowed to decrease and the pH to reacidify such that cell survival and PLDR at this new metabolic equilibrium could be compared to cell survival and PLDR from an unperturbed, metabolically quiescent cell population.

4.2 MATERIALS & METHODS

The human glioma cell line U-87MG was cultured as described in section 2.2. Cells were irradiated with 750 cGy ^{60}Co (120-200 cGy/min) while suspended in an ice

water bath then trypsinized and assayed for survival either immediately (IP survival) or after a 24 h delayed plating period (DP survival). Recovery ratio (RR) is expressed as DP survival/IP survival.

For all experiments 10^5 cells were plated in 25 cm² flasks. For experiments on exponentially growing cultures progressing to a plateau growth phase, no further medium changes were made. Cell survival was assayed on days 4, 7 and 10 post plating. For experiments commencing on confluent plateau growth phase cultures, the medium was changed on days 4 and 7 post plating with cell survival assayed on days 10, 13, and 16

Cell survival was assayed by the colony formation assay (See appendix II). For delayed plating (DP) experiments the flasks were immediately warmed post-irradiation for 2 minutes in a 37°C water bath and then placed, medium unaltered, in a 37°C tissue culture incubator for 24 hours.

Medium pH was raised to ~ 7.4 with the addition of 1N NaOH (5-10% of total volume) on days 4 and 7 for the exponential experiments and on days 10 and 13 for the extended plateau experiments. Medium pH was measured at room temperature with an ATC pH electrode, and the values shown represent the pH of the medium from control and IP irradiated flasks sampled just after irradiation, at the time of trypsinization. Survival values were normalized to the plating efficiency of control flasks which were treated with the same pH adjustment protocols as those which received radiation. The

plating efficiency of this cell line under these conditions was approximately 30% with an observed range of 20-40%.

Glucose utilization and lactate production was monitored by measuring the residual concentrations in the medium. Glucose was measured by the spectrophotometric determination of oxidized o-Dianisidine at 450 nm in the presence of excess o-Dianisidine di-HCl, glucose oxidase and peroxidase. Lactate was measured by the spectrophotometric determination of NADH formation at 340 nm in the presence of excess NAD⁺ and lactate dehydrogenase. Concentration values for pH adjusted samples were corrected for any dilution from the addition of NaOH. Both assays are based on the Sigma Diagnostics kit protocols.

Cell cycle distributions of samples fixed in 70% ethanol were measured by standard flow cytometry techniques using an Ortho Cytofluorograf 50H and propidium iodide staining of the nuclear DNA. The resulting distributions of fluorescence were segmented into regions of interest to determine the percentage of cells in the phases of the cell cycle.

All data points on all graphs represent the average of 3-4 independent experiments with error bars signifying the Standard Error of the Mean.

4.3 RESULTS

Growth curves for the human glioma cells are shown in figures 14 and 15. With no additional medium changes after plating the cells entered a plateau growth phase at about day 7. With two medium changes during exponential growth phase the cells did not enter a plateau growth phase until approx. day 10 at which time the plateau cell density was approximately 10^5 cm^{-2} , a factor of 2 higher than the plateau cell density in the former case. Raising the pH of the existing medium on days 4 and 7 resulted in some increase in plateau cell number but not to the extent that occurred with 2 medium changes. Figure 15 shows the plateau cell number relative to day 10 for cultures held in plateau phase with no further medium changes. These cultures were grown to plateau phase with medium changes on days 4 and 7. From day 10 to day 16 the plateau cell density remained fairly constant with minimal loss in cell number. Some loss in cell number may have occurred due to the lifting off of dead cells. Increasing pH on days 10 and 13 did not result in a significant increase in cell number, especially considering that these pH increases may have resulted in fewer cells lifting off.

The corresponding pH of the culture media is shown in figures 16 and 17. In both sets of experiments the cells entered a plateau growth phase when the medium pH decreased below ~6.7 after which the pH slowly reached a constant value of ~6.6.

Glucose utilization and lactate production, monitored by measuring the respective concentrations in the medium, is shown in figures 18 and 19. The initial concentrations

agree with those calculated from the media and sera specifications. With no pH adjustment the rates of glucose utilization and lactate production approached zero as the cells entered a plateau growth phase with 40-50 % of the glucose remaining, and a plateau lactate concentration of ~ 1.6 mg/ml or ~ 18 mM. For both the exponential and plateau experiments, raising the pH of the medium to ~ 7.4 twice during the incubation period caused an increase in the rate of glucose utilization and lactate production such that the final glucose concentration was < 10 % of its initial value and the final lactate concentration approached 2.6 mg/ml or 29 mM. After each of these pH increases, significant reacidification of the medium occurred within the next 3 days. In each case the pH fell to 6.8 or less, except after the second pH increase in extended plateau in which case the pH fell to only 7.0.

When all measured glucose concentrations shown in figures 18 and 19 were plotted as a function of the corresponding lactate concentrations, the data showed a linear correlation ($r^2 = .93$) throughout the entire concentration range present in these experiments (data not shown). This result indicated that for every molecule of glucose used, 0.76 molecules of lactate were produced. Galarraga [106] measured the utilization of glucose and the production of lactate and pyruvate in the growth medium of 7 human glioma derived cell lines and found a lactate plus pyruvate concentration divided by glucose used concentration ratio of approximately 1.0. If glycolysis were entirely anaerobic, 2 molecules would be present as either lactate or pyruvate for every molecule

of glucose used, and therefore in Galarraga's experiments ~ 50 % of the glucose was consumed via the glycolysis pathway. Since glucose was metabolized only as far as lactate and pyruvate even in the presence of O_2 , this has been termed aerobic glycolysis [107] as opposed to anaerobic glycolysis. If a similar amount of pyruvate as in the study by Galarraga is assumed for this glioma cell line (~ 10% of the lactate concentration), then ~ .85 molecules of lactate plus pyruvate were produced for every 1 molecule of glucose consumed. This translates to ~ 42 % of the glucose being consumed via aerobic glycolysis.

The distribution of cells within the cell cycle for the two growth protocols is shown in figures 20 and 21. For exponential cells approaching a plateau growth phase the proportion of cells in G_1/G_0 increased from 70% on day 4 to 85% on day 10. Increasing the pH on days 4 and 7 resulted in a slight increase in the number of cells in S and G_2/M with a corresponding decrease in the number of cells in G_1/G_0 . In the more dense plateau cultures the distribution of cells within the cell cycle was 87% G_1/G_0 , 11% G_2/M , 2% S on day 10. This did not change during the 6 day plateau period. In addition these confluent plateau cultures showed no significant change in cell cycle distribution after the two pH increases. These results are consistent with the relative cell number data shown in figure 15.

IP and DP survival levels for 7.5 Gy irradiation under the various growth conditions are shown in figures 22 and 23. With no pH change IP survival showed two

distinct features, an initial decrease as the cells progressed from an exponential growth phase to a plateau growth phase (2.3 % survival on day 4 to 0.7 % on day 7), and secondly, a further decrease to 0.22 % as cells remained in a plateau growth state from day 7 to day 10. A similar decrease in IP survival occurred to a greater extent in the extended plateau phase cultures (figure 23) with IP survival decreasing from 0.8 % on day 10 to 0.11 % on day 16. Note there was no significant change in plating efficiency of control cultures over the 6 days. This decrease in IP survival as cells were held in a plateau phase was not accompanied by any change in cell cycle phase distribution, and the pH, glucose and lactate curves were leveling off during this period as the rate of glycolysis approached zero.

In contrast, survival following a DP period of 24 h for experiments with no pH change was initially ~10 % for both exponential cultures (day 4) and extended plateau cultures (day 10) and showed a more moderate decrease to 4.5 % and 2.5 % respectively over the next 6 days. PLDR, expressed in terms of recovery ratios, increased from 4.2 on day 4 to 19 on day 10 for the exponentially growing cultures moving into a plateau phase, and from 13 on day 10 to 22 on day 16 for the extended plateau phase cultures.

Increasing medium pH 3 days prior to assaying for survival resulted in increases in both IP and DP for cells in plateau growth phase. At the time of irradiation the pH and rate of glycolysis were comparable to that in control experiments which had no pH change.

Increasing medium pH on day 7 for cells moving from an exponential to a plateau growth

phase resulted in increases in both IP and DP survival and a final RR (day 10) of 14 compared to 19 in control experiments which had no pH change. In the extended plateau growth phase experiments increasing medium pH on days 10 and 13 also resulted in increases in both IP and DP survival. The final RR on day 16 was 23, the same as in experiments with no pH change.

4.4 DISCUSSION

Radiosensitivity is typically defined by IP survival responses. This study shows that the IP radiosensitivity of human glioma cells increased as cells aged in a plateau growth phase culture. DP survival decreased to a lesser extent than IP survival, resulting in an amplitude of PLDR which increased as the radiosensitivity increased. These findings agree with a previous study with rodent cells [81]. During this period the rate of glycolysis in these cells approached zero concurrent with an acidic pH and lack of cell proliferation. This is the first indication in a human glioma cell line that chronic exposure to a metabolically quiescent state is not advantageous for absolute values of IP and DP survival. Since the recovery ratio (RR) actually increased during this period, chronic exposure to a metabolically quiescent state did not affect the cells ability to repair potentially lethal damage. The relative decreases in IP and DP sensitivity occurred as the pH decreased slightly which is in agreement with the study by Rottinger [91], except in this case the cells are known to be in a non-proliferative state during the entire period of

observation and therefore the increase in sensitivity cannot be explained by a decrease in proliferation status or cell cycle redistribution. It is also interesting to note that in contrast to the study by Rottinger, chronic exposure to low pH did not significantly decrease the plating efficiency. Although the decreases in IP and DP survival in plateau growth phase cells were not due to changes in proliferative status, the decrease in IP survival from day 4 to day 7 was probably due to the change in proliferative status as the cells moved from an exponential growth phase to a plateau growth phase (figures 14 and 20), in agreement with other studies [81,82].

As in a previous study [94], increasing the pH of the medium caused some additional cell growth in exponentially growing cell cultures (figures 14 and 20). However, increasing the pH of conditioned medium in plateau growth phase cultures did not result in an increase in cell proliferation (figures 15 and 21). This was also found by Varnes [108]. In addition, no increase in DNA synthesis was detected in that study. In conditioned medium, high ammonia and lactate concentrations, the waste products of glutaminolysis and glycolysis respectively, have been shown to inhibit cell growth [109,110]. A reduced level of L-glutamine which is present in conditioned medium also inhibits cell growth [111]. Factors other than acidic pH such as cell crowding and serum deprivation also inhibit DNA synthesis [96]. Given these inhibitory factors an increase in pH to stimulate glycolysis in conditioned medium on plateau growth phase cells is probably not sufficient to cause an increase in cell proliferation.

Stimulating glycolysis 3 days prior to assaying for survival increased both IP and DP survival in plateau growth phase cells as compared to control experiments which had no glycolytic stimulus. At the time of assaying for survival the rate of glycolysis, pH, cell cycle distribution and proliferative status were comparable to control experiments.

Although the pH was not exactly the same as in control experiments, a recent study with this cell line under similar conditions showed that the amplitude of recovery did not change significantly with a pH between 6.6 and 7.0 during the DP period [83]. Since the RR did not change significantly from control experiments, periods of increased metabolic activity prior to assaying for survival did not alter PLDR. The fact that the metabolic conditions were relatively static and similar to control conditions is an important factor. A previous study with plateau phase A549 lung carcinoma cells [108] reported that increasing the rate of glycolysis post-irradiation caused a decrease in the amplitude of PLDR. This is also examined in the next chapter.

Studies with the glycolytic inhibitor 2-deoxy-D-glucose have demonstrated that reduced ATP levels concomitant with decreased glycolysis resulted in a reduction in the number and the rejoining rate of strand breaks, which in turn lead to decreased radiation survival [112,113,114]. Reduced aerobic metabolism as a result of reduced levels of L-glutamine will also lead to a decrease in the levels of ATP [115] and a decrease in IP cell survival [111]. This may partially explain why there is less decrease in IP and DP survival after a period of increased metabolic activity as compared to a period of low

metabolic activity. A wide variety of solid tumours has been shown to maintain ATP levels by accelerated aerobic glycolysis as a consequence of impaired respiration [116]. Consistent with other studies [106] this glioma cell line also relied heavily on aerobic glycolysis (42 %). Glycolysis is widely accepted as the most important process for ATP and lactate production in tumour cells [117] although glutaminolysis is also thought to be responsible for much of the ATP production especially if glycolysis is inhibited [115,118].

These data show that the RR was virtually unaffected by the increased glycolysis protocol. It has been shown that PLDR requires energy from either aerobic metabolism or less effectively from anaerobic glycolysis [119] and both nutrient deprivation and lack of O_2 are required to inhibit sublethal and potentially lethal damage repair [120,121]. Since PLD repair prefers energy from the aerobic pathway, changes in glycolysis in the presence of O_2 should have little effect on its expression, which is what we observed in this study.

These experiments also indicate that low constituent levels of glucose and high levels of lactate do not inhibit the PLDR pathway, provided metabolic conditions favourable for PLDR remain static during the DP period. This may reflect conditions which exist in metabolically quiescent regions of solid tumours. With concentrations of lactate reaching 29 mM, 2-fold higher than in control experiments, the RR was unchanged. Exogenous lactate added post-irradiation at concentrations between 10-50

mM has been shown to suppress PLDR expression in three human tumour cell lines [122] and in normal diploid fibroblasts [123]. Since in the present experiments the lactate was produced by the cells and over a long period of time the cells may have adapted to this condition, its effect on metabolic processes that pertain to repair may be minimal.

The results presented in this paper are significant for studies of recovery in human cell lines for several reasons. Firstly, assessment of survival under long term irradiation conditions such as fractionation or continuous low dose-rate irradiation (~ 1 cGy/min) requires several days to deliver a dose that will reduce survival by several orders of magnitude. A decrease in both IP and DP survival would suggest increasing radiosensitivity as a function of time to be an important factor to consider in any low dose-rate or protracted fractionation studies *in vitro*. Furthermore, since repair processes are underway during low dose-rate irradiation, the decrease in DP survival may be the most significant result to consider. Secondly, these results lead to a RR which increased as the cells moved from an exponential to a plateau phase and with the amount of time the cells spent in a plateau growth phase without a medium change. This observation is significant to the use of RR's as an expression of repair capacity and radioresistance especially when comparing PLDR *in vitro* with PLDR *in vivo*. This study in a human glioma cell line shows that IP survival is not only sensitive to proliferation status, but is also sensitive to the amount of time spent in a metabolically quiescent condition. It is also interesting to note that DP survival after a dose of 7.5 Gy was maximal at 10% and

was less sensitive to proliferative and metabolic status although this level was best maintained with a period of increased metabolic activity prior to irradiation. It has been suggested that this maximum recovery potential (MRP) is a better predictor of radioresistance and may predict clinical outcome in radiotherapy [124]. Since the increase in RR was primarily due to IP survival decreasing at a faster rate than DP survival, these findings may explain why measured PLDR levels are lower in tumour cell lines which are increasingly radioresistant as classified by their IP survival response [10,21].

This study suggests that metabolically quiescent regions of a tumour may be more radiosensitive than other regions. This effect may only dominate in smaller tumours or in oxic regions of large tumours, since it is well known that if these regions are also hypoxic, they are much more radioresistant. In terms of the response of metabolically stimulated regions in a tumour to subsequent fractions of radiotherapy, this study indicates that these cells may develop a radioresistance similar to more metabolically active regions while retaining their ability to repair potentially lethal damage to a significant degree.

Figure 14: Cell growth summary: Exponentially growing cultures with no medium change and no pH change (squares), with no medium change, pH raised to 7.4 on days 4 and 7 (triangles), and with medium changes on days 4 and 7 (circles)

Figure 15: Relative cell number Plateau growth phase cultures having had medium changes on days 4 and 7, with no further medium or pH changes (squares), and with pH raised to 7.4 on days 10 and 13 (triangles).

Figure 16: Medium pH summary: Exponentially growing cultures with no medium change and no pH change (squares), with no medium change, and pH raised to 7.4 on days 4 and 7 (triangles).

Figure 17: Medium pH summary. Plateau growth phase cultures having had medium changes on days 4 and 7, with no further medium or pH changes (squares), and with pH raised to 7.4 on days 10 and 13 (triangles).

Figure 18: Glucose depletion (triangles) and lactate production (squares) Exponentially growing cultures with no medium change and no pH change (closed symbols), with no medium change, and pH raised to 7.4 on days 4 and 7 (open symbols)

Figure 19: Glucose depletion (triangles) and lactate production (squares): Plateau growth phase cultures having had medium changes on days 4 and 7, with no further medium or pH changes (closed symbols), and with pH raised to 7.4 on days 10 and 13 (open symbols).

Figure 20: Cell cycle phase summary: G_1/G_0 (squares), S (triangles), G_1/M (diamonds) phases. Exponentially growing cultures with no medium change and no pH change (closed symbols), with no medium change, and pH raised to 7.4 on days 4 and 7 (open symbols).

Figure 21: Cell cycle phase summary: G₁/G₀ (squares), S (triangles), G₂/M (diamonds) phases. Plateau growth phase cultures having had medium changes on days 4 and 7, with no further medium or pH changes (closed symbols), and with pH raised to 7.4 on days 10 and 13 (open symbols).

Figure 22: PLDR summary: IP survival (squares) and DP survival (triangles). Exponentially growing cultures with no medium change and no pH change (closed symbols), with no medium change, and pH raised to 7.4 on days 4 and 7 (open symbols). Recovery Ratio's defined as S_{DP}/S_{IP} ; given by numbers within rectangular bars. The DP period was 24 h.

Figure 23: PLDR summary: IP survival (squares) and DP survival (triangles). Plateau growth phase cultures having had medium changes on days 4 and 7, with no further medium or pH changes (closed symbols), and with pH raised to 7.4 on days 10 and 13 (open symbols). Recovery Ratio's defined as S_{DP}/S_{IP} ; given by numbers within rectangular bars. The DP period was 24 h.

5.0 Inhibition of Potentially Lethal Damage Recovery by increased metabolic activity and proliferation post-irradiation in plateau growth phase cells

5.1 INTRODUCTION

It is well established that many types of cells can recover from a certain portion of radiation-induced damage *in vitro* by holding the cells in conditions favourable for this recovery. Recovery measured in this manner has been operationally defined as potentially lethal damage recovery (PLDR) [60]. Studies with post-irradiation holding or delayed plating (DP) have shown that PLDR is maximal under environmental conditions which inhibit cell proliferation [61-63]. Replacing existing conditioned medium on plateau growth phase cells with fresh medium at the time of irradiation stimulates progression through the cell cycle and leads to a decrease in the amplitude of PLDR [108,125]. Although medium pH plays a role in cell cycle progression [94,95] it also regulates glycolysis [92,93]. Increased glycolysis post-irradiation, stimulated by raising medium pH, has been shown to inhibit PLDR [108]. In that study the cells were in a plateau growth phase under depleted medium conditions and the pH increase did not result in cellular proliferation.

In this study, using U-87MG cells grown to a plateau growth phase, PLDR was measured under four post-irradiation medium conditions. This study extends previous studies by examining further the extent to which increased glycolysis and cellular proliferation play a role in the inhibition of PLDR. Modification of pH from acidic to

basic (7.4) in the case of conditioned medium and from basic to acidic (6.8) in the case of fresh medium was utilized to manipulate the amount of cellular proliferation and glycolysis post-irradiation compared to conditioned and fresh medium with no pH adjustment, respectively. In addition, these four medium conditions were examined as the cells were held in a plateau growth phase for an extended period of time to see if changes in the amount of glycolysis or proliferation induced post-irradiation affects the amount of PLDR inhibition. It has been shown in chapter 4 that these cells enter a state of extreme metabolic and proliferative quiescence if held in a plateau growth phase for several days.

The findings of this study may have implications in the radiotherapy of solid tumours since they may contain regions of transient acidity and nutrient supply [18,90] and therefore the capacity for PLDR in these regions may vary. PLDR in human tumour cell lines has been associated with tumours which are clinically very radioresistant [20,21]. Glioblastoma are considered clinically to be very radioresistant [2,4] and *in vitro*, glioblastoma cell lines have shown a large degree of PLDR [33].

5.2 MATERIALS & METHODS

The human glioma cell line U-87MG, was cultured as described in section 2.2. Cells were irradiated with 750 cGy ^{60}Co (120-200 cGy/min) while suspended in an ice

water bath then trypsinized and assayed for survival either immediately (IP survival) or after a 24 h. delayed plating period (DP survival).

For all experiments 10^5 cells were plated in 25 cm² flasks. For all experiments the medium was changed on days 4 and 7 post plating. The cells entered a confluent plateau growth phase on day 10 with a cell density of $\sim 10^6$ cm⁻². Cells were irradiated on days 10,13, and 16 as the cells aged in conditioned medium. Just prior to irradiation the following adjustments were made to the medium: A) no change, B) pH of existing medium raised to ~ 7.4 , C) medium exchanged with fresh medium- pH lowered to ~ 6.8 , and D) medium exchanged with fresh medium- pH unaltered. Survival values were normalized to the plating efficiency of control flasks which were treated with the same medium change or pH adjustment protocols as those which received radiation. The plating efficiency of these cells under these conditions was approximately 30% with an observed range of 20-40%.

Cell survival was assayed by the colony formation assay (See appendix II). For delayed plating (DP) experiments the flasks were immediately warmed post-irradiation for 2 minutes in a 37°C water bath and then placed, medium unaltered, in a 37°C tissue culture incubator for 24 hours.

The pH of the existing or 'old' medium was raised to ~ 7.4 with the addition of 1N NaOH (5-10% of total volume). The pH of the fresh or 'new' medium was lowered with the addition of 1 N HCl. (5-10% of total volume) Medium pH was measured at

room temperature with an ATC pH electrode. The pH values labelled 'initial' represent the pH of the medium just after irradiation and were averaged from control and IP survival flasks sampled at the time of trypsinization. The pH values labelled 'final' represent the pH of the medium from DP survival flasks only.

Glucose utilization was measured as described in section 4.2

Cell cycle distributions were measured as described in section 4.2

All data points on all graphs represent the average of 3-4 independent experiments with uncertainty ($\pm$) values signifying the Standard Error of the Mean.

5.3 RESULTS

Figure 24 shows initial pH values on day 10, 13, and 16 post-plating and the final pH after a 24 hour DP period for the four medium conditions investigated. In all cases there was a decrease in pH during the DP period and in general this pH change decreased as the cells aged in the conditioned medium. The largest pH changes were seen when the initial pH was ~ 7.4 . This occurred when the pH of the existing or 'old' medium was raised and when the old medium was replaced with fresh or 'new' medium. Lowering the pH of the new medium to ~ 6.8 at the time of irradiation resulted in a very small pH change, similar to that observed when no pH adjustment was made to the old medium.

On day 10 the concentration of glucose in the medium was ~ 1.4 mg/ml ($\sim 50\%$ of that in fresh medium). By day 16 this value was ~ 1.0 mg/ml. Figure 25 shows the

utilization of glucose during the DP period corresponding to the four medium conditions. It is immediately obvious that very little glucose was used by the cells when they remained in the old medium which had no pH adjustment. This indicates that very little glycolysis occurred under these conditions. In contrast, increased glycolytic activity occurred in the three cases of altered medium conditions as indicated by the significant utilization of glucose. It is interesting to note that significant glucose utilization occurred under the new medium- pH lowered condition even though the pH was acidic and the pH gradient was comparable to that observed when no pH adjustment was made to the old medium.

Recovery from potentially lethal damage has been assessed by calculating recovery ratios (RR) as DP survival / IP survival. Figure 26 shows IP and DP survival values as well as the RRs on day 10, 13, and 16 for the four post-irradiation medium conditions. The largest amount of recovery was seen with no change made to the old medium. Compared to this level of recovery, raising the pH of old medium, exchanging with new medium- pH lowered, and exchanging with new medium- no pH adjustment, resulted in increasingly less recovery, respectively. PLDR can be thought to be inhibited under the latter three medium adjustment conditions as compared to the maximum recovery achieved under the old medium- pH unaltered condition. The extent of PLDR inhibition was quantified according to:

$$\begin{aligned}
 \text{PLDR inhibition} &= \frac{(\text{RR}_{\text{old med. - no adj.}} - 1) - (\text{RR}_{\text{med. adj.}} - 1)}{(\text{RR}_{\text{old med. - no adj.}} - 1)} \\
 &= \frac{(\text{RR}_{\text{old med. - no adj.}} - \text{RR}_{\text{med. adj.}})}{(\text{RR}_{\text{old med. - no adj.}} - 1)} \quad (12)
 \end{aligned}$$

Subtracting 1 from the RR was necessary since the minimum value for a RR is 1. This gives maximum and minimum values to PLDR inhibition of 1 and 0 respectively. The degree of PLDR inhibition on days 10, 13, and 16 is shown in figure 27. Inhibition was maximum when the old medium was exchanged with new medium- pH unaltered. Inhibition was almost complete on day 10 but decreased progressively on days 13 and 16, as the cells aged in a plateau phase. Figure 27 reveals that in all three cases the amount of PLDR inhibition was maximum on day 10 and progressively decreased on days 13 and 16.

To assess whether or not cellular proliferation occurred during the 24 h DP period, cellular redistribution within the cell cycle was measured. The plateau growth phase cell cycle distribution on day 10 was 85% G₀/G₁, 10% G₂/M, and 5% S. This distribution changed little as the cells were held in old medium- pH unaltered. On day 16 the distribution was 87% G₀/G₁, 11% G₂/M, and 2% S. The change in cell cycle distribution that occurred during the DP period as a result of the three adjusted medium protocols is shown in figure 28. No detectable change was observed for the old medium-

pH unaltered case. There was a dramatic decrease in the percentage of cells in G_0/G_1 when new medium at either initial pH was substituted compared to only a slight decrease for the old medium- pH raised condition. For the new medium- pH lowered case these cells moved primarily into S phase. For the new medium- pH unaltered case the cells moved further within the cell cycle with many of the cells redistributing to G_2/M . In all three cases the change in % cell cycle phase was maximal on day 10 and in general decreased on days 13 and 16.

The large shift in the cell cycle phase distribution that occurred when new medium at either initial pH was substituted is indicative of cellular proliferation. Indeed, following these two protocols an increase in the total cell number, compared to the old medium- pH unaltered case, was detected. The change in cell number relative to day 10 is shown in figure 29. Relative cell numbers are shown for new medium- pH lowered and new medium- pH unaltered at two and three days post medium change. Consistent with greater redistribution within the cell cycle in the new medium- pH unaltered case, the increase in relative cell number was also greater as compared to the new medium- pH lowered case. The old medium- pH raised case is not shown in figure 29 since it did not result in a significant change in cell number compared to the old medium- pH unaltered case.

5.4 DISCUSSION

The recovery ratio for the four post-irradiation medium conditions increased as the cells aged in a plateau growth phase. This is due to the fact that IP survival decreased significantly from day 10 to day 16. The decrease in IP survival under these conditions has been discussed in chapter 4 and may be related to the cells becoming increasingly metabolically and proliferatively quiescent. As seen in figure 24 the medium became increasingly acidic from day 10 to day 16. This has also been shown to decrease IP survival [91]. These plateau growth phase conditions are however favourable for maximal expression of PLDR [61-63,81,91]. As seen in figure 26 following 7.5 Gy γ -irradiation the cells expressed a large amount of recovery.

Compared to this control, PLDR was inhibited by post-irradiation manipulation of the medium. The degree of inhibition increased for old medium- pH raised, new medium- pH lowered, and new medium- pH unaltered, respectively. Under these three altered medium conditions significant glucose utilization occurred during the 24 h DP period as compared to the control case of old medium- pH unaltered. In the two cases where new medium was applied to the cell cultures there was significantly more glucose utilization than in the old medium- pH raised case. This may be related to the fact that there was increased re-distribution within the cell cycle during the DP period (figure 28) which eventually led to increased proliferation. (figure 29).

This study confirms in human glioma cells the result in A549 human lung carcinoma cells [108] that a post-irradiation increase in glycolysis, without an increase in proliferation, is sufficient to cause PLDR inhibition. PLDR inhibition by exchanging the old medium for new medium has been noted previously and correlated to increased proliferation [108,125]. In the study by Varnes [108], the amount of inhibition by alkalization of old medium and by exchanging the old medium with new medium was the same. In a human glioma cell line we found that the inhibition of PLDR by increased glycolysis was less than the inhibition produced by both increased glycolysis and increased proliferation. We also found that these two inhibitory stimuli may interact to produce almost total inhibition of PLDR (figure 27, day 10)

In addition, this study shows that the degree of inhibition may be related to the rate of glycolysis during the DP period (compare figure 27 with figure 25) and/or related to the amount of cellular proliferation induced during the DP period (compare figure 27 with figures 28 and 29). The latter point is supported by the fact that as the cells aped in a quiescent plateau growth phase, the decrease in the amount of inhibition correlated to the general decrease in the amount of re-distribution within the cell cycle

This study also shows that an acidic pH is not sufficient to insure that the amount of PLDR is maximal. Although the pH in the new medium- pH lowered case was 6.8 and decreased only slightly during the DP period (similar to the control case), a large amount of inhibition of PLDR occurred. Despite the low pH, increased glycolysis and

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increased proliferation occurred during the DP period, resulting in the inhibition of PLDR. Fresh medium must contain sufficient nutrients and growth stimulating factors to overcome the fact that a low pH tends to inhibit glycolysis and proliferation. It has been noted that many tumour cell lines actually prefer a low pH for optimal cell growth [95]. The tendency for low pH to inhibit glycolysis and proliferation is evident however by the difference in the utilization of glucose and the degree of proliferation which occurred between the new medium- pH lowered and the new medium- pH 7.4 cases. Less glucose utilization and less proliferation did result in less inhibition of PLDR than in the new medium- pH lowered case.

Following alkalization of the old medium, the increased rate of glycolysis resulted in very little glucose remaining in the medium at the end of the DP period. In another study we have shown that PLDR is not affected by these low levels of glucose in the medium as long the metabolic processes remained static during the DP period [chapter 4]. In the study by Varnes [108] it was concluded that pH and glucose concentration are important factors when studying PLDR.

This study suggests that perhaps the rates of glycolysis and proliferation are the most important factors to consider when measuring the capacity of cells to recovery from PLD. It is noteworthy however that extracellular pH may affect these two processes differently in different cell lines, thus playing a role in the expression of PLDR.

Examining the range in PLDR achieved by post-irradiation manipulation of the growth medium pH may provide some indication of the range in PLDR that may be expected in the different regions of solid tumours. In addition, this study suggests that in the treatment of solid tumours with fractionated radiotherapy, increasing glycolysis post-irradiation may decrease the amount of PLDR taking place after each fraction. A recent study has indicated that the chemotherapy drug verapamil increases glycolysis in some cell lines [126], and therefore it may be useful as an adjuvant to radiotherapy by decreasing PLDR in the irradiated tumour.

Figure 24: Medium pH summary: pH values for days 10, 13 and 16 post-plating. Initial values (triangles) are at the time of irradiation. Final values (squares) are after a 24 h delayed plating (DP) period under the four medium conditions given in the figure. The four medium conditions are: A) old medium-pH unaltered, B) old medium- pH raised, C) medium exchanged with new medium-pH lowered, and D) medium exchanged with new medium- pH unaltered. The term 'old' refers to the existing conditioned medium.

Figure 25: Glucose utilization summary: Glucose utilization [mg/ml] during the 24 hour DP period for days 10, 13 and 16 post-plating, under the four medium conditions given in the figure.

Figure 26: PLDR summary: IP survival (squares) and DP survival (triangles) for days 10, 13 and 16 post-plating, under the four medium conditions given in the figure. Recovery ratios defined as S_{DP}/S_{IP} ; given by numbers within rectangular bars. The DP period was 24 h.

Figure 27: PLDR Inhibition summary: PLDR inhibition for days 10, 13 and 16 post-plating, under the three adjusted medium conditions given in the figure. PLDR inhibition is given by: $(RR_{\text{old med - no adj}} - RR_{\text{med - adj}}) / (RR_{\text{old med - no adj}} - 1)$.

Figure 28: Change in cell cycle summary: Change in % cells in the cell cycle phases during the 24 hour DP period for days 10, 13 and 16 post-plating, under the three adjusted medium conditions given in the figure. The change is given as a difference in percentage points from the old medium- pH unaltered condition at the time of irradiation

Figure 29: Relative cell number with new medium added at the time of irradiation. The cell number relative to day 10 is shown for old medium- pH unaltered (circles) and for new medium added on days 10, 13, and 16 post-plating. Relative cell number is shown for new medium- pH lowered (triangles) and new medium- pH unaltered (diamonds) at two and three days post medium change.

6.0 LOW DOSE-RATE IRRADIATION AND THERMORADIO-SENSITIZATION BY MILD HYPERTHERMIA

6.1 INTRODUCTION

In spite of current treatment modalities the prognosis for patients with a brain tumour classified as glioblastoma multiforme (GM) or anaplastic astrocytoma (AA) is poor, with localized recurrence within a few years cited as the main cause for failure. Interstitial radiotherapy or Brachytherapy has yielded modest improvements in the time to recurrence, but long term survival has not changed significantly, especially for patients with GM [6,7]. Typical dose-rates employed with Brachytherapy are from 0.3 to 1 Gy per hour. In this range of low dose-rate irradiation (LDR) increased cellular recovery occurs due to repair of sublethal damage [8]. A recent review has implicated the intrinsic radioresistance of these tumours caused by repair of sublethal damage as a major cause for failure with Brachytherapy [2]. LDR studies *in vitro* have shown that human glioma cell lines are at the radioresistant end of the radiation sensitivity spectrum [11-13].

Hyperthermia has been shown to be an effective radiosensitizer *in vitro* [26,28] and *in vivo*, especially when given simultaneously with radiation [27]. There have been many animal tumour studies of hyperthermia (> 42°C) given before or after LDR that have shown varying degrees of sensitization [127-129]. One of these studies indicated that hyperthermia given during LDR provided greater thermal enhancement [127]. In many clinical trials heating either before or after Brachytherapy has shown varying degrees of improvement [130,131]. Clinical trials with brain tumours have shown only moderate

(AA) to no (GM) improvements in long term survival with external or interstitial heating before or after Brachytherapy [35,36]. The above studies have typically involved hyperthermic temperatures $> 42^{\circ}\text{C}$ and heating times up to 1 hour. Less is known about the interaction mechanisms of heat delivered chronically at temperatures $< 42^{\circ}\text{C}$ and radiation delivered at low dose-rates. One of the reasons for the overall lack of significant improvement with these treatments is thought to be the failure to heat the entire tumour volume to high enough temperatures to fully realize the thermoradiosensitization of temperatures $> 42^{\circ}\text{C}$. Another reason may be the fact that the hyperthermia treatment only interacts with a portion of the LDR treatment.

Recently brachytherapy and simultaneous mild hyperthermia ($< 42^{\circ}\text{C}$) has been utilized in Phase I/II clinical trials with pelvic and brain malignancies and has shown promising results [37,38]. *In vitro* studies on exponentially growing rodent cell lines have shown significant thermal enhancement with simultaneous LDR and mild hyperthermia ($\leq 41^{\circ}\text{C}$) [132-137]. Results with human cell lines in exponential growth phase [138] and plateau growth phase [139] have also shown a wide degree of thermoradiosensitization when 41°C was delivered continuously during LDR.

In this study we examined the thermoradiosensitization effect of mild hyperthermia ($\leq 41^{\circ}\text{C}$) on LDR with two human glioma cell lines in a stationary growth phase. LDR of a stationary cell population allows the examination of the LDR effect due to the repair of sublethal damage without significant contributions from cellular

redistribution within the cell cycle and repopulation [8]. A specific aim of this study was to assess the degree of thermo- radiosensitization with LDRI at 39, 40, and 41°C under these *in vitro* conditions. The direct cytotoxicity of these heat treatments was also evaluated.

The effect of two pre-irradiation heating regimes on LDRI delivered at 37°C and 41°C was also examined. Specifically the degree of thermoradiosensitization of pre-heating on a single dose of LDRI at 37°C was assessed, as well any additional thermoradiosensitization that pre-heating may have on the thermoradiosensitization achieved by a single dose of LDRI at 41°C. Two pre-irradiation heating regimes, 8h at 41°C and 1h at 43°C, were chosen since they involve temperatures that may have been achieved with previous clinical trials and they represent pre-irradiation heating regimes that could be used in future trials employing LDRI. In addition, these two heating regimes alone produced only a modest amount of cell kill (approximately 25%)

6.2 MATERIALS AND METHODS

The cell lines U-87MG and U-373MG, were cultured as described in section 2.2. For all experiments 4×10^4 cells per cm^2 were plated in 25 cm^2 flasks for HDRI and in 1.3 cm^2 glass vials for LDRI. The medium was changed (4 ml in flasks and 0.5 ml in vials) on days 4 and 7 post-plating with experiments commencing on day 10 at which time the monolayers were in a stationary growth phase as verified by growth curves and cell cycle

distributions (85%, and 80% G_0/G_1 respectively). Cell population double times were approximately 28 hours for both cell lines. Cell densities at a plateau growth phase were $1-1.5 \times 10^5$ and $2-2.5 \times 10^5$ per cm^2 respectively. Medium pH at the start of experiments for both cell lines was 6.90 ± 0.5 . The plating efficiencies for these cell lines under these conditions were approximately 25-35% and 18-25%, respectively, in either flasks or vials.

For HDR, cells were irradiated on ice with a ^{60}Co therapy unit at a dose-rate of 150- 200 cGy/min. For LDR, cells were irradiated by an array of ^{226}Ra sources (total activity 800 mCi) contained in a temperature controlled incubator (See appendix I). Two low dose-rates were used, LDR_1 at 0.88 cGy/min. and LDR_2 at 0.41 cGy/min. For LDR at all temperatures, accurate temperature control ($\pm 0.1^\circ\text{C}$) was achieved by monitoring thermocouples placed in the irradiation chamber. Hyperthermia treatment alone was accomplished by water bath heating ($\pm 0.05^\circ\text{C}$).

Cell survival was assayed by the colony formation assay (See appendix II).

Dose reduction factors ($\text{DRF}_{1\%}$) are defined as the ratio of the LDR dose and the HDR dose at 37°C to achieve a survival of 1%. Thermal Enhancement Ratios (TER) at the survival levels were calculated by dividing the dose of radiation at 37°C by the dose of radiation at the specified hyperthermic temperature to achieve the same survival level with survival at the hyperthermic temperature corrected for the cell kill due to heating alone. Survival reduction factors (SRF) were calculated from the % survival values

normalized for the cell kill due to heat alone by dividing the survival without pre-heating by the survival with pre-heating

All data points for U-87MG results represent the average of a least 4 independent experiments while data points for U-373MG results represent the average of 2-3 independent experiments. Error bars on data points are shown only when they extend further than the data symbol. All uncertainty estimates ($\pm$ values) represent the Standard Error of the Mean.

6.3 RESULTS

The cell survival response of these two human glioma cell lines to HDR and LDRI was shown in chapter 2. While their HDR response was similar, with U-373MG being slightly more resistant, their LDR survival varied at the two low dose-rates used. The Linear-Quadratic fits of these data have been re-drawn in figures 30 and 31. Listed in table 5 are the Dose Reduction Factors (DRF) at the 1% survival level. These LDR survival responses were not complicated by any measurable redistribution within the cell cycle or repopulation, as measured by flow cytometry and growth curves, respectively.

The results of LDRI and continuous concurrent 41°C hyperthermia are shown in figures 30 and 31. The corresponding 10% and 1% isosurvival TFRs are given in table 5. For both glioma cell lines LDR_1 at 41°C produced almost complete inhibition of the sparing effects of LDRI. If inhibition were complete the TFR_{10} values should equal the

DRF₁₀ values. At the lower dose-rate, LDR₂, heating at 41°C resulted in even greater thermoradiosensitization for U-87MG. However there was less thermoradiosensitization for U-373MG with LDR₂ at 41°C, as indicated by a lower TER. For both cell lines thermoradiosensitization with LDR₂ at 39°C and 40°C was similar and was much less than with LDR₂ at 41°C (figures 32 and 33). There was some thermal enhancement with 39°C and 40°C with U-87MG however with U-373MG thermoradiosensitization was essentially zero as indicated by TER₁₀ values of 1.0.

The TERs have been normalized to the survival response due to these mild hyperthermic temperatures alone. The heat survival response curves are shown in figures 34 and 35. These two glioma cell lines show several interesting differences in their response to 39, 40, and 41°C. Firstly, U-87MG show an initial sensitivity at short heating times (see inset figure 34) for all three temperatures which was not seen with U-373MG. Apart from this difference the response to 39°C was similar for both cell lines. The second interesting difference is their response to 40 and 41°C at the longer heating times. The response of U-87MG to 41°C, if plotted on a linear scale, appears quite linear ($R^2 = .99$) from 30 min. to 72 hours with cell survival decreasing by ~ 1% per hour. This data set has the most data points and an average of 6 independent experimental values per data point. In comparison the U-373MG response to 41°C was not quite as linear ($R^2 = .79$), showing increased resistance from ~ 20 hours to 56 hours. This deflection may not be statistically significant however. At 40°C the response is similar in both cell lines up to ~

30 hours. For heating times greater than 30 hours survival continues to decrease for U-87MG, while for U-373MG survival tends to level off.

Table 6 shows the cell survival results for U-87MG after 9 Gy LDRI at 37 and 41°C with the addition of two pre-irradiation heat sequences. Pre-heating for 8h at 41°C resulted in little direct heat cell kill and no thermoradiosensitization of LDRI at 37°C. This is indicated by a Survival Reduction Factor (SRF) of 1.0. While 1h at 43°C produced the same amount of heat cell kill as 8h at 41°C, pre-heating at 43°C followed by LDRI at 37°C did result in some thermoradiosensitization (SRF 1.4). When the cells were exposed to LDRI at the mild hyperthermic temperature of 41°C, pre-heating for 8h at 41°C and 1h at 43°C resulted in significant additional thermoradiosensitization as indicated by SRF's of 1.6 and 3.5, respectively. The 'HEAT ALONE' values also indicate significant synergism with the 'step-down' heating sequence of 1h at 43°C followed by 36.4 h at 41°C.

The pre-irradiation heat sequences of 8h at 41°C and 1h at 43°C produced similar cell survival levels when plated immediately (74.8 ± 2.2 and 74.6 ± 1.4 , respectively). As indicated from the values in table 6, after 36.4h of incubation at 37°C some recovery of heat damage occurred (recovery factors of 1.16 and 1.14, respectively)

6.4 DISCUSSION

The DRF_{10} values for LDRI at 37°C are indicative of a large amount of LDR sparing as compared to other human tumor cell lines [47], and are in the range of values reported for human glioma cell lines [11,12].

If the sparing achieved by irradiating at low dose-rates is totally inhibited by concurrent mild hyperthermia, then the TER_{10} values should equal the DRF_{10} values. One would expect that if the level of inhibition is maintained as the dose-rate is lowered from LDR_1 to LDR_2 , then the TER_{10} should increase as the DRF_{10} increases. Although this was the case with the U-87MG cell line it was not the case with the U-373MG cell line. It has been observed that the degree of thermoradiosensitization by mild heating concurrent with LDRI may be related to the heat sensitivity of the cell line [139,140]. U-87MG showed a linear decrease in survival through to 72 hours while U373MG showed a change in heat sensitivity at heating times greater than 24 hours (figure 35).

The possibility of thermotolerance development in the case of the U-373MG cells at the longer heating times may explain the lack of continual thermoradiosensitization. Similar to the response of the U-87MG cell line, excellent thermoradiosensitization and no thermotolerance expression with chronic heating at 41°C has been observed in other human cell lines [139]. Currently it is not known whether thermotolerance development occurs in plateau growth phase cultures with chronic mild hyperthermia and whether its

expression or lack of expression plays a role in thermoradiosensitization of concurrent with LDRI.

The thermoradiosensitization seen previously with concurrent mild hyperthermia and LDRI in rodent [132-137] and human cell lines [138], was with cells in an exponential growth phase. In the human cell studies 41°C also inhibited proliferation of the cell populations [141]. Thus in exponentially growing cell systems, changes in proliferation status and cell cycle distribution may not only affect the amount of LDR recovery as discussed in section 2.4 but also the degree of thermoradiosensitization. In this study these factors remained fairly static during the treatments and therefore it is thought that these TERs reflect to a greater extent the ability of mild hyperthermia to inhibit LDR recovery. The fact that these monolayers were in a stationary growth phase does not preclude the existence of a small subpopulation of cycling cells which may, to some extent, affect both the degree of LDR sparing and the degree of radiosensitization.

The results with pre-heating indicate that the sparing effects achieved with LDRI at 37°C are not inhibited by pre-heating up to 8 hours at 41°C. This supports the lack of thermoradiosensitization seen with *in vivo* studies with temperatures less than 42°C. Pre-heating for 1 hour at 43°C showed some thermoradiosensitization (SRF 1.4) and supports *in vivo* results in which target temperatures closer to 43°C were achieved. These data are in partial agreement with *in vitro* studies with exponentially growing 9L rat astrocytoma cells [136]. In that study, neither heating protocols enhanced cell sensitivity

to LDRI. The lack of thermoradiosensitization with 41°C pre-heating in these human cells disagrees with earlier data with rodent cells [133,134].

When LDRI is delivered with concurrent continuous 41°C the results of this study suggest that pre-heating may provide additional thermoradiosensitization. This occurred most strongly with a step-down protocol (SRF 3.5). Enhanced thermoradiosensitization by pre-irradiation step-down heating has been observed in rodent cells [142] and in animal tumour systems with LDRI at 37°C [143] and HDRI [144]. In the animal studies maximal thermoradiosensitization was achieved when heat was given simultaneous with radiation. In this study the step-down protocol also resulted in synergism in heat alone survival values consistent with previous *in vitro* studies [145,146].

In the present study with human glioma cell lines we have shown good thermoradiosensitization with concurrent 41°C heating even though the direct heat cytotoxicity during these treatments was not very severe. Thermoradiosensitization at temperatures less than 41°C was less encouraging.

With regard to *in vivo* clinical trials with brachytherapy and hyperthermia, this study emphasizes that temperatures > 41°C are necessary for significant thermoradiosensitization with pre-irradiation heating if brachytherapy is conducted at 37°C. If brachytherapy is delivered with concurrent 41°C hyperthermia, results of this study indicate that additional thermoradiosensitization may be realized by pre-heating with temperatures as low as 41°C. In addition, significant additional thermoradiosensitization may be realized with a step-down protocol

Table 5: Dose Reduction Factors and Thermal Enhancement Ratios.

CELL LINE	DOSE-RATE	DRF _{1.0} *	TEMP (°C)	TER _{10.0} ** (± .05)***	TER _{1.0} (± .05)
U-87MG	LDR ₁	1.8	41	1.6	1.6
	LDR ₂	2.6		1.9	2.1
	LDR ₂		40	1.4	
	LDR ₂		39	1.3	
U-373MG	LDR ₁	1.9	41	1.6	1.8
	LDR ₂	2.0		1.2	1.5
	LDR ₂		40	1.1	
	LDR ₂		39	1.0	

*DRF_{1.0} is the Dose Reduction Factor defined as the ratio of the LDR dose and the HDR dose to achieve a survival of 1%.

**TER's at the 10%, and 1% isosurvival levels were calculated by dividing the dose of radiation at 37°C by the dose of radiation at the specified hyperthermic temperature to achieve the same survival level with survival at the hyperthermic temperature corrected for the cell kill due to heating alone.

***Average uncertainty estimate.

LDR₁ = .88cGy/min.

LDR₂ = .41 cGy/min.

Table 6: Percent cell survival values for U-87MG after 9 Gy LDRI at 37 and 41°C with 2 pre-irradiation heat sequences.

	PRE-IRRADIATION HEAT SEQUENCES					
	37°C DURING IRRAD. TIME			41°C DURING IRRAD. TIME		
	NONE (+/-)	8h-41°C (+/-)	1h-43°C (+/-)	NONE (+/-)	8h-41°C (+/-)	1h-43°C (+/-)
9 Gy LDR₂ (36.4 h)	14.1 (0.9)	12.4 (1.1)	8.4 (1.3)	0.41 (0.04)	0.21 (0.03)	0.056 (0.005)
HEAT ALONE*	100	86.6 (3.6)	84.7 (4.2)	47.8 (1.5)	39.7 (1.3)	22.8 (4.5)
NORMALIZED SURVIVAL	14.1 (0.9)	14.3 (1.4)	9.9 (1.6)	0.85 (0.09)	0.52 (0.07)	0.24 (0.05)
SURVIVAL** REDUCTION FACTORS	—	1.0 (0.1)	1.4 (0.2)	—	1.6 (0.3)	3.5 (0.9)

*For HEAT ALONE controls, cells were incubated for the pre-heat time and temp. indicated plus further incubation for 36.4h at either 37° or 41°C.

**Survival reduction factors were calculated from the normalized %survival values as (survival without pre-heating) / (survival with pre-heating).

Figure 30: Cell survival following LDRI at 41°C: U-87MG LDRI₁ (circles), LDRI₂ (squares).

Figure 31: Cell survival following LDRI at 41°C: U-373MG. LDR₁ (circles), LDR₂ (squares).

Figure 32: Cell survival following LDR₂ at 39 (circles), 40 (triangles), and 41°C (squares). U-87MG.

Figure 33: Cell survival following LDR₂ at 39 (circles), 40 (triangles), and 41°C (squares). U-373MG.

Figure 34: Cell survival following heating at 39 (circles), 40 (triangles), and 41°C (squares). U-87MG.

Figure 35: Cell survival following heating at 39 (circles), 40 (triangles), and 41°C (squares) U-373MG

7.0 INHIBITION OF POTENTIALLY LETHAL DAMAGE RECOVERY BY 41°C HYPERTHERMIA

7.1 INTRODUCTION

Hyperthermia has been shown to be an effective radiosensitizer *in vitro* [26,28]. Its use as an adjuvant therapy with high dose-rate radiotherapy is well documented [27]. There have been many animal tumour studies [127-129] and clinical trials [36,130,131] with hyperthermia given before or after low dose-rate irradiation (LDRI) that have shown varying degrees of sensitization. One of the reasons for the overall lack of significant improvement with these treatments is thought to be the failure to heat the entire tumour volume to high enough temperatures to fully realize the thermoradiosensitization effect of hyperthermic temperatures $> 42^{\circ}\text{C}$. Another reason may be the fact that the hyperthermia treatment only interacts with a portion of the brachytherapy treatment.

It has been demonstrated that human cell lines which express recovery from sublethal damage during LDRI in a stationary growth phase also express significant PLDR [10,16]. PLDR is thought to play a role in the resistance of human tumours to radiotherapy [20,21].

In previous chapters a large degree of PLDR has been demonstrated in human glioma cells under stationary growth conditions and this has been effectively reduced by hyperthermic temperatures $> 42^{\circ}\text{C}$ [33]. In this study, we examined the effect of a sub-optimal hyperthermic temperature, (41°C), on the recovery of PLD in a human glioma cell line. Specifically we measured the degree of inhibition by heating prior to

irradiation and heating after irradiation. In the post-irradiation experiments we examined the effect of heating during the period of rapid recovery, and heating after the recovery was complete.

7.2 MATERIALS AND METHODS

The cell line U-87MG was cultured as described in section 2.2.

For all experiments 4×10^3 cells per cm^2 were plated in 25 cm^2 flasks. The medium was changed (4 ml) on days 4 and 7 post plating with experiments commencing on day 10 at which time the monolayers were in a stationary growth phase as verified by growth curves and cell cycle distributions (85% G_0/G_1). Cell density at this time was $1-1.5 \times 10^5$ per cm^2 . Medium pH at the start of experiments was $6.80 \pm .05$ and did not change by more than 0.1 unit during experiments. The plating efficiency of this cell line under these conditions was approximately 25-35%.

The cells were irradiated on ice with a ^{60}Co therapy unit at a dose-rate of 150- 200 cGy/min. Hyperthermia treatment alone was accomplished by water bath heating (± 0.05 °C)

For delayed plating (DP) experiments the flasks were immediately warmed post-irradiation for 2 minutes in a 37°C water bath and then placed, medium unaltered, in a 37°C tissue culture incubator for the required period of time.

Cell survival was assayed by the colony formation assay (See appendix II).

All data points on all graphs represent the average of 3-4 independent experiments with error values signifying the Standard Error of the Mean

7.3 RESULTS

Figure 9 showed the result of a PLDR time course experiment at a dose of 7.5 Gy. This data was fit to a single exponential function yielding a half time for recovery of 3.5 hours. To examine the effect of mild hyperthermia on PLDR, 41°C was applied for heating times (HT) from 30 min. to 24 hours before irradiation, 'pre-heating', and after irradiation, 'post-heating'. Figure 36 shows the result for pre-heating. Survival values for immediate plating (IP) and a 24 hour delayed plating (DP) period (S0 and S24, respectively) have been normalized for the direct cell kill due to the heat treatments. S24 values were also corrected for a certain amount of recovery from heat damage since in this case cells were pre-heated and then incubated for the DP period. Control experiments to measure the degree of heat recovery yielded an average heat recovery factor of 1.24 ± 0.03 with heating times from 30 min. to 24 hours and post-heating incubation periods at 37°C from 16 to 24 hours. It is immediately evident that 41°C sensitizes HDRI or 'acute' irradiation to a greater extent than it did LDRI (chapter 6). However, the question remains, is cellular recovery from PLD inhibited by pre-heating with 41°C? Recovery Ratio's (RR's) defined as S_{24}/S_0 relative to $HT = 0$ are also shown in figure 36. It can be seen from the relative RR's that cellular recovery of PLD was not inhibited by

pre-heating with 41°C hyperthermia. In fact the relative RR actually increased with pre-heating time

Figure 37 shows the results of 41°C heating after irradiation or 'post-heating'. For these experiments two protocols were used in which heating at 41° C was a fraction of the total post-irradiation DP period, which was kept constant at 24 hours. This enabled RR's calculated as S_{24}/S_0 to be compared. The first protocol involved heating the cells immediately after irradiation with heating times (HT) from 30min to 24 hours and then returning the cells to 37°C for a time 't' equal to the remainder of the 24 hour DP period, i.e. $HT + t = 24h$. For the second protocol the sequence of heating and incubation at 37°C was reversed ($t + HT = 24h$). The first protocol examined the effect of heating while the cellular recovery processes were occurring, as indicated in figure 9, and the second, the effect of heating after cellular recovery has reached a maximum. Since the S_{24} values in both protocols include the effects of both radiation and heat, they were normalized to the cell survival due to the heat alone. Note that for the 'HT + t' protocol, normalization to heat survival also included the heat recovery factor mentioned previously, except for the case $HT = 24, t = 0$, in which no heat recovery was allowed. The results for the RR's relative to $HT = 0, t = 24$ (maximal recovery), are shown in Figure 37. It is immediately obvious that recovery from PLD is inhibited by post-irradiation heating at 41°C, only if the heat is delivered while the cellular processes responsible for this recovery are active. These experiments also demonstrate that 41°C hyperthermia when delivered during the

entire PLDR period (in this case 24 hours) is sufficient to totally inhibit this type of cellular recovery (Total inhibition of recovery = relative RR of approx 0.1)

7.4 DISCUSSION

A large degree of PLDR was seen in this cell line when assayed under plateau growth phase conditions. Under these conditions PLDR should be maximal [61,62]. HDR sensitization was seen with respect to both IP and DP survival values by pre-heating at 41°C, but there was no inhibition of PLDR. Post-irradiation incubation at 41°C during the various segments of the PLDR period suggest that PLDR can be totally inhibited by 41°C only if heat is delivered while the recovery processes are taking place. If the recovery of PLD and the recovery of sublethal damage which occurs during LDRI are in fact recovery from the same type of repairable lesion [59,65] then this result supports the large degree of thermoradiosensitization seen in this cell line with concurrent 41°C and LDRI (chapter 6). Post-irradiation incubation at 41°C did not inhibit PLDR if cellular recovery was complete prior to heating.

This study supports the lack of thermoradiosensitization seen with *in vivo* studies when temperatures less than 42°C were delivered before LDRI and before or after LDRI. Although previous work with rodent cells has shown that 41°C has inhibited PLDR [147], this is the first demonstration in a radioresistant human glioma cell line that 41°C can inhibit PLDR.

One of the mechanisms of radiosensitization is thought to be inhibition of radiation damage repair processes. Many studies with temperatures $> 42^{\circ}\text{C}$ have shown that hyperthermia can inhibit the repair of DNA strand breaks [28-30] and that there is correlation between thermoradiosensitization and heat inactivation of DNA polymerases α and β [31,32]. At temperatures $< 42^{\circ}\text{C}$ however, the mechanisms of the inhibition of cellular recovery are less understood.

Figure 36: Cell survival following 7.5 Gy HDR1 with pre-irradiation heating (HT) at 41°C. Survival after immediate plating - 'S0' (triangles) Survival after a 24 hour delayed plating period at 37°C - 'S24' (squares). Relative recovery ratio's (S24/S0) relative to HT = 0 (circles). S24 values have been normalized to heat survival

Figure 37: Relative recovery ratios for post- irradiation heating (HT) at 41°C. Recovery ratio = S_{24}/S_0 . S_0 = survival after immediate plating. S_{24} = survival after a 24 hour delayed plating period under the following conditions: HT + t (triangles), t + HT (squares), with t = incubation at 37°C such that HT + t = 24 hours. , Irradiation dose = 7.5 Gy HDR. Recovery ratios relative to HT = 0. S_{24} values have been normalized to heat survival.

8.0 SELECTED COMPLEMENTARY EXPERIMENTS

In this chapter several noteworthy experiments are presented which, on their own, did not warrant a separate chapter. They are however significant in their relevance to the studies presented in this thesis. The results of these experiments are discussed with reference to the previous chapters only.

8.1 ON THE NATURE OF A PLATEAU GROWTH PHASE

Unlike normal cells or transformed normal cell lines, tumour cell lines when grown *in vitro* do not express contact inhibition. It is well known that most tumour cell lines enter a plateau growth phase (see figures 1 and 2) largely because the growth stimulating factors contained in the medium have been depleted and the waste products of the cells metabolic processes have accumulated. This topic was discussed in chapter 4. Chapter 4 also examined several factors which changed as cells were held in a plateau growth phase and how these factors affected radiation survival and PLDR. Although increased proliferation was observed in exponentially growing cells with increases in medium pH, it was concluded that cells in plateau phase were not stimulated to proliferate by increases in medium pH, and that for many reasons, plateau phase medium did not support further growth by the cells. These two points were examined further in a series of experiments with U-87MG cells.

'Conditioned' medium from day 11 plateau growth phase cultures was collected, sterile filtered, and exchanged with the existing medium on day 2 cultures. Figure 38 shows the result of this experiment. Within two days the cells approached a plateau growth phase at a much lower cell density (factor of 10) than if fresh medium was added on days 4 and 7 (repeated from figure 1, circles). The pH of this conditioned medium was raised on day 4, which resulted in a significant increase in proliferation compared to no pH increase. The relative increase was much greater than in the exponentially growing cultures which has pH increases to the existing medium on days 4 and 7 (repeated from chapter 4 for comparison). Figure 39 shows the cell cycle distributions for these cells. This analysis confirms that when the conditioned medium was added, the cells moved from an exponential growth phase to a plateau growth phase. In addition, the cell cycle analysis shows that raising the pH of the medium tended to keep the cells in a more proliferative state. Immediate and delayed plating survival was also measured on days 4 and 6 (figure 40). By day six these growth arrested cultures showed survival and PLDR characteristics of the plateau phase cells in chapter 4.

These experiments confirm that medium overlying plateau growth phase cells does not support proliferation. In addition they support the statement that a plateau growth phase in tumour cell lines is more a function of the condition of the growth medium, and not the cell density. Unlike the situation with extended plateau phase cultures (chapter 4), raising the pH of low density growth arrested cultures caused

increased proliferation. This increase in proliferation manifested itself as an increase in IP-survival on day 6 (figure 40). This result supports the results in chapter 4 with regard to the increase in IP-survival in exponentially growing cultures as the pH was increased, but does not explain the increase in IP and DP survival seen in chapter 4 with plateau phase cells as the pH was increased.

8.2 CELL CYCLE DISTRIBUTION WITH LDRI AND 41°C

Although the results of section 8.1 indicated that plateau growth phase medium did not support further cellular proliferation, the possibility of growth in the low dose-rate vials during LDRI with/without 41°C hyperthermia was examined. If proliferation occurred during the LDRI at 37°C or at 41°C, it may have affected the shape of the survival response curves. The cell cycle distributions were measured for cells which received LDRI for 3 days at 37°C or 41°C and were compared to those from cells which received no irradiation grown in either vials or flasks. Figure 41 shows the result of this study. It is immediately obvious that the cell cycle distribution characteristic of a plateau growth phase in flasks is also reflected by the cells grown in vials. This distribution was maintained during the 3 day period and was not affected by either LDRI at 37°C or LDRI at 41°C. Heating at 41°C alone also had no effect. These results are for the U-87MG cell line.

8.3 LDR SURVIVAL VALUES - INFLUENCE OF HYPOXIA AND PH

In the LDR experiments the vials containing the cells were irradiated with the caps on the vials closed for the duration of the experiment. This was necessary to insure sterility. Keeping the caps closed during the chronic exposure to LDRI raised two concerns. The first was the possibility that hypoxia (low O_2 concentration) may have developed as the cells used up the remaining O_2 in the medium. If hypoxia had developed, then the LDR survival responses would be an overestimate of the survival of cells in oxic conditions. It is well known that cells in the presence of O_2 are 2-3 times more sensitive to radiation as compared to irradiation under hypoxic conditions [40]. The second concern was the possibility that a closed system would soon be without the CO_2 which is present in the incubators where the cells are grown. CO_2 is required by the sodium bicarbonate in the medium to buffer the pH. A lack of CO_2 would cause the pH to increase. It is known from the studies in chapters 4 and 5 that pH changes can affect the survival outcome.

To address these concerns a series of experiments were conducted in which vials were subject to 5 different treatments every 12 hours for up to 60 hours of LDRI. These conditions are briefly as follows: A) no medium change, caps closed; B) no medium change, caps opened for 5 minutes in the tissue culture incubator; C) no medium change, medium pipetted up and down and caps left opened for the remainder of the 5 minutes; D) medium replaced with similar well oxygenated medium, pH 6.75; and E) same as D), pH

raised to 7.4. Cell survival was measured following 9 and 15Gy LDRI (0.41 cGy·min), and is shown in figure 42. These results show that a significant degree of hypoxia was probably not occurring in the LDR vials with the caps closed. If it were occurring the %survival under condition A) would have been significantly higher than under conditions B) and C), respectively. These results also show that a large variation in pH throughout the LDRI period did not affect the survival outcome. The pH values throughout the LDRI period for conditions A), D), and E) are shown in figure 43 for the irradiated vials and for the unirradiated control vials.

The insensitivity of cell survival to pH during LDRI is surprising given the variation in IP- and DP-survival that was seen in chapter 4 when the pH was raised 3 days prior to irradiation, and the variation in PLDR seen in chapter 5, when the pH was raised at the time of irradiation. Although not explicitly measured, one would assume, based on the work in chapters 4 and 5, that the pH response shown in figure 43 is the result of increased glycolysis between pH adjustments. This raises the question of whether the recovery of sublethal damage during LDRI is subject to the same influences with regard to changes in metabolic status, that affect the recovery of PLD. This area is worthy of further investigation.

Figure 38: Cell growth summary: Exponentially growing cultures with conditioned medium added on day 2 (diamonds) with no pH change (closed symbols), and with pH raised on day 4 (open symbols). In addition, exponentially growing cultures with no medium change and no pH change (squares), with no medium change, pH raised to 7.4 on days 4 and 7 (triangles), and with medium changes on days 4 and 7 (circles).

Figure 39: Cell cycle phase summary: Exponentially growing cultures with conditioned medium added on day 2 with no pH change (closed symbols) and with pH raised to 7.4 on days 4 (open symbols). G_1/G_0 (square:), S (triangles), G_2/M (diamonds) phases.

Figure 40: PLDR summary: Exponentially growing cultures with conditioned medium added on day 2 with no pH change (closed symbols), and with pH raised to 7.4 on day 4 (open symbols). IP survival (squares) and DP survival (triangles). Recovery Ratio's defined as S_{DP}/S_{IP} ; given by numbers within rectangular bars.

Figure 41: Cell cycle phase summary: Cells in plateau growth phase in flasks and vials. Cells in vials subject to LDR1 for 3 days at 37°C, and at 41°C compared to 41°C alone and no treatment at all.

Figure 42: Cell survival following 9 and 15Gy LDRI, having been subject to 5 medium conditions. Medium conditions given in figure.

Figure 43: Medium pH variations during 60 hours of LDRI - pH values given for irradiated and control vials. Medium conditions given in figure.

9.0 DISCUSSION AND CONCLUSIONS

9.1 DISCUSSION OF FINDINGS

In this thesis, cell survival and cellular recovery following radiation damage were measured under a variety of conditions in human glioma cells. Recovery during low dose-rate irradiation (LDRI) was examined in a non-proliferating cell population. Recovery was also investigated following high dose-rate irradiation (HDRI) in proliferating and non-proliferating cell populations, under various environmental and physiological conditions. The kinetic nature of this recovery was also measured. Once the conditions for maximal recovery were established, the inhibition of recovery by mild hyperthermia was investigated.

In this study we have shown that the recovery of radiation damage following HDRI in exponential and plateau growth phase U-87MG cells is mono-exponential in nature and occurs at a similar rate when iso-recovery values are compared. We have also shown that recovery is saturated within the observed dose range in that the recovery half-time increases as a function of dose. Although there were not enough data points to rule out a linear relationship, the data did suggest that the recovery half-time increased according to an exponential function of dose. We have presented evidence that over a limited dose range, this exponential function may be linear-quadratic in nature, implying that the recovery half-time and the recovery ratio increase with dose in a similar fashion. Recently, it has been proposed that recovery reflects the incidence of recoverable lesions

not the ability of a cell to repair them [148]. If this is the case, and recovery is saturated, then the time it takes for full recovery should increase in a similar manner as the amount of recoverable damage is accumulated. Lastly, this study indicates that significant recovery of radiation damage, whether it be termed potentially lethal or sublethal, occurs between dose fractions and that this recovery proceeds at a slower rate than recovery following a single dose of radiation.

Radiation cell survival and cellular recovery were measured in U-87MG cells as they progressed from an exponential to an extended plateau growth phase. Immediate plating (IP) survival following 7.5 Gy ^{60}Co irradiation decreased from 2.3% for cells in exponential growth phase to 0.11% for cells held in an extended plateau growth phase with no change or adjustments made to the medium. Delayed plating (DP) survival decreased from 10% to 2.5%, respectively. IP survival was found to be more sensitive to both proliferation status and the amount of time the cells spent in a metabolically quiescent state. Recovery ratios (DP surv./ IP surv.) increased from 4.3 to 23, due to IP survival decreasing at a greater rate than DP survival. Compared to these control conditions, IP and DP survival were measured 3 days following an increase in the metabolic activity. Glycolysis was increased by increasing the pH of the existing medium. Survival was measured 3 days after each pH adjustment to allow a new metabolic equilibrium to establish with a pH and rate of glycolysis comparable to that in control experiments which had no pH adjustments. Both IP and DP survival showed only

slight decreases compared to control experiments, while the recovery ratio at the end of a six day plateau period remained the same as in control experiments. No additional cell growth or redistribution of cells within the cell cycle occurred. Thus a period of increased metabolic activity prior to irradiation was advantageous for both IP and DP survival as compared to a period of low metabolic activity. No change occurred in the expression of PLDR even though after several periods of increased glycolysis minimal glucose and twice the lactate was present in the medium.

Cellular recovery following HDRI was also measured in U-87MG cells in a plateau growth phase under four post-irradiation medium conditions. Recovery was maximal in depleted medium at an acidic pH, conditions which inhibit cellular proliferation. Compared to this control, recovery was increasingly inhibited by alkalization of the existing medium (to 7.4), exchanging the old medium with fresh medium- pH acidified (to 6.8), and exchanging the old medium with fresh medium- pH unaltered (7.4), respectively. These three medium adjustments were made at the time of irradiation. Increased glycolysis was detected post-irradiation in all three cases, while increased proliferation was detected only when fresh medium was exchanged for old medium. Thus inhibition of recovery has been correlated to increased glycolysis and increased proliferation during the recovery period. When acting together, these two processes provided almost complete inhibition. This study also revealed that the degree

of inhibition may be related to the rate of glycolysis and/or the rate of proliferation occurring during the recovery period.

The inhibition of recovery observed by post-irradiation manipulation of medium pH contrast the results observed when the medium pH was manipulated 3 days prior to irradiation. In the latter case little change was observed in the recovery level. Together these results indicate that the rate of glycolysis and rate of proliferation must be low during the recovery period to achieve maximal recovery. This supports the theory of Wheeler et al. [149] who hypothesize that the degree of recovery is a function of competing post-irradiation biochemical and/or metabolic processes.

At the two low dose-rates employed, cellular recovery in U87MG and U-373MG was almost complete as indicated by the parameter β in the linear-quadratic survival model being almost zero (chapter 2). This recovery was not complicated by any measurable redistribution of cells within the cell cycle or significant proliferation of the cell population (section 8.2). In section 8.3 the recovery during 60 hours of LDRI was not affected by increases in medium pH every 12 hours. This contrasts the recovery observed following HDRI. Perhaps the fact that the recovery processes are saturated following HDRI, as indicated in chapter 3, governs whether the competing biochemical and/or metabolic processes play a role in determining the final recovery level observed. Under LDRI, the dose is delivered in an infinite number of small fractions and therefore

the rates of damage induction and damage removal may occur at similar rates, as suggested by Curtis [59]. This would result in a recovery process that is not saturated..

The large degree of recovery observed at 0.88 cGy/min (LDRI) was almost completely inhibited by concurrent heating at 41°C (TER of 1.6 and 1.8 at the 1% survival level for U-87MG and U-373MG, respectively). At 0.41 cGy/min concurrent heating at 41°C resulted in greater thermoradiosensitization for U-87MG (TER 2.1) and less thermoradiosensitization for U-373MG (TER 1.5). These results were not a result of a change in cell cycle distribution during the treatment as shown in section 8.2.

Thermoradiosensitization at 39 and 40°C was similar and sub-optimal in both cell lines compared with 41°C. For U-373MG, thermoradiosensitization was virtually absent at both 39 and 40°C. The direct cytotoxicity of 39,40, and 41°C heating was in the same cell survival range in both cell lines. Survival after 48 hours of heating at 41°C was not less than 30% for either cell line. Mild hyperthermic treatments prior to LDRI resulted in varying degrees of additional sensitization. Pre-heating with 8h at 41°C did not sensitize 9 Gy LDRI at 37°C, however 1h at 43°C did result in some thermoradiosensitization (SRF of 1.4). With 9 Gy LDRI delivered at 41°C, pre-heating provided additional thermoradiosensitization (SRF of 1.6, 3.5, respectively).

The inhibition of cellular recovery following HDRI by pre- and post-irradiation heating at 41°C was also examined. The considerable PLDR that occurred under stationary growth conditions following 7.5 Gy was not inhibited by pre-irradiation

heating up to 24h at 41°C. Post-irradiation heating at 41°C, however, completely inhibited this PLDR when the heat was administered immediately post-irradiation. If recovery was complete prior to heating, post-irradiation heating had little effect on PLDR

9.2 CONCLUSIONS

A range in recovery levels was observed in proliferating and non-proliferating cells due to relative changes in IP and DP survival levels brought about by pre- and post-irradiation manipulation of the growth medium. The degree of metabolic and proliferative quiescence affected by a change in pH was found to play a dominant role in determining the amount of recovery observed. Perhaps this range in recovery levels observed *in vitro* may provide some indication of the range in recovery that may occur *in vivo*. In addition, it may explain the observed variations between recovery measured *in vitro* and recovery measured *in vivo* with the same cell type

Recovery post-HDRI follows mono-exponential kinetics. The recovery half-time increases as a function of dose in a similar manner that the amount of recovery increases with dose. This implies that the recovery processes are saturated. At an iso-recovery level the rate of recovery is similar in proliferating and non-proliferating cells. Half-times should be compared at iso-recovery levels in light of the observations and conclusions in the last paragraph. Assessing the kinetic nature of recovery is vital for mathematical

models which utilize this information to compare the survival outcome of different treatment protocols.

The ability of cells to recover from radiation damage may be quantified *in vitro* by measuring the increase in cell survival following LDRI or by the increase in cell survival following HDRI by delayed plating techniques. While the recovery in either case is maximal under the same conditions (non-proliferating cells, low pH, low metabolic activity), under different conditions (increased metabolic activity) the recovery may be different. This fact is important when the expression of recovery *in vitro* is used as a measure of the inherent radioresistance or radiosensitivity of human tumours *in vivo*. It also implies that recovery measured by these two techniques should only be compared when the cells are under the same conditions (proliferative and metabolic state) while the recovery processes are taking place.

The findings of this study indicate that mild hyperthermia may be an effective inhibitor of the cellular recovery processes which occur during LDRI and following HDRI only if applied while the recovery processes are taking place. These findings have implications in clinical trials of combined hyperthermia and radiation in which hyperthermia is administered before or after irradiation, especially when only mild hyperthermic temperatures are achieved. The large sensitization seen with concurrent 41°C heating and LDRI supports the role of inhibition of cellular recovery with mild hyperthermia as an effective way to improve brachytherapy.

9.3 AREAS FOR FUTURE RESEARCH

One area for further research to enhance the findings reported in chapters 4 and 5 is the measurement of ATP levels following periods of varied metabolic activity, to see if the IP or DP survival levels correlate with the constituent levels of ATP. In addition, the role of cell crowding in inhibiting proliferation in tumour cells warrants further investigation. Recall in chapter 4 that an increase in pII did not lead to increased proliferation in densely populated plateau phase monolayers, however as indicated in section 8.1 when a plateau growth phase was induced in a sparsely populated exponentially growing population, an increase in pII did cause increased proliferation.

With regard to recovery half-times following large dose fractions (chapter 3), additional $T_{1/2}$ values are needed at low doses to validate the hypothesis that recovery half-times increase according to a linear-quadratic function of dose. In addition, recall that the half-time reached an upper limit of approximately 3.5 h in plateau growth phase cells. Therefore half-times should be calculated at higher doses to determine if in fact this trend continues. An upper limit to the recovery half-time may imply that there is a rate determining process that is independent of dose. Recovery half-times could be obtained by irradiating cells at several low dose-rates and fitting the survival curves to a survival model which parameterizes the recovery half-time such as the Incomplete Repair model of Thames [66]. Since this model has three parameters, to obtain a reasonable estimate of the recovery half-time, at least 3 low dose-rate survival responses are needed.

which fall between the high dose-rate response and the maximal low dose-rate response [150]. Such an analysis should provide an estimate of the lower limit for the recovery half-time and would be useful as an estimate of the recovery half-time at infinitely small dose fractions.

The lack of sensitivity of the low dose-rate survival data to a fluctuating pH (section 8.3) warrants further investigation. The rate of glycolysis should also be measured under these conditions to further understand this phenomenon. In addition, it would be interesting to observe whether the thermoradiosensitization of LDRI and concurrent mild hyperthermia is affected by a fluctuating pH.

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APPENDIX I

LOW DOSE-RATE CELLULAR IRRADIATOR

A low dose-rate cellular irradiator was designed and constructed at the Ottawa Regional Cancer Centre for use in the irradiation of the glioma cell lines. Briefly, this consists of 8 radium (^{226}Ra) pencils housed in a lead container which itself was fastened inside of a temperature controlled tissue culture incubator.

The Radium pencils consist of approximately 100 mg or 100 mCi radium sulphate (RaSO_4) each, housed in a stainless steel tube with lead plugs and brass end caps. These were obtained from Atomic Energy of Canada Ltd., Chalk River. A lead irradiation chamber, which was also obtained from AECL, was modified to hold the irradiation vial holder (figures 1 and 2). The irradiation vial holder was designed to securely hold 14 irradiation vials in such a manner that when inserted into the irradiation chamber the bottom of the vials rested on an iso-dose contour.

The dose distribution within the irradiation chamber was estimated with the use of THERAPLAN (Theratronics), a treatment planning program. The iso-dose contour chosen for the irradiation vials was estimated at 0.5 Gy/h in air or 0.83 cGy/min. Actual absorbed dose measurements were made with the irradiation vial holder positioned in the irradiation chamber and the radium pencils in place. A single TLD chip was placed in each of the vials and exposed for 300 minutes. The absorbed dose was

estimated by comparing the response of these TLD chips to those exposed to a similar dose of ^{60}Co γ -rays from a THERATRON 780C clinical irradiator.

To estimate the dose-rate to the cell monolayer covered with 0.5 ml of growth medium, the TLD chips were placed on the bottom of the vials and covered with 0.5 ml of distilled water. The dose-rate at this level was determined to be 0.88 ± 0.03 cGy/min. This value is an average from 1 TLD chip placed in each of the fourteen vials. With a half-life of 1604 years, this dose-rate will not change significantly over the time that this cellular irradiator would be in use. To achieve a lower dose-rate a removable lead sleeve was designed to be placed in the irradiation chamber such that it surrounded the vial holder, positioned between it and the radium pencils (not shown in figure 1). The thickness of the lead sleeve was chosen to reduce the dose-rate by approximately one half. The half value layer (HVL) thickness for lead is approx. 1.05 cm assuming an average energy of 1.25 MeV for Radium. The radioactive decay scheme of radium actually consists of a spectrum of γ -ray energies from 0.18-2.2 MeV. The absorbed dose estimation using TLD chips resulted in a dose-rate of 0.41 ± 0.01 cGy/min in the outer ring of 10 vials. A slightly higher dose-rate (0.47 ± 0.02 cGy/min) was observed in the inner 4 vial slots due to the difference in the angle between these slots and the radium pencils (more perpendicular), resulting in a lower thickness of lead to attenuate the radiation.

For radiation protection purposes an extra tenth value layer (TVL) of lead, 4.5 cm, was fastened to the side of the lead irradiation chamber such that it subtended the angles covered by the immediate laboratory (The back side of the chamber faced an outside wall on the second floor). An additional TVL was also placed under the incubator to reduced the exposure to personnel on the 1st floor. Table I shows the results of an exposure survey in the surrounding area. It is obvious that radiation safety standards have been met.

Table I.1: Radiation protect survey

POSITION FROM IRRADIATOR	EXPOSURE (mR/h)*	DOSE EQUIVALENT per year (mSv/y)**
Outside radiator room (2m)	1.5-2.5 (2.0 avg)	40
Personnel Bench (3m)	0.04-0.08 (0.06 avg)	1.2
Personnel Bench (6m)	0.02-0.05 (0.035 avg)	0.7
Outside lab door (~ 7m)	0.01-0.03 (0.02 avg)	0.4
In Secretary's office (~ 20-25 m)	0.01-0.03 (0.02 avg)	0.4
DOSE LIMIT - GENERAL POPULATION		5
DOSE LIMIT - RW (radiation worker)		50
BACKGROUND		1

* mR/h = milli-Roentgen/hour

** mSv/y = milli-Sieverts/year = mR/h x .97 rad/R (for H₂O) x 0.01 Gy/rad x 1 Sv/Gy x 2000 h/y.

2000 h/y is based on 8h/d x 250 d/y and represents a maximum occupancy factor (Reference: Johns HE, Cunningham JR, The physics of radiology, 4th ed. Thomas CC publisher, Springfield, Illinois, 1983).

Figure 1.1: Cellular Irradiator

large circle dia. 8cm
small circle dia. 1.6cm

Figure I.2: Irradiation vial holder

APPENDIX II

THE COLONY FORMATION ASSAY

The colony formation assay is a measure of the proportion of cells which remain reproductively viable following an experimental treatment. Following treatment, the cell monolayers were rinsed with citrate saline (KCl 134 mM, citric acid 17.6 mM) to remove cellular debris and trypsin inhibiting substances. Citrate saline is also a mild chelator of divalent cations which can inhibit monolayer dispersal and cause cell aggregation. Trypsin (0.2% w/v in citrate saline) was then rinsed over the cells, and the excess aspirated. After several minutes at 37°C the cells were pipetted up and down until the cells were in a single cell suspension. The suspensions were then diluted in fresh medium and counted using an electronic cell counter (Particle Data). Appropriate cell numbers were plated in either 60 or 100 mm dishes, depending on the number of cells required to achieve 30-80 colonies per dish. Fourteen days later the cells were stained with crystal violet (0.2% w/v in 70% ethanol) and surviving colonies containing > 50 cells were enumerated. Surviving colonies were visualized with a x10 binocular dissecting microscope. Cell survival was normalized to the plating efficiency of untreated cells.

Typically 5 dishes were used to score the number of surviving colonies for experimental treatments and 6 dishes for control plating efficiency (PE). This kept the standard deviation for the number of colonies scored between 5 and 10%. Percent survival was calculated according to the following relationship:

$$\% \text{ SURVIVAL} = (\text{mean \# col./dish}) \div (\# \text{ col. seeded}) \div (\text{PE}) \times 100$$

An uncertainty estimate for #colonies seeded of ~ 10% was estimated from the precision of successive dilutions and cell counting. Survival values in any one experiment typically had an uncertainty estimate of 12-15%.

This uncertainty estimate was found to be conservative since on many occasions the difference between %survival values of duplicate flasks (separate dilution/plating) within the same experiment was < 5%. Of far more importance in these studies were the systematic errors which affected results of entire experiments. Examples of such errors are: variation in incubator temperature and humidity, age of cell culture medium, and sensitivity of the cell line over time in culture. Even though these factors were kept to a minimum, the results of any two experiments of the same treatment separated by several weeks often differed by a factor of 2. Without elaborating further on the limitations of the colony formation assay or any in vitro assay for that matter, suffice it to say that with 4 replicate experiments, the inter-experimental standard error of the mean (SEM) could be kept at approx. 15%.

(Reference: Freshney RI: Culture of animal cells. A manual of basic technique. 2nd ed. Liss AR publisher, New york, NY, 1988)

END

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FIN