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Radiobiology of Radioresistant Glioblastoma

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1. Introduction

Therapy of glioblastoma has been very problematic with disappointing results using multiple therapeutic approaches. In general, glioblastomas are considered radioresistant tumors with different radiation modalities failing to control them in the clinic. However a comprehensive and detailed analysis of the radiosensitivity of glioblastoma cells has not been performed. We now present such an analysis in this chapter seeking a better definition of patterns of radiosensitivity in glioblastomas compared to other tumor cells. These data show that some glioblastomas have unusual responses to radiation that may render them more resistant to some forms of radiotherapy but also render them amenable to exploitation by other forms of radiotherapy.

Multiple mechanisms have been proposed to be associated with radioresistance in human glioblastoma cells: Bao et al (1) have suggested increased DNA damage response. Karim et al (2) have proposed differential cyclo-oxygenase response in radioresistant glios. Brandani et al (3) have suggested HSP 70 elevation. Akuguka et al (4) have suggested increased rates in DNA double strand break rejoining association with micronuclei. Scmidberger et al (5) observed variation interferon-induced β associates with increased radiosensitivity in four out of five glioblastomas. Yao et al (6) suggest variation in cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. Streffer et al (7) showed BCL- family proteins modulate radiosensitivity in human malignant glioma cells. Kraus et al (8) showed aberrant p21 regulation in radioresistant primary glioblastoma multiforme cells bearing wild-type p53. Haas-Kogan (9) et al showed p53 function influences the effect of fractionated radiotherapy on glioblastoma tumors. Hsiao et al (10) showed functional expression of human p21(WAF1/CIP1) gene in rat glioma cells suppresses tumor growth in vivo and induces radiosensitivity. Yount et al (11) showed cell cycle synchrony unmasks the influence of p53 function on radiosensitivity of human glioblastoma cells. Britten et al (12) showed differential level of DSB repair fidelity effected by nuclear protein extracts derived from radiosensitive and radioresistant human tumour cells. Guichard et al (13) suggest potentially lethal damage repair as a possible determinant of human tumour radiosensitivity including glioblastoma. Kal et al (14) have suggested rhabdomyosarcomas, similar to glioblastomas are sensitive to low dose-rate irradiation.

These studies used multiple types of glioblastoma cells, but they did not define glioblastoma cells based on essential cellular response mechanisms. We will identify classes of glioblastoma cells that exhibit distinct mechanisms of radiosensitivity in vitro and in vivo. In general there is an overall correlation between radiosensitivity of tumor cells in vivo, radiosensitivity of xenograft tumors as measured in the laboratory and radiosensitivity of tumors in the clinic, although it is clear more studies between these three forms of radiosensitivity is needed. One purpose of this article is to provide such data for radioresistant glioblastoma

Radiosensitivity as assayed by clonogenic inactivation is a precise and accurate endpoint measurable over a wide range of inactivation levels (circa 10^5) induced by a wide range of radiation doses (circa 10^3). Over this dynamic range, clonogenic inactivation can be measured with acceptable variation. Further, clonogenic inactivation is a dichotomous endpoint based on whether individual cells are either clonogenically inactivated or not. Mathematically, this enables the application of Poisson statistics to estimate the probability of inactivation for each increment of dose. Since 1956 when Puck and Marcus (15) published the first "survival curve" such patterns have been examined to discern underlying mechanisms that produce cellular inactivation. Although "hit-target" theory did not identify exact "hits" defined as patterns of ionizations or exact "targets" that once induced inactivates cell, these studies demonstrated a continuing concept that improves such estimates over specific dose-segments. The observation of a log-linear relationship over a specific dose-segment, e.g. logarithm of cells inactivated are a linear function of dose, indicates a constant rate of inactivation over that dose segment.

From these early data a common pattern of inactivation was usually observed for tumor cells: a low rate of cell inactivation below circa 2 to 3 Gray (Gy) followed by increased rates of inactivation at higher doses. Further the patterns of inactivation at higher doses could be approximated as a log-linear response and the slope of such a dose-segment could be calculated as a Poisson probability of inactivation, usually expressed as the parameter Do, the dose needed to inactivate a single cell.

Results from the huge empirical data base obtained in the clinic for the relative effect of different doses and protocols that induced both tumor regression and normal tissue toxicity, clearly demonstrated radiotherapy of some tumors was more successful when multiple doses below 3 Gy were used. Mathematical models were proposed to explain the rate of inactivation of tumors at lower doses circa 1 to 3 Gy and the most successful model was the "linear-quadratic (LQ) model" as proposed by Hendry (16) and by Fowler (17, 18). The LQ model was based on the concept that tumor cell inactivation was induced at a linear rate at lower doses (the alpha response) but reflected a quadratic component at higher doses determined by a coefficient beta times the square of the dose (the beta response). These efforts failed to identify specific targets or hits, which in retrospect, was partially due to the complex processes involved in cellular inactivation. However one basic observation from these analyses is useful: identification of a dose-segment over which inactivation is log-linear, is still valid in identifying the rate of inactivation over such a dose-segment, represented by a single Poisson coefficient.

The pioneering work of Joiner and his colleagues in identifying "low-dose hyper-radiosensitivity" (19) demonstrated that at lower doses (< 0.5 Gy) there were additional changes in rates of clonogenic inactivation that could not be well explained by the linear-quadratic model.

We have recently proposed that patterns of inactivation in tumor cells are expressed as two general responses, the alpha response and the omega (or quadratic) response, are in fact actually comprised of four distinct components induced sequentially at increasing doses (20). Three of these responses can be well fitted to a log-linear relationship and a Poisson coefficient could be calculated over each of these log-linear responses over distinct dose-segments that represented the rate of inactivation. We will discuss these results subsequently but our data suggested that radiosensitivity of any cell line can be described as coefficients that describe four sequentially induced responses in each cell line. Some glioblastoma cells, not others, show specific values for these four responses.

1.1 Concept of a "radiosensitivity phenotype"

Our studies suggest that radiosensitivity of tumor cells cannot be expressed as a single parameter or histological type, but should be analyzed on the basis of descriptors of multiple responses that are specific to human tumor cell lines. We argue that the radiosensitivity phenotype of each cell line should be defined by a set of coefficients including: 1) relative rates of inactivation over four distinct, sequentially-induced components that comprise radiosensitivity of each cell; 2) the general radiosensitivity group into which cell lines segregate non-randomly based on the values of exceptional coefficients; these groups associated with tumor cell genotype; 3) modulation of inactivation by reduced dose-rates; and 4) modulation of inactivation by the effect of ionization density of delivered radiations; and 5) modulation by in vivo mechanisms that are particular to genotype.

In the next several paragraphs we identify those coefficients that together define the "radiosensitivity phenotype" of human tumor cells. One overall goal is to equate radiosensitivity phenotypes of tumor cells to genetic or epigenetic properties.

1.2 Coefficients that describe four sequentially-induced responses in each human tumor cell

We have measured clonogenic inactivation in multiple cell lines over different dose-segments (20). **T**hese studies showed that response of tumor cells to radiation in vitro can be resolved into two general responses, termed the alpha response and omega response (or quadratic response) that represent the overall rate of inactivation over the dose-segment from 0 to circa 2 Gy (alpha response) and over doses greater than circa 3 Gy (omega response). These two general responses can be approximated by the linear and quadratic components of the linear-quadratic model. These two general responses vary not only between glioblastoma cell lines and other tumor types but also vary between different glioblastoma lines.

Our data show these two general responses are actually comprised of four more specific responses induced sequentially in each cell line. Thus one goal of this chapter is to measure coefficients that describe these four responses in tumor cells and to suggest their relative importance in clinical radiotherapy.

As stated above we have defined four sequentially-induced responses in ten tumor cell lines by increasing doses from 0.0 to 10.0 Gy. These responses are common to all tumor cells, induced at the same doses but vary in the rates of inactivation over these common dose-segments. These four responses are:

The hypersensitive (H) response is observed over the dose-segment from 0.0 to 0.10 Gy and is characterized by highest rates of clonogenic inactivation observed in tumor cells. This

response is related to low-dose radio-hypersensitivity as described by Joiner and his colleagues (19). In each cell line in which the H response is observed, it is expressed over the dose segment from 0.0 and 0.10 Gy with a very low threshold if any but ends at 0.10 Gy. The survival level at 0.10 Gy can be used to calculate the slope of the H response between 0.0 Gy and 0.10 Gy as α (SF.1). The H response varies strongly in different types of tumor cells with some genotypes not expressing this response. Thus each cell line can be classified as to whether the H response is induced and the slope of the rate of inactivation. Interestingly, some glioblastoma cells express the H response at a high rate but are very radioresistant at higher doses.

The resistant (R) response is observed over the dose-segment from 0.1 to 0.2 Gy and is characterized as increased resistance to clonogenic inactivation exhibited in each cell line that expresses the H response. The R response appears to be induced at circa 0.10 Gy in all cells and persists until it is terminated when the alpha* response is induced. The R response is coupled strongly to the H response. In our studies shown below the R response is not always expressed as a log-linear response across the dose-segment from 0.0 to 0.1 Gy and hence the change in rates of inactivation over the R response varies strongly with genotype. The expression of the H and R responses do not correlate with the expression of the alpha* and omega* responses.

The alpha* (repair) response is induced at 0.20 Gy in all cells and once induced extends to all higher doses. The alpha* response is a protective response preventing excessive loss of irradiated somatic cells. The rate of inactivation over the alpha response is more resistant than rates evidenced over the H response and also more resistant than increased sensitivity observed subsequently when the omega* response is induced. The alpha* response is characterized by transiently suppressed apoptosis, induction of repair responses and perturbation of cell cycle progression. We refer to this induced response specifically as the alpha* (α*) response and it is the only response determining inactivation between 0.2 and 2.0 Gy. The slope of the alpha* response is a correlate of the general alpha response observed between 0.0 and circa 3 Gy measured either by the slope of inactivation estimated between 0.0 and 2.0 Gy, α (SF2) or by linear component of the linear-quadratic model α (LQ). We will show examples of this relationship subsequently.

The omega* (triage) response is induced at circa 3.0 Gy in all cells and extends to all higher doses. We refer to this induced response as the omega* (ω*) response and consider it a triage response that results is increased inactivation of damaged cells. It can be approximated mathematically by the quadratic response of the linear-quadratic model. The omega response is determined by linear regression of data above circa 3.0 Gy and its slope designated as omega (ω). The omega response is the combined effect of the omega* response induced as circa 3 Gy and an extension of the alpha* response. The omega* response is characterized by increased rates of clonogenic inactivation and chromosomal aberrations but decreased rates of tumorigenesis, carcinogenesis and mutation. Thus the increased inactivation of cells at doses above 3 Gy preferentially removes cells more likely to express mutation or cancer and thus can be considered a triage process that preferentially eliminates cells by detecting radiation-induced properties and eliminating cells by post-repair apoptosis.

1.3 Coefficients that describe the rates of inactivation over the alpha and omega responses segregate human tumor cells into only four statistically-valid cellular radiosensitivity groups

In a broad survey of clonogenic inactivation in multiple tumor cells we observed that the rate of inactivation over the alpha response was the major determinant of overall cellular

radiosensitivity Williams et al (21, 22). The values of the coefficients that describe the alpha response derived over doses from 0 to circa 2.5 Gy segregated all cell lines non-randomly into four distinct, statistically-valid radiosensitivity groups. Each radiosensitivity group is inactivated over the alpha response at different rates and the statistical variation is significant. While the alpha response, that is in reality the combined effects to the H, R and alpha* responses, is related to the omega response in the four radiosensitivity groups, measurements of the general alpha response as will be shown subsequently, correlates with the alpha* response as measured using the linear-quadratic model. Thus the radiosensitivity groups listed below are dependent on the values of the alpha* response. Our work showed these four radiosensitivity groups segregate with specific genotypes, one of which was a group of some glioblastoma cells that as stated earlier express what we refer to as the "glio" response.

All tumor types we examined segregated into only four cellular radiosensitivity groups:

A **VS (very sensitive) radiosensitivity group** was comprised of only a single hypersensitive tumor line that was mutated in the ataxia telangiectasia mutated (ATM) gene. This cell line is hypersensitive to radiation on the basis of clonogenic inactivation, expression of apoptosis, cell cycle progression and susceptibility to chromosomal aberrations.

An **S (sensitive) radiosensitivity group** was comprised of 17 cell lines all but one expressing wild type tumor protein 53 (wtTP53). The coefficients that describe the alpha response for these cell lines were intermediate between the VS cell line and other more resistant lines.

An **R (resistant) radiosensitivity group** was comprised predominantly of
cell lines that expressed a mutant form of TP53 although the exact form of mutation that renders cells more resistant has not been defined. R cells are intermediate in their radiosensitivity between S and R cells.

A **VR (very radioresistant) radiosensitivity group** was identified that was comprised of only three human glioblastoma cells. For descriptive purposes, we will refer to the factor or gene that leads to this exceptional resistance as "glio". This is in contradistinction to the S and R groups that contain tumor cells that derive from multiple histological types.

1.4 Coefficients that describe radiosensitivity of tumor cells to low dose-rate irradiation

We also defined rates of clonogenic inactivation of glioblastoma cell lines to low dose-rates (0.25 Gy per hour) compared to high dose rate (circa 50 Gy per hour) and again find significant differences in some radioresistant glioblastoma cell lines 21, 22. Importantly, these data suggest that some glioblastoma cell lines are distinctly different in their response to low dose-rate irradiation compared to their resistance to radiation delivered at higher doses. We will show analysis of these data subsequently below, but they do demonstrate that some glioblastoma cell lines that are very resistant as measured over the alpha* responses have a unique response to low dose-rate radiation that perhaps can be exploited in the clinic. Therefore a broad assessment of the radiosensitivity phenotype of a human tumor cell should include response to protracted irradiation.

1.5 Coefficients that represent the susceptibility of radiosensitivity to differences in ionization-density

While we will publish data elsewhere on the effects of dose-rate and ionization density, here we can make the general statement here that these data show the H and R responses are

generally not modified by either by dose-rate or ionization density. In contradistinction, the alpha* and omega* responses are highly susceptible to dose-rate and ionization density.

1.6 Coefficients that represent the modulation of in vivo radiosensitivity by genotype and dose

We demonstrated variation in the response of tumor xenografts to radiotherapy protocols based on genotype and dose-schedule. In these studies, Williams et al 21, we showed genotype of tumor cells influenced both in vitro radiosensitivity of tumor cells and also, by a different mechanism, influenced xenograft response in vivo. We attributed this effect, that was substantial in some cells, as an interaction between tumor genotype and the in vivo tumor microenvironment. Importantly one glioblastoma line that was in the VR cellular radiosensitivity group, expressed surprising sensitivity when irradiated as xenograft tumors in vivo.

2. Methods and materials

We have published in detail the exact protocols that we have used in these studies (21, 22, 23, 24).

2.1 Cell lines

We study 3 radioresistant glioblastoma cell lines (U251, T98G and U87) two other lines classified as glioblastoma but are more sensitive (GL-13, JW-1T). We compare them in detail with two human colorectal tumor cell lines (DLD-1 and 19S184), both cells expressing mutTP53 but 19S186 has been abrogated in CDKN1A (p21) and this abrogation while not effecting in vitro radiosensitivity causes increased radiosensitivity in xenograft tumors (Waldemann, 24).

2.2 Cell and culture techniques

The basic media for colon tumor cell lines was McCoy 5A, supplemented with 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine; Human glioma cell lines were cultured in DMEM/F12 with 10% FBS, 1% L-glutamine and 1% Penicillin and streptomycin. All cells were sub-cultured twice a week to maintain exponential growth.

2.3 Cell survival assay

Cells were plated ~18 hours before irradiation. Surviving colonies were determined 10-14 days after irradiation depending on the cell line. Cells were stained with crystal violet and colonies counted (>50 cells/colony). Additional plates for each experiment were used as microcolony controls. Special care was taken in dispersing cell cultures to obtain single cell suspensions with high plating efficiencies.

2.4 Irradiation

Cells were irradiated in complete media in a Gammacell 40 (Nordion Ottawa ONT Canada) at approximately 0.7 Gy/min. Cells were plated 15 to 18 hours before irradiation with careful measurement of plating efficiency and multiplicity. After exposure, plates were incubated for 8-14 days depending on specific growth and colonies stained with crystal violet. Colonies with more than 50 cells were counted. For each cell line we performed

controls to account for possible proliferation during the period between plating and irradiation. This control consisted of plating 10^5 cells in separate plates when replicates of cells were plated for colony formation. When irradiation was performed on the plates for colony formation, the microcolony plates were stained and the number of cells per colony measured. The average number of cells per colony was below 1.20 cells per microcolony for all cell lines and did not vary significantly between cell lines.
Low dose rate irradiation was carried out in a specially constructed Cs-137 irradiator with temperature control and the ability to irradiate cells with constant or exponentially-decreasing dose-rates.

2.5 Regrowth delay in xenograft tumors

Tumors were established by subcutaneous injection of 5 million cells suspended in PBS into the upper thigh of nude mice. Each cohort included 6 to 13 tumors. Tumor growth rate was determined by measuring three orthogonal diameters of each tumor twice a week and the tumor volume estimated as π/6 [D1 x D2 x D3], when individual tumor volumes reached ~0.1-0.3 cm^3, radiation treatment was initiated. Modal specific growth delay (mSGD) was measured for all cohorts in which a majority of tumors reached a volume four times the initial volume. Response was normalized to growth of unirradiated cells. We chose not to use the mean of specific regrowth delay patterns since a significant proportion of our cohorts included one or more tumors that did not regrow. Thus the mean became limited as a regrowth parameter. For cohorts for which some tumors did not regrow we estimated mSGD based on the regrowth pattern for the minority of tumors that did regrow. When we tested the sensitivity of modal to mean growth delay in selected cohorts in which all tumors regrew, the modal value always fell within one standard deviation of the mean. These methods share some characteristics of the methods described by Schwatchofer [25]. To provide an overview of the dichotomous response when some tumors regrow but some do not, we indicated such cohorts with an arrow showing this value, in terms of overall tumor response, was the common minimum response.

3. Analyses

3.1 Clonogenic inactivation of radioresistant glioblastoma cell lines

In our previous studies (21, 22) we identified three glioblastoma cell lines (U251, T98G, U-87) that were the most resistant of 39 cell lines examined as defined by comparison of clonogenic inactivation between circa 2 Gy and 10 Gy. These three radioresistant cell lines expressed two forms of TP53, with U251 and T98 expressing mutTP53 and U87 expressing wtTP53. For designation purposes we will refer to these three cell lines as expressing a VR radiosensitivity phenotype and expressing either a glio$^+$mutTP53 genotype (U251 and T98G) or a glio$^+$wtTP53 genotype. In figure 1 we compare clonogenic inactivation curves for these three VR (very radioresistant) glioblastoma cell lines compared to two colorectal cancer cell lines that fall into the R (radioresistant) radiosensitivity group wtTP53 (HCT116) and its subline abrogated in p21 (19S186) .
The data in figure 1 show relative radiosensitivity between the five cell lines but it is important in our interpretation of these data to show them in the context of overall radiosensitivity of human tumor cell lines. In figure 1 there are clear differences between the three glioblastoma cell lines and the two more sensitive colorectal tumors. These differences vary with the dose-segment over which the data are presented.

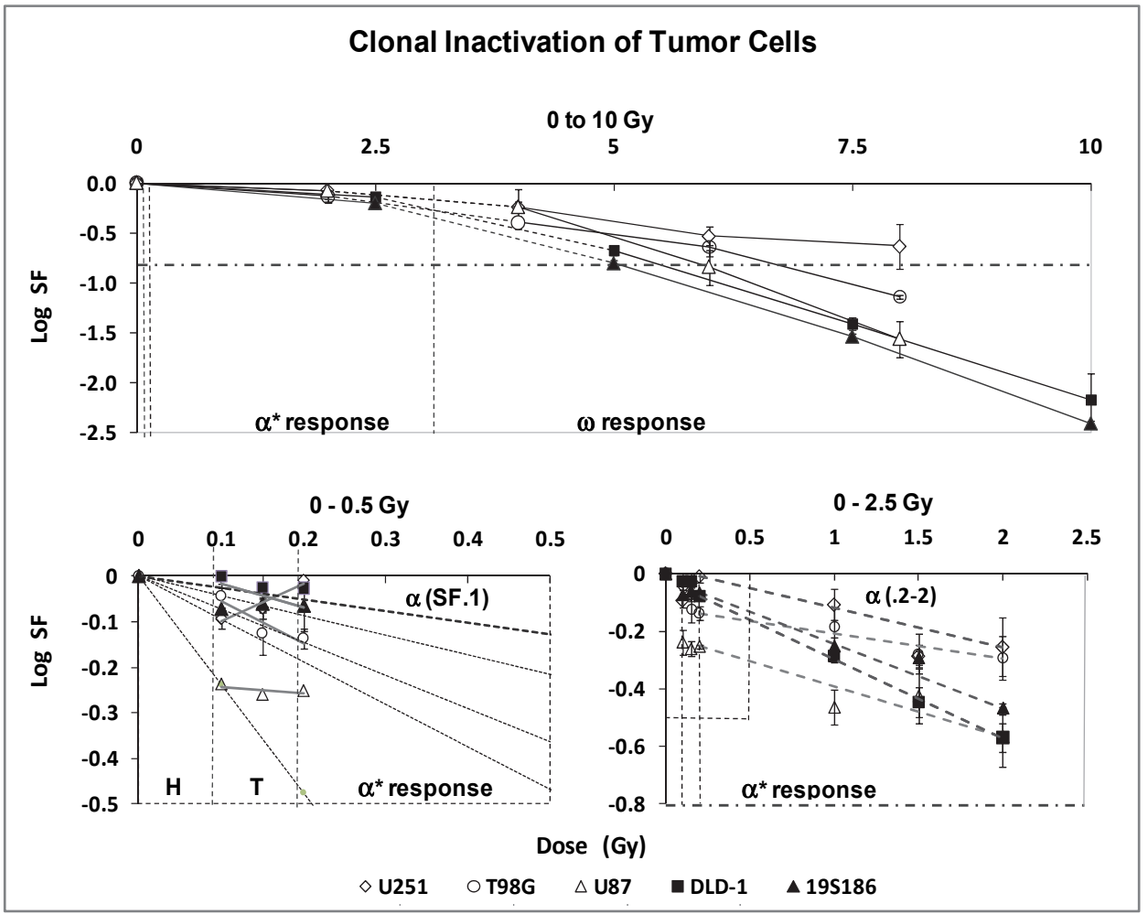


Fig. 1. Radiosensitivity curves (2 to 10 Gy) for five cell lines: three radioresistant human glioblastoma cell lines, U251, T98G, U87, and two human colorectal tumor cell lines, HCT116 and DLD-1. These data show standard survival measurements in the upper panel expressed as two general responses, the α response from 0.0 to circa 3.0 Gy and the ω response for doses greater than circa 3 Gy. The lower two panels show three components that together constitute the alpha response: the H response, the R response and the α* response.

In the top panel the overall responses are shown between 0 and 10 Gy for the five cell lines and these responses can be analyzed by measuring the slope of inactivation between 0.0 Gy and 2.0 Gy and defined as the alpha response. The values for the alpha response calculated in this manner are significantly higher for VR cells than R cells but these differences are difficult to visualize at the scale used in this panel so the dose-response patterns are expanded in the lower panels.

Similarly an omega response can be calculated for all five cell lines using linear regression of all data points above 4 Gy and the slopes of the five lines do not segregate between the two radiosensitivity groups, with two VR lines U251 and T98G showing a more resistant response than the third line U87. This dichotomy in response corresponds to the differences in these three lines in their expression of TP53. U251 and T98G express mutTP53 while U87 expresses wtTP53. These differences are shown more clearly subsequently.

In the bottom left panel the responses of all five cell lines are shown for their detailed responses over doses between 0.0 and 0.20 Gy. In this panel, the dashed lines are the slopes for each cell line defined by connecting 0.0 dose points to the points at 0.10 Gy, α (SF.1), extended to illustrate the strong variation in slope for the H responses.
All cell lines change in their rates of inactivation at 0.10 Gy that represents the induction of the R response. Note that the rates of inactivation over the R responses (0.1 to 0.2 Gy), varies between cell lines with U87 showing a marked increase compared to the other four lines. .
In the lower right panel, the rates of inactivation over the alpha* responses are shown. The rates for the slopes of the alpha* response are calculated by the slopes between 0.20 and 2.0 Gy indicated as α (.2-2.). The slopes of the For this dose-segment, all three VR cell lines are more resistant than the two R lines.
In a larger cohort of cells we have previously shown that the alpha responses of the three VR lines are distinctly more resistant (22). In our studies of multiple components (20) we showed that the alpha response is comprised of the "average" slope for the H, R and alpha* responses. This work also showed there is correlation between the alpha* response measured over the dose-segment from 0.2 Gy to 2.0 Gy an the general alpha response shown in the upper panel.

3.2 Coefficients that define the alpha and omega responses segregate human tumor cells into four radiosensitivity groups

In figure 2 we show a scatter diagram based on our data from Williams et al (21) expressed as values of the coefficients derived for the alpha and omega responses measured as shown in figure 1 and measured as the slope of the general alpha response and the omega response. This figure also specifically identifies the five cell lines that are the subject of our present analysis: U251, T98G, U87, DLD-1 and shows they are distinctly different in their radiosensitivity compared to the lines JW-1T and GL-13 purported also to be glioblastoma cells.
There are important implications of these data. First the values of the alpha response and omega response segregate all cell lines into four statistically distinct radiosensitivity groups: VS, S, R and VR. The alpha response is the predominant determinate of radiosensitivity group. Note that the five cell lines that we study in this chapter are distributed in two clusters: U-251 and T98G are clustered in cell lines that express extreme resistance based on their alpha and omega responses. Three cell lines cluster in patterns with the lowest values for both alpha and omega responses, but three cell lines, DLD-1, 19S186 and U-87 while showing resistance to lower doses (alpha response) have significantly larger values of the omega response are also determined as that are also resistant (alpha response) but show elevated values of their omega responses. Hence the three glioblastoma cells in the VR group share the smallest values for their alpha response but vary significantly in their omega responses. Two cell lines classified in the literature as glioblastoma GL-13 and JW-1T fall into distinctly different radiosensitivities segregating with the S radiosensitivity groups
The omega response for U87 cells is distinctly higher, reflecting, we hypothesize, the role of wtTP53 in "glio" cells. We hypothesize that over the alpha response, "glio" confers radioresistance beyond that characterized by expression of mutTP53. At higher doses, wtTP53 modulates radiosensitivity as shown for U87 cells. The data in this figure show three distinct clusters of glioblastoma cell lines.

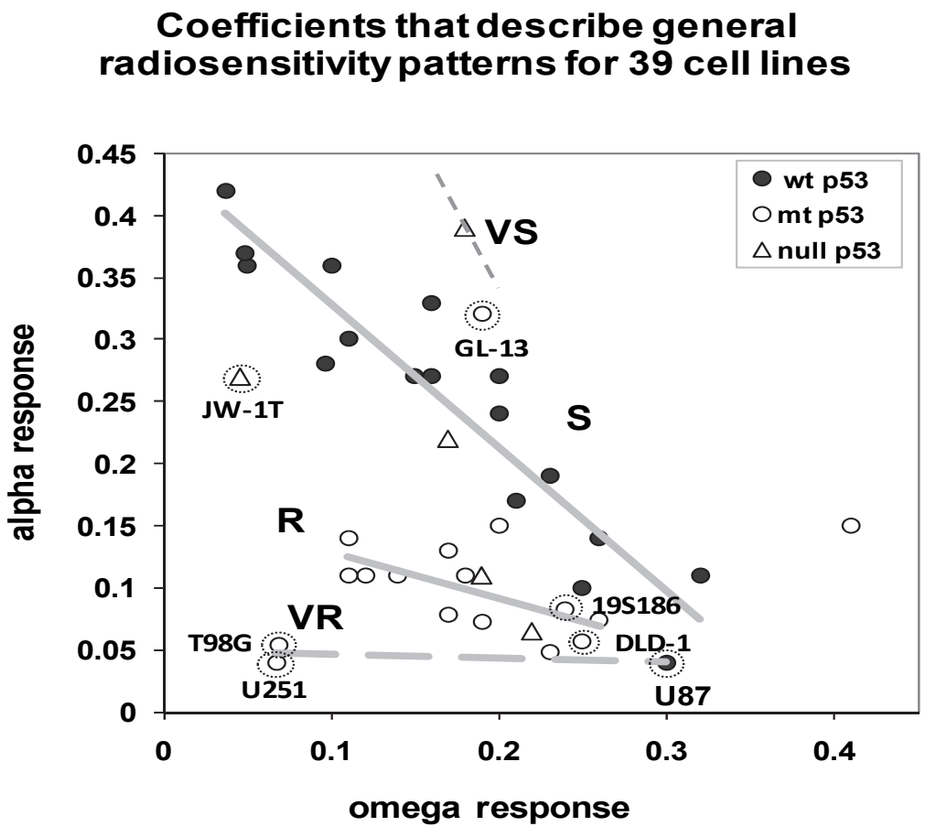


Fig. 2. Radiosensitivity of five glioblastoma cell lines in relationship to 34 other human tumor cell line data extracted from Williams et al 2008a. The ordinate is the coefficient that describes the slope of the alpha responses and the abscissa is the coefficient that describes the slope of omega responses as shown in figure 1. The diagonal lines are best fit estimates of regression and identify four distinct radiosensitivity groups (VS, S, R and VR). The "VR" (very resistant) group is comprised of only three radioresistant glioblastoma cell lines (U251, T98G, U87). In this figure we two cell lines that are also classified ad glioblastoma cell lines (JW-1T, GL-13) that do not segregate into the VR group. We also identify two R (resistant) cell lines DLD-1 and its subclone abrogated in p21, 19S186.

3.3 Radiosensitivity of radioresistant glioblastoma cell lines and human colorectal tumor cells to low dose-rate irradiation

We have previously measured the response of 27 human tumor lines to low dose-rate ionizing radiation (26) and in figure 3 we show the general responses of a VR cell line (U-251) and a less resistant colorectal tumor cell line (DLD-1).

These data show two important differences in these two cell lines. First the VR line is more resistant than the R line for both rates of radiation. Second, it is clear that within each line the differences between irradiation at HDR and LDR are markedly different for the two types of cells with the VR line showing a significant increase in inactivation by LDR.

These differences in rates of clonal inactivation between LDR and HDR are shown in more detail in the data in figure 4.

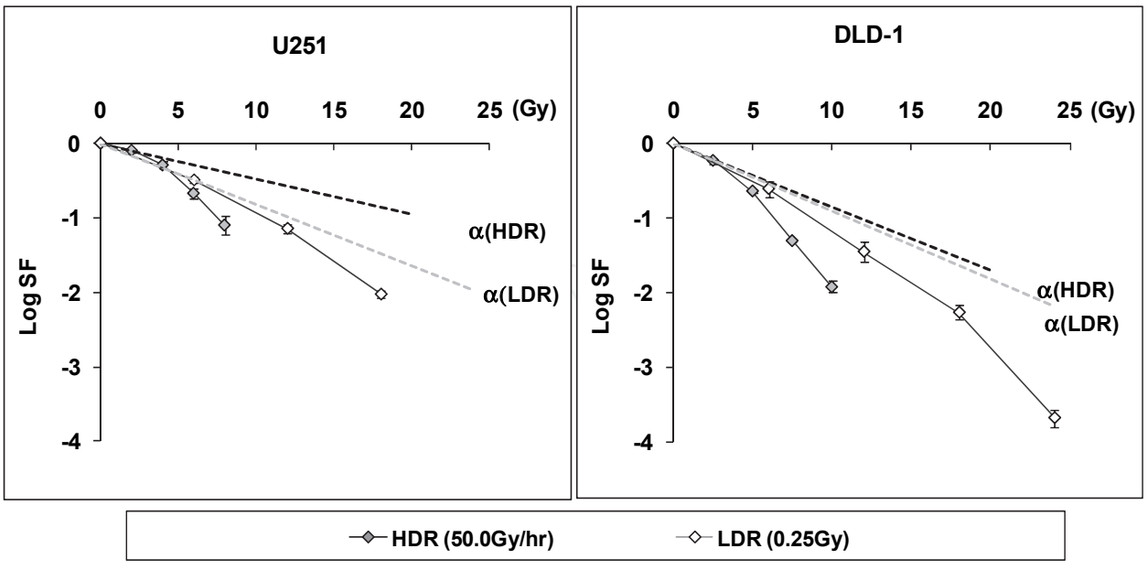
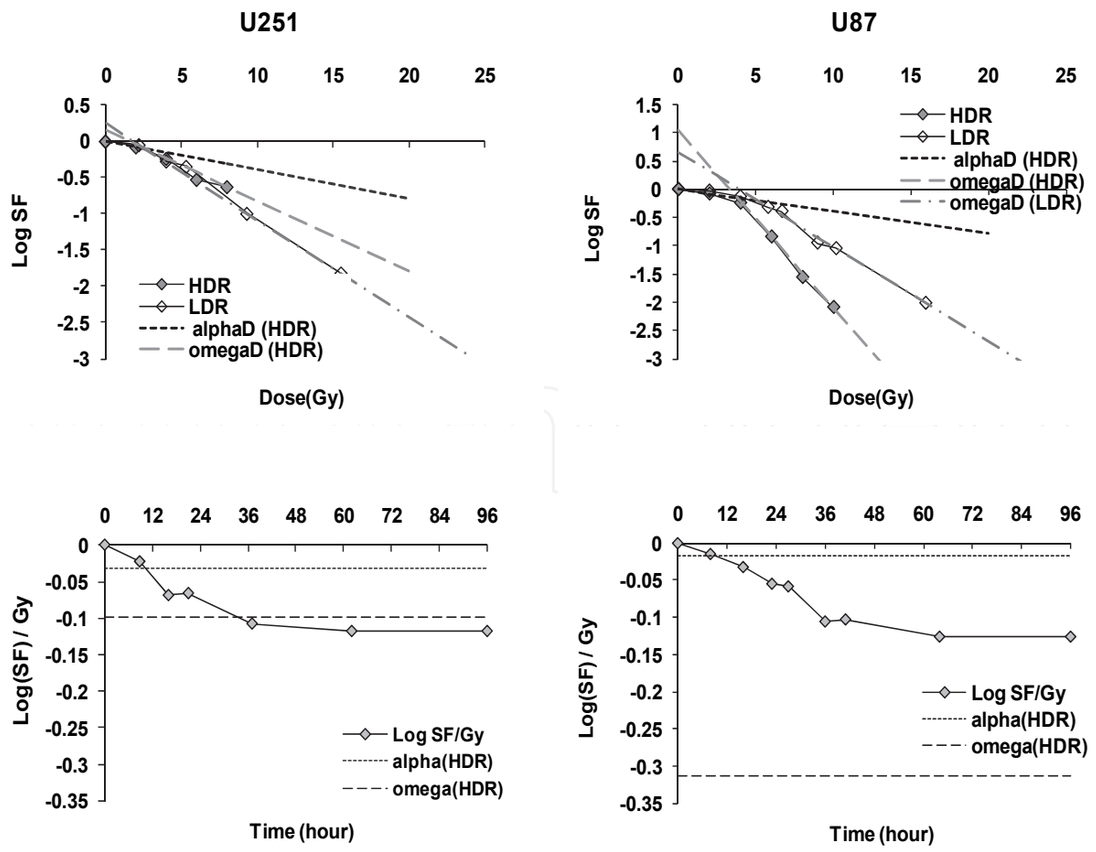


Fig. 3. Comparison of clonogenic inactivation induced by acute high dose-rate HDR (50 Gy/hr) and protracted irradiation LDR (0.25 Gy/hr). The dashed lines represent the extrapolation of the rate of inactivation at lower doses based on the slopes of inactivation by LDR and HDR.



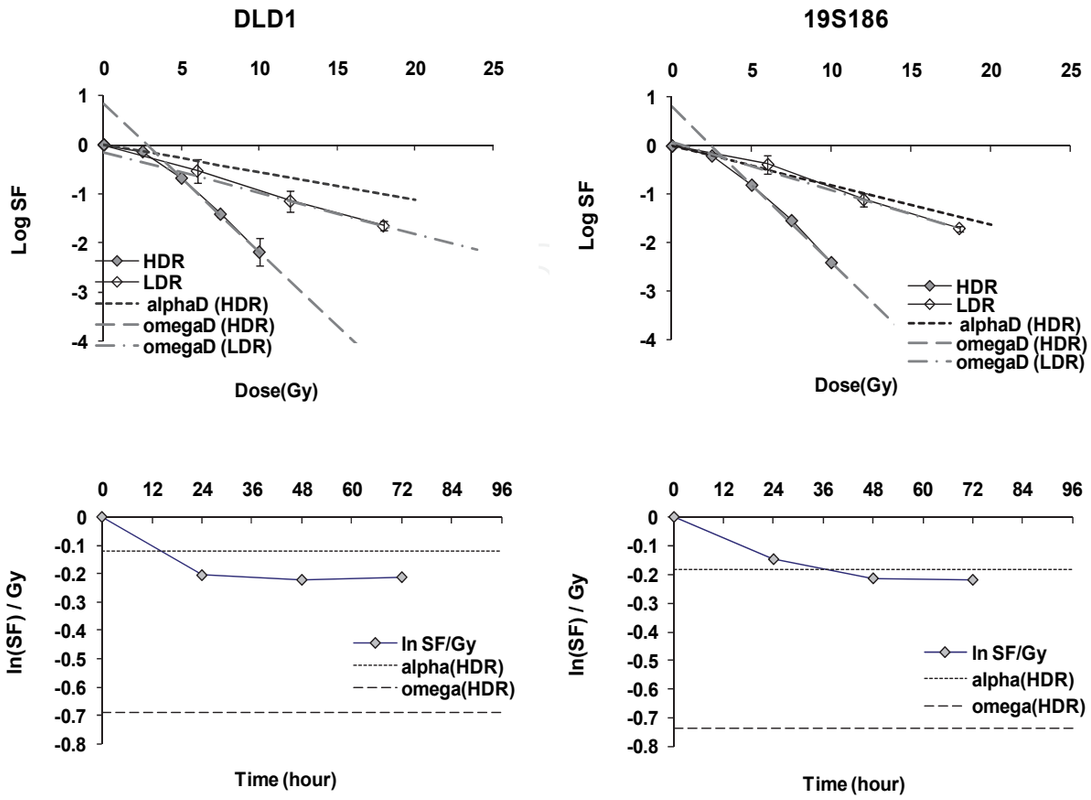


Fig. 4. Clonogenic survival patterns for two glioblastoma cell lines (U251, U87) and two colorectal tumor cell lines (HCT116 and DLD-1) irradiated with either HDR (50 Gy/hr) and LDR (0.25 Gy/hr). Data for each cell line are shown as two panels, the upper panel shows surviving fraction, lower panels show the rate of cell killing calculated as logs killed per Gy between sequential time points. The slopes of cell killing curves are represented by: α(HDR) which is measured from the slope of the line from 0.0 to surviving fraction at 2.0 Gy HDR; α (LDR) which is the slope of the line from 0.0 to 6.0 Gy LDR; the slope of the cell killing curve at doses greater than 4 Gy HDR determined by linear regression, ω (HDR); and the slope of cell killing at LDR doses greater than 6 Gy, ω (LDR). In the lower panels, the rate of cell killing by HDR is indicated by dotted, dashed lines.

These data show that both glioblastoma cell lines are inactivated by low dose-irradiation at approximately the same rates similar to rates for both colorectal tumor cells, showing a clear increase in rate that surpasses the levels of high dose-rate inactivation (omega response) of both cell types. This rate of LDR inactivation surpasses the rate of inactivation for the omega response for U-251 but the elevated level of U-87 cells for the omega response is not achieved.

These data suggest strongly that for radioresistant glioblastoma cells, the rate of inactivation by LDR irradiation can surpass the rate of inactivation for by multiple fractions that induce inactivation along the alpha response.

This in turn suggests using LDR radiotherapy for glioblastoma tumors and we synthesize our data to demonstrate the relative effect of different dose rates for induction of clonogenic inactivation. These data are shown in figure 5.

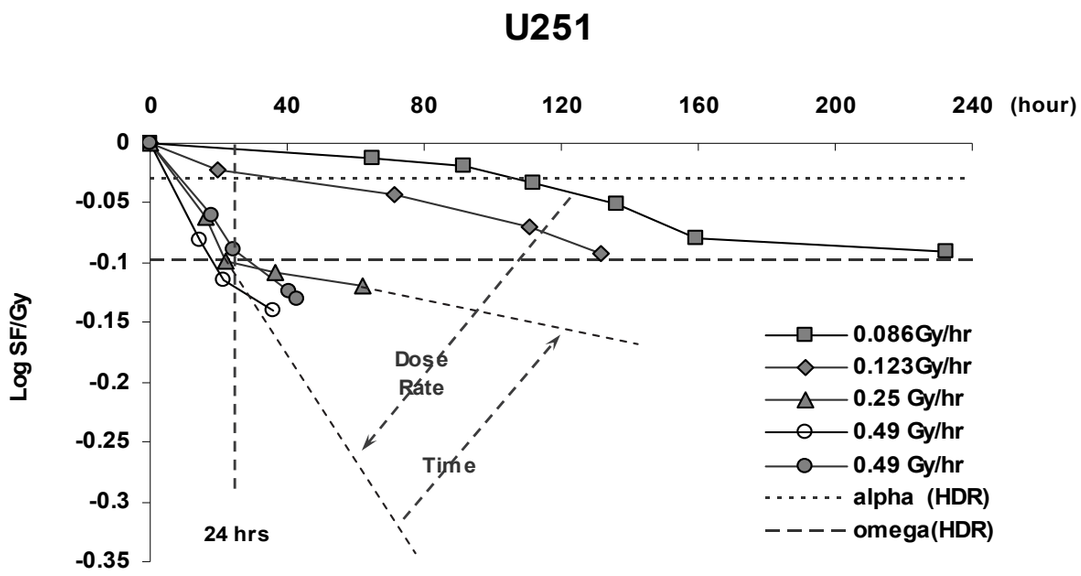


Fig. 5. Changes in the rate of cell killing expressed as Log10 of cells killed per Gy as a function of time of protracted irradiation in U251 cells irradiated with 4 different constant dose-rates and one that begins at 0.49 Gy/hr and decays with a half life of 2.7 days. The horizontal dotted and dashed lines represent the rate of cell killing for HDR irradiation at lower doses (alpha) and higher doses (omega).

These data show that increasing dose-rate increases the rate at which cells are inactivated until dose-rates reach approximately 0.12 Gy/hr when there is a relatively common response for higher dose-rates including our chosen LDR of 0.25 Gy/hr. The diagonal dashed lines in figure 5 are a general indication of the relative effects of dose-rate compared to the relative effects of duration of exposure (time). In our previous studies of LDR irradiation in multiple tumor cells we show that all cell lines change in their rates of inactivation at circa 20 to 24 hours, so duration of exposure and dose-rate are both factors in achieving changes in clonogenic inactivation.

The data in figure 5 suggests that dose rates in the range of 0.25 to 0.49 Gy/hr increase tumor cell inactivation to rates that exceed that can be achieved by the alpha response induced by HDR irradiation. Thus, these patterns of inactivation show that glioblastoma cells while resistant to radiotherapeutic protocols that use multiple fractions below circa 3 Gy, are more sensitive to protracted irradiation.

Together with J.A. Williams, we have shown that combining irradiation delivered by an implanted radioactive seed with concomitant external beam fractionated radiotherapy produces significant increases in tumor response (Williams JA et al 1998). This study established the feasibility of combining brachytherapy and external beam radiotherapy to achieve good responses in radioresistant glioblastoma cells.

3.4 Radiosensitivity of xenograft tumors comprised of two R cells compared to a radioresistant glioblastoma cell line

The response of xenograft tumors that differ in their susceptibility to clonogenic inactivation in vitro also vary in their radiosensitivity to different radiotherapy protocols delivered in vivo.

We performed a large set of experiments that compared the response of eight different cells that vary in their in vitro radiosensitivity to different radiotherapy protocols in vivo (Williams et al 2010). These studies showed a strong correlation between the total cells killed in vitro with tumor response but also showed a new in vivo effect that resulted from an in vivo interaction between tumor cell genotype and tumor microenvironment. Response of xenograft tumors comprised of two R cell lines, DLD-1 and 19S186 cells are compared to the very resistant VR line U-251 in figure 6.

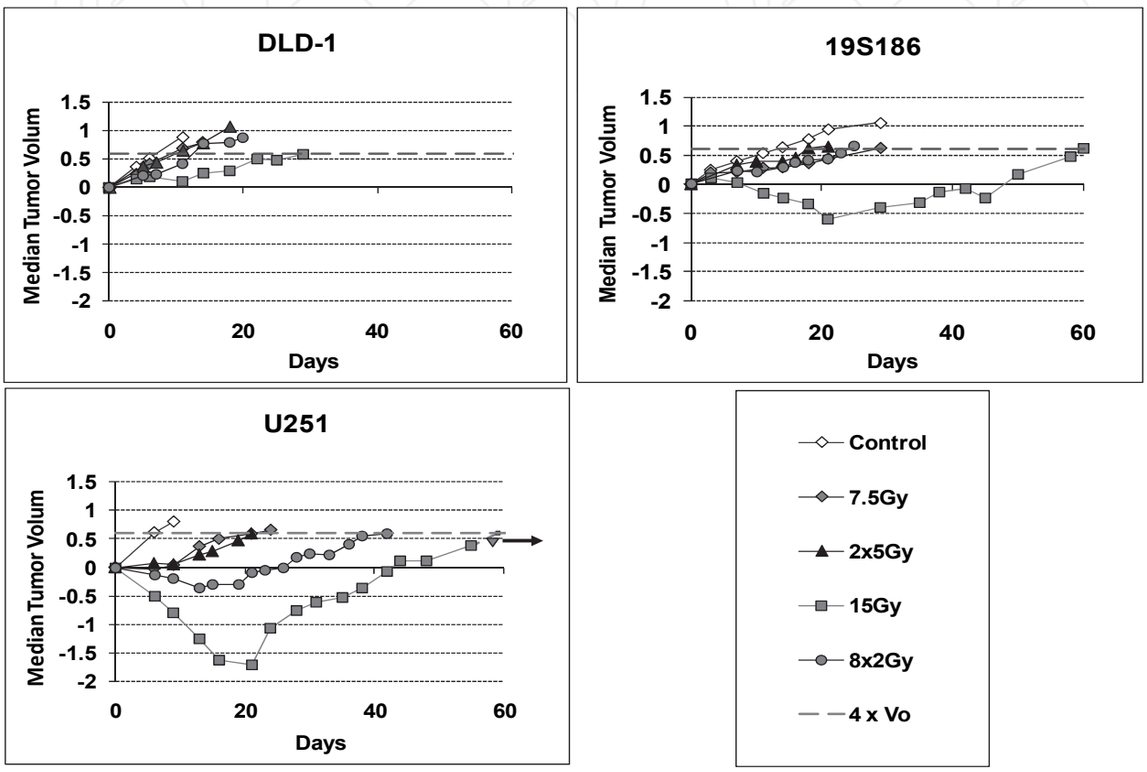


Fig. 6. Growth of cohorts of xenograft tumors comprised of DLD-1, 19S186 and U-251 glioblastoma cells to four radiation protocols: 8 x 2 Gy, 2 x 5Gy, 1 x 7.5 Gy and 1 x 15 Gy. The ordinate in these figures represent the median log V/Vo. Tumors that were irradiated at approximately 0.2 gm and their volumes measured with time. Tumor size is expressed as median tumor volume as a function of days after irradiation.

The data in figure 6 show an increase in tumor radiosensitivity compared to their in vitro radiosensitivity for glioblastoma cells.

In detailed analysis of 40 experiments similar to those shown in figure 6, we showed tumor response could be resolved into two independent sensitivity factors: **τ** and **ρ** (23). The factor τ is related to total cells inactivated in vitro by each protocol and is dependent on genotype, fraction-size ant total dose. The factor ρ is dependent on genotype, fraction-size and total dose, but is independent of τ. We showed that for each protocol and genotype tumor response was dependent on the product of τ and ρ.

The relationship between τ and ρ is a useful comparison for radiosensitivity of xenograft tumors induced in tumor that vary in genotype and treated with different protocols. These coefficients are shown in figure 7 for DLD-1, 19S186 and U-251 cells.

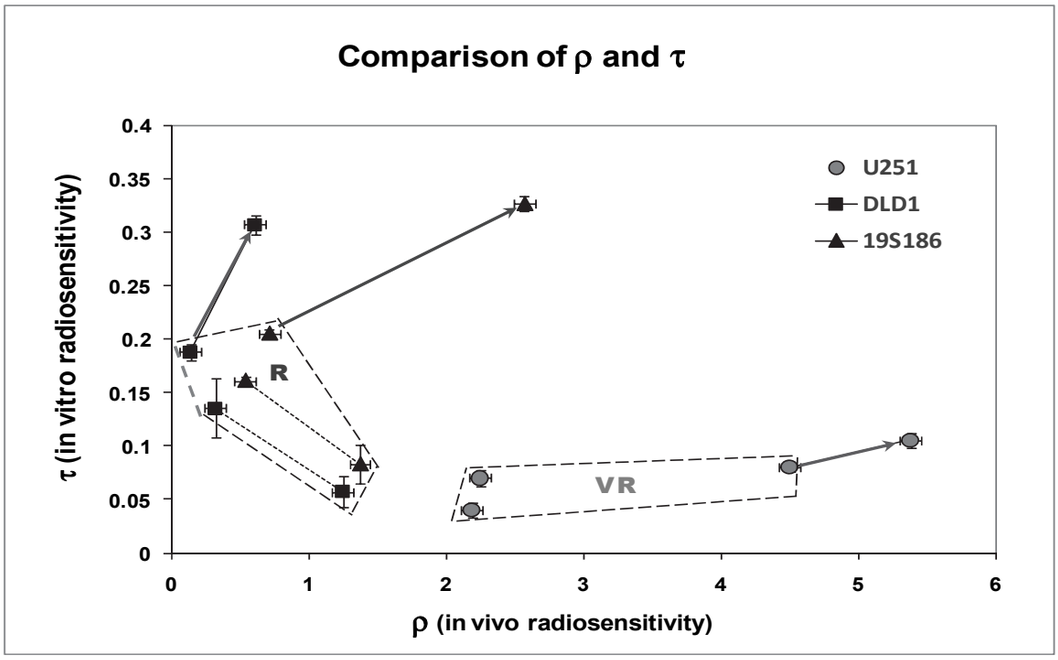


Fig. 7. Relative in vitro radiosensitivity (τ) and in vivo radiosensitivity (ρ) for two R cells, DLD-1 and 19S186 and a radioresistant glioblastoma cells U-251. Each data point represents tumor response of each cell line to one of four protocols. Data point representing the two fractionated protocols, 8 x 2 and 2 x 5, are connected by a dashed lines and these responses are very similar for U-251 cells but are markedly different for the two R cell lines. The response for each cell line to a single dose of 7.5 Gy is connected by an arrow to response of the same tumor to a single dose of 15 Gy. These increased responses are significant beyond the differences observed in fractionated protocols.

These data show important differences between the response of glioblastoma cell line and the two R cell lines. First, the contribution of in vivo radiosensitivity represented by ρ to responses of the glioblastoma line is remarkably greater than the contribution of this sensitivity in R cells. In contradistinction, the values for the in vitro component of tumor radiosensitivity in vitro, τ, are diminished, this diminution similar to differences predicted by in vitro clonogenic inactivation. The effects shown in this figure are large up to a factor of 50 to 100 in doses to induce equivalent regression or in doses needed to induce the same levels of regression.

4. Conclusions

4.1 Glioblastoma cells express a diverse radiosensitivity phenotype

Our studies show that cell lines designated as "glioblastoma" in the literature are diverse in their radiosensitivity phenotypes.

4.1.1 Some glioblastoma cell lines express a low rate of inactivation over the alpha* response

In our studies these reduced rates underlay the observation that such cell lines are refractory to doses over the alpha response. Specifically we hypothesized that these cell lines, express

"glio", an unidentified genetic or epigenetic factor, that renders such glioblastoma cells resistant to radiation delivered at doses below circa 3 Gy. This in turn suggests that tumors comprised of cells that express glio and fall into the VR radiosensitivity group will be refractory to radiotherapy that is based on multi-fraction of cells when fractions are below 3.0 Gy.

4.1.2 The alpha* response can be uncoupled from the omega response in VR resistant glioblastoma cells

Our data show that while two radioresistant glioblastoma cell lines (U-251 and T98G) show resistance to inactivation at higher doses (omega response), one cell line (U-87) shows a more sensitive response. We hypothesize that this difference is associated with expression of TP53 in these cell lines, U-251and T98G express mutTP53 while U-87 expresses wtTP53. These differences are consistent with the ration between alpha and omega responses for cells that express mutTP53 versus wtTP53 as shown in figure 3 and are consistent with all VR cells expressing glio but susceptible to the effect of expression of TP53.

4.1.3 Glioblastoma cells that express VR radiosensitivity after high dose-rate irradiation show an unpredicted sensitivity to low dose-rate irradiation for dose-rates circa 0.25 to 0.49 Gy/hr

Glioblastoma cell lines that show VR radiosensitivity secondary to their expression of glio when irradiated with high dose-rate show a relatively more sensitive response to low dose-rate irradiation for dose-rates circa 0.25 to 0.49 Gy/hr. The data in figures 5 and 6 show that two VR lines, U-87 and U-251 show rates of inactivation when irradiated with lower dose-rates that are more sensitive than the rates of inactivation over the alpha response igh dose-rate irradiation, response to LDR irradiation by exhibiting a more sensitive response show elevated rates of inactivation.

4.1.4 Protocols that combine brachytherapy with external beam are highly effective in treating xenograft tumors of glioblastoma cells

Led by JA Williams (27) we have shown that xenograft tumors of U-251 glioblastoma cells are highly susceptible to the combined effects of brachytherapy using single seeds and external beam radiotherapy. These data shows that xenograft tumors that are resistant to lower fractions (2 and 5 Gy) and protracted radiation from implanted radioactive seeds are highly response to their response to protocols that combine these two modalities.

5.1 Implications for new approaches to radiotherapy of glioblastoma cell lines based on their radiosensitivity phenotype

Our data suggest relationships between the radiosensitivity phenotypes of glioblastoma cells and their predicted response to different radiotherapy protocols.

5.1.1 Cells that express the VR radiosensitivity phenotype will be refractory to protocols that use multiple fractions of doses lower than 3.0 Gy

Cells the express the VR radiosensitivity phenotype have low rates of inactivation by doses below 3 Gy (alpha response) and should not respond well to protocols that use smaller fraction sizes.

5.1.2 VR glioblastoma cells that express wtTP53 should be more responsive to protocols that use higher doses per fraction (> 3 Gy)

The data in figures 1, 2 and 3 show that U87 cells that express the VR phenotype but also express wtTP53 are more sensitive to doses that elicit the omega response (> 3 Gy) than the other two VR cells. This would suggest that tumors comprised of this form of VR cells would show significantly more inactivation than VR cells that express mut TP53 when doses are used that elicit the omega* response.

5.1.3 The VR cell line U 251 shows increased radiosensitivity of its xenograft tumors irradiated in vivo compared to other cell types

This increased response in vivo is observed for all protocols for glioblastoma cell line U-251 but is significantly elevated for protocols that use large fractions of 7.5 Gy and 15.0 Gy. This in turn suggests that some forms of VR glioblastoma cells will respond to hypofractionation with fractions circa 7.5 to 15.0 Gy.

6. Our observations suggest certain studies are needed to design protocols to exploit the VR radiosensitivity phenotype observed in some glioblastoma cell lines

Our research suggests several studies would improve selection of radiotherapy protocols that could improve therapeutic results from specific protocols.

6.1 Better markers are needed to define the VR radiosensitivity phenotype from tumor biopsies

Our studies overall suggest there is no single protocol that would be predicted to provide maximum improvement in tumor radiotherapy for all variations in the radiosensitivity phenotypes that we have observed in cells believed to be "glioblastoma" cells.

6.2 The data base on the VR radiosensitivity phenotype need to be extended to include more cells presumed to be glioblastoma cells

It seems clear from our work and that of others that a larger number of presumed glioblastoma cells need to be examined for their radiosensitivity phenotype including response to high dose-rate, to low dose-rate and their response as xenograft tumors to selected protocols.

6.3 The mechanisms that underlay increased in vivo response of tumors comprised of U-251 glioblastoma cells needs to be extended to other glioblastoma cells

Our data show this is a significant increase in radiosensitivity and the mechanisms that underlay it need to be studied in detail. The data we have presented offers a useful range of responses in different genotypes to study the role of genotype, fraction-size and total dose on this effect.

7. Overall conclusions

Radiosensitivity phenotypes of tumor cells are comprised of distinct, multiple responses to radiation. Glioblastoma cells exhibit responses that are both sensitive and resistant

compared to other tumor cells. Specific protocols can be designed to exploit these differences in radiosensitivity.

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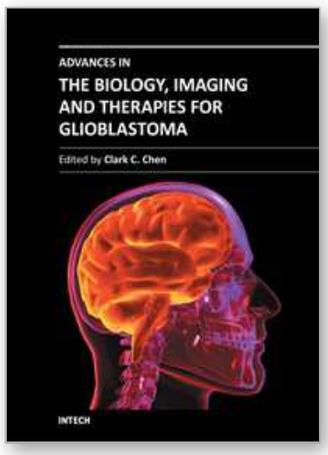

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This book is intended for physicians and scientists with interest in glioblastoma biology, imaging and therapy. Select topics in DNA repair are presented here to demonstrate novel paradigms as they relate to therapeutic strategies. The book should serve as a supplementary text in courses and seminars as well as a general reference.

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