Analysis of radiation-induced changes to human melanoma cultures using a mathematical model

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Received 6 May 2009; revision accepted 29 July 2009

Abstract

Objectives: Tumour cells respond to ionizing radiation by cycle arrest, cell death or repair and possible regrowth. We have developed a dynamic mathematical model of the cell cycle to incorporate transition probabilities for entry into DNA replication and mitosis. In this study, we used the model to analyse effects of radiation on cultures of five human melanoma cell lines.

Materials and methods: Cell lines were irradiated (9 Gy) prior to further culture and harvesting at multiple points up to 96 h later. Cells were fixed, stained with propidium iodide and analysed for G₁-, S- and G₂/M-phase cells by flow cytometry. Data for all time points were fitted to a mathematical model. To provide unique solutions, cultures were grown in the presence and absence of the mitotic poison paclitaxel, added to prevent cell division.

Results: The model demonstrated that irradiation at 9 Gy induced G₂-phase arrest in all lines for at least 96 h. Two cell lines with wild-type p53 status additionally exhibited G₁-phase arrest with recovery over 15 h, as well as evidence of cell loss. Resumption of cycling of surviving cells, as indicated by increases in G₁/S and G₂/M-phase transitions, was broadly comparable with results of clonogenic assays.

Conclusions: The results, combined with existing data from clonogenic survival assays, support the hypothesis that a dominant effect of radiation in these melanoma lines is the induction of long-term cell cycle arrest.

Introduction

Ionizing radiation is one of the main treatment modalities for human cancer. It causes a variety of forms of DNA damage and while much of this is rapidly repaired, induction of DNA double-stranded breaks leads to important cell responses. Double-strand breaks are recognized by the MRE11/RAD50/NBS1 protein complex (1), which then recruits ATM (ataxia telangiectasia mutated kinase) to modify chromatin around the DNA breakage site, preparing it for repair and resumption of the cell cycle (2). ATM activates a network of proteins to promote cell cycle arrest and allow time for DNA repair (3). For example, ATM phosphorylates the CHK1 and CHK2 checkpoint kinases, which in turn phosphorylate CDC25A/C protein phosphatases, preventing activation of cyclin-dependent kinase-1 (CDK1) and causing arrest in G₂-phase. ATM also phosphorylates the p53 transcription factor, leading to activation of p53 transcription, induction of p21 and cell cycle arrest in G₁-phase (4) while activation of proteins such as BAX, PUMA and NOXA, as well as other stress kinase pathways, can lead to p53-dependent and -independent programmed cell death (5).

Various groups have developed mathematical models to interpret flow cytometric data in cell lines exposed to radiation and chemotherapeutic agents (6–10). Our model, shown schematically in Fig. 1, incorporates probabilities for transitions from G₁-phase to S-phase (r_{G_1S}), from G₂-phase to M-phase (r_{G_2M}), from M-phase to mitotic cell death (r_{MD}) and for cell division (r_{MG_1}). These probabilities reflect intrinsic variability of the cell division cycle and have been used previously to analyse the effect at a number of time points of paclitaxel, a drug which arrests cells in mitosis, on melanoma cell lines. The resulting dynamic picture of drug action suggested that paclitaxel-induced transition to mitotic cell death involved a process whereby cell DNA content decreased with time over several days (11). In this communication, we have extended this approach to analyse the effects of radiation on cultured cells. To provide sufficient information for unique solutions,

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Figure 1. Model of the effect of radiation on the cell division cycle incorporating transitions for entry from G₁-phase to S-phase (r_{G1S}) and from G₂-phase to mitosis (r_{G2M}). The model also has terms for S- and M-phase duration, as well as terms for cell loss.

we have determined flow cytometry profiles for cell population growth for various times after irradiation in the presence and absence of paclitaxel, as well as in the presence of paclitaxel alone. Five melanoma lines developed in this laboratory (12) were used in this study. Four of these, NZM3, NZM5, NZM6 and NZM13, are known to have functional wild-type p53, while NZM4 has mutant p53 protein arising from serine to threonine mutation at position 241 (13). Radiation was applied at one of two doses (2 or 9 Gy) and cells were subsequently grown for 1–4 days before harvesting and flow cytometric analysis. The mathematical model was used to generate theoretical profiles that were compared with the experimental profiles. The main result emerging from this analysis is that long-term cycle arrest is a more significant component of radiation response of the melanoma cell lines than induction of cell death.

Materials and methods

Cell lines

Early passage melanoma cell lines were derived from surgical melanoma samples after appropriate Human Ethics Committee approval. The origins of NZM3, NZM4, NZM5, NZM6 melanoma lines used in this study have been published (12–15), while the NZM13 line was derived from a lymph node of a patient with metastatic melanoma. Cell lines were grown in α -modified minimal essential medium supplemented with 5% foetal bovine serum (Moregate Pty Ltd, Hamilton, New Zealand), insulin (10 μ g/ml), transferrin (10 μ g/ml) and sodium selenite (10 ng/ml) (Boehringer, Mannheim, Germany). Cells were recovered from plates using trypsin (15).

Flow cytometry

Logarithmic phase cells were grown on 100 mm plates (10 ml) at 10^4 cells/ml. Irradiation (2 or 9 Gy) was car-

ried out at room temperature using a ^{60}Co source (12). Paclitaxel (200 nM) was added to some cultures to prevent cell division. Cultures were incubated at 37 °C for various times and cells were harvested and counted using a Coulter counter. Cells were washed, fixed overnight in 100% methanol at –20 °C at a density of 5×10^5 cells/ml, rehydrated by washing in PBS plus 2% FBS and incubated with RNAase A (0.25 mg/ml) at 37 °C for 30 min. Cells were then washed and resuspended in PBS containing 0.1 mM EDTA and 25 μ g/ml propidium iodide. Analysis for cell DNA content was performed using a Becton Dickinson (Mountain View, CA, USA) FACScan flow cytometer and data for 10 000 events used for analysis.

Mathematical model analysis procedure

This is based on conservation equations for cells in each phase of the cell cycle, with transition rates between phases (7,11,16,17). For example, if one counts the number of cells in each phase every hour, then the number of cells in G₁-phase at $t + 1$ h is equal to the number of cells that were in G₁-phase one hour previously, that is, at time t hours, minus those that transferred to S-phase during the last hour, plus those newly divided cells that have arrived from M-phase. This gives rise to the differential equation for conservation of cells in G₁ phase at time $t + 1$:

$$n_{G_1}(t + 1) = n_{G_1}(t) - r_{G_1S}n_{G_1}(t) + 2r_{MG_1}n_M(t)$$

where $n_{G_1}(t)$ and $n_M(t)$ are the numerical density of cells in the G₁ and M-phase respectively at time t hours, r_{MG_1} is the division rate per hour and r_{G_1S} is the transition rate of cells from G₁ to S-phase per hour. The model incorporating time steps Δt less than 1 h gives rise to the differential equation

$$n_{G_1}(t + \Delta t)\Delta t = n_{G_1}(t)\Delta t - r_{G_1S}\Delta t n_{G_1}(t)\Delta t + 2r_{MG_1}\Delta t n_M(t)\Delta t.$$

Transition rates between phases may depend on the length of time that cells have been in a particular phase. If $n_{G_1}(t, \tau_{G_1})$ is the numerical density of cells in G₁, at time t , that have been in G₁ for τ_{G_1} hours, the conservation equation becomes

$$n_{G_1}(t + \Delta t, \tau_{G_1} + \Delta\tau_{G_1})\Delta t\Delta\tau_{G_1} = n_{G_1}(t, \tau)\Delta t\Delta\tau_{G_1} - r_{G_1S}\Delta t n_{G_1}(t, \tau_{G_1})\Delta t\Delta\tau_{G_1}$$

with a renewal condition

$$n_{G_1}(t + \Delta t, \tau_{G_1} = 0)\Delta t\Delta\tau_{G_1} = \sum_{\tau_M=0, \Delta t, 2\Delta t, \dots} 2r_{MG_1}\Delta t n_M(t, \tau_M)\Delta t\Delta\tau_M$$

where $n_M(t, \tau_M)$ is the numerical density of cells in M, at time t , that have been in M for τ_M hours. For arbitrarily

small time steps, the differential equation becomes a partial differential equation with the associated renewal condition:

$$\frac{\partial n_{G_1}(t, \tau_{G_1})}{\partial t} + \frac{\partial n_{G_1}(t, \tau_{G_1})}{\partial \tau} = -r_{G_1S} n_{G_1}(t, \tau_{G_1})$$

$$n_{G_1}(t, \tau_{G_1} = 0) = 2 \int_0^\infty r_{MG_1} n_M(t, \tau_M) d\tau_M.$$

One can obtain similar equations in other phases of the cell cycle using the same principles. Using these transition rates, one can observe that cells spend a variable amount of time in each phase. As the average life expectancy of an individual cell is the reciprocal of its death rate, so is the average length of time T_{G_1} that it spends in G_1 -phase the reciprocal of transition rate from that phase i.e. $T_{G_1} = 1/r_{G_1S}$ hours. Cells are assumed to spend a fixed time T_S hours in S-phase before transferring to G_2 -phase.

With constant or age-dependent transitions between phases, independent of how many cells are in each phase at time $t = 0$ h, eventually proportions in each phase become constant with increasing time. This situation represents a cell line unperturbed by cancer therapy. One can obtain the flow cytometric DNA distribution of such a cell population at any time by superimposing a Gaussian distribution and comparing model to data. For example, the flow cytometric DNA distribution F_{G_1} of the G_1 -phase population at time t hours would be

$$F_{G_1}(t) = \frac{N_{G_1}(t)}{\sqrt{2\pi\theta_1^2}} \exp\left(-\frac{(x - \mu_1)^2}{2\theta_1^2}\right)$$

where μ_1 is the mean of the G_1 peak and θ_1^2 is its variance. The area under this curve is the numerical density of cells in G_1 phase at time t hours namely $N_{G_1}(t)$.

DNA distributions in the other phases can be generated similarly using appropriate means and variances in each phase. Mean and variances in G_2 /M phase are denoted μ_2 and θ_2^2 respectively. As cells increase their DNA content during S-phase, it is important to keep track of how long a cell spends in S-phase. The mean of the DNA distribution of a group of cells that have been in S-phase for τ_S hours is assumed to be $\mu_1 + g_S\tau_S$ where g_S is the rate of DNA

synthesis during S-phase. When a group of cells has been in S-phase for $\tau_S = T_S$ hours, they transfer to G_2 phase and therefore the mean of their DNA distribution should be that of the G_2 peak namely $\mu_1 + g_S T_S = \mu_2$. Rearranging this latter expression gives a formula for time a cell spends in S-phase $T_S = (\mu_2 - \mu_1)/g_S$ hours. Variance of the DNA distribution of cells in S-phase is chosen so that variances increase linearly from the G_1 peak to G_2 /M peak.

Three transition rates are defined as the proportions of cells per unit time (here chosen as 30 min) that are transferred from G_1 -phase to S-phase (r_{G_1S}), from G_2 -phase to M-phase (r_{G_2M}) and from M-phase to mitotic cell death (r_{MD}). Perturbations of transition probabilities can be made by altering both rate constant for transition and time before perturbation is relaxed. It is assumed that cell loss has a minimal effect on relative sizes of the G_1 , S and G_2 -M peaks in the flow cytometric profiles.

The mathematical model was used to generate theoretical flow cytometric profiles, and model parameters were adjusted to minimize differences between experimental flow cytometric data and the model, using the least squares optimization function *fmincom* (MATLAB; The Mathworks).

Results

Analysis of cell lines

Flow cytometry profiles were collected for the five cell lines following addition of paclitaxel (200 nM), following exposure to radiation at 2 and 9 Gy, or following exposure to radiation at 2 and 9 Gy and addition of paclitaxel. Profiles were collected at multiple time points up to 96 h. The following sequence of analysis was used. First, flow cytometric data from cultures treated with paclitaxel alone were used to determine the model parameter values for transition rates from G_1 -phase to S-phase (r_{G_1S}), from G_2 -phase to mitosis (r_{G_2M}), from mitosis to cell death (r_{MD}) as described previously (11,18). Values for r_{G_1S} and r_{G_2S} , as well as derived cell cycle times, are shown in Table 1, which also shows DNA content and p53 status (13); because of refinements in the model, values differ slightly

Table 1. Properties of the melanoma lines

Cell Line	p53 status	Cycle time (h)	DNA content (x diploid)	r_{G_1S} (% G_1 -phase cells/30 min)	r_{G_2M} (% G_2 -phase cells/30 min)
NZM3	Wild type	53.5	1.65	2.6%	15%
NZM4	Mutant	44.4	2.4	2.6%	5.7%
NZM5	Wild type	40.8	0.9	1.0%	2.0%
NZM6	Wild type	35.4	1.76	2.6%	13.1%
NZM13	Wild type	77.2	1.08	1.63%	3.4%

from those previously published. Secondly, cultures treated with radiation or with a combination of paclitaxel and radiation were used to determine changes in r_{G_1S} and r_{G_2M} as a function of time after radiation. As the proportion of S-phase cells in treated cultures remained low over the culture period, it was assumed that the main radiation-induced perturbations were for r_{G_1S} and r_{G_2M} and changes in S-phase duration were not included. Although it was clear from the profiles that cell cycle perturbations were occurring following radiation at 2 Gy, they were of low magnitude and the model was fitted only for the 9 Gy data. For these cell lines, radiation did not cause accumulation of cells with DNA content higher than G₂/M. Values for transition rates from mitosis to cell death (r_{MD}), as well as values for cell division rate (r_{MG_1}) (16) were not significantly incorporated for each of the datasets. For each of the cell lines, the r_{G_1S} and r_{G_2M} transition rates were found to drop initially to a minimum value, then to undergo recovery after delay. A sigmoidal recovery curve was assumed for this analysis, although linear recovery was also tested and had little effect on the final result. Results for individual lines are shown below.

NZM3 cell line

Examples of experimental data and the best fit model are shown in Fig. 2. The model indicated no change in r_{G_1S} over the 4-day measurement period and a 99.7% initial reduction in r_{G_2M} (Table 2). Best fit recovery of r_{G_2M} started at 18.5 h and reached a value of 23% of unperturbed r_{G_2M} value by 80.5 h (Fig. 3). This fit was not quite

Figure 2. Flow cytometry profiles of the NZM3 cell line. Dashed line indicates experimental data, while the solid line indicates fit of the mathematical model. Dotted peaks to the left of the profile for NZM3 cells (approximately 0.1 and 0.2 times the G₁-phase DNA content) originate from DNA of chicken erythrocytes, which was added as an internal standard.

Table 2. Effects of radiation at 9 Gy within a 96-h time-frame

Cell line	Transition	Initial reduction (%)	Initiation of recovery (h)	Fraction of unperturbed value at 96 h (%)
NZM3	r_{G_1S}	0	None	100
	r_{G_2M}	99.7	18.5	23
NZM4	r_{G_1S}	15	None	85
	r_{G_2M}	93	4.5	85
NZM5	r_{G_1S}	0	None	100
	r_{G_2M}	97.5	6.5	44
NZM6	r_{G_1S}	96.3	0.5	48
	r_{G_2M}	91.4	60	12
NZM13	r_{G_1S}	90	0.5	92
	r_{G_2M}	84	13.5	33

ideal as the proportion of G₁-phase cells was somewhat over-estimated at 18 h in the paclitaxel data, leading to over-estimation of combination with paclitaxel plus 9 Gy data at 18 h. The slow increase of r_{G_2M} with time was interpreted as representing growth of a recovering population, and assuming that this population had the same kinetic properties as the original provided an estimate for the recovering fraction of 6%. Radiation at 9 Gy was previously found to induce 97% loss of clonogenic survival (12).

NZM4 cell line

Best fit model for response (Fig. 4) indicated a small initial 15% reduction in r_{G_1S} that was maintained for the entire 96 h. The initial 93% reduction in r_{G_2M} was followed by a rise to 90% of the unperturbed value by 96 h (Table 2; Fig. 3). This rise was consistent with the growth of a recovering 20% proportion of cells. In comparison, radiation at 9 Gy induced 90% loss of clonogenic survival (12).

NZM5 cell line

The best fit model for response to radiation at 9 Gy (Fig. 5) included no change in r_{G_1S} and initial 98% reduction in r_{G_2M} . The subsequent rise in r_{G_2M} commenced at 6.5 h and reached 44% of unperturbed value after 96 h (Fig. 3). This increase was consistent with growth of recovering 20% fraction of cells. In comparison, radiation at 9 Gy induced 96% loss of clonogenic survival (12).

NZM6 cell line

The best fit model for response to radiation at 9 Gy (Fig. 6) included initial 96% reduction in r_{G_1S} and a 91% reduction in r_{G_2M} . Reversal of reduction of r_{G_1S} was rapid,

Figure 3. Values for the (r_{G1S}) and r_{G2M} cell cycle transitions (using the model outlined in Fig. 1) as a function of time after irradiation for each of the cell lines.

Figure 4. Flow cytometry profiles for the NZM4 cell line. Dashed line indicates experimental data and solid line indicates fit of the mathematical model. Other details are as in Fig. 2.

commencing within the first hour and reaching a new value after 14.5 h, of 48% of unperturbed value (Fig. 3). In contrast, the subsequent rise in r_{G2M} was slow, commencing after 60 h and reaching 12% of unperturbed value after 96 h. This was consistent with a recovering fraction of 2%. Number of cells in the cultures, as measured after 48 h, was approximately 30% of original value, consistent with induction of cell loss as well as of cell cycle arrest. In comparison, radiation at 10 Gy

Figure 5. Flow cytometry profiles for the NZM5 cell line. Dashed line indicates experimental data and solid line indicates fit of the mathematical model. Other details are as in Fig. 2.

induced $97 \pm 1.5\%$ loss of clonogenic survival (E.S. Marshall and B.C. Baguley, unpublished result).

NZM13 cell line

The best fit model for response to radiation at 9 Gy (Fig. 7) included an initial 90% reduction in r_{G1S} and 84% reduction in r_{G2M} . Recovery of r_{G1S} was rapid, starting in the first hour and attaining a new equilibrium after 18 h of

Figure 6. Flow cytometry profiles for the NZM6 cell line. Dashed line indicates experimental data and solid line indicates fit of the mathematical model. Other details are as in Fig. 2.

Figure 7. Flow cytometry profiles for the NZM13 cell line. Dashed line indicates experimental data and solid line indicates fit of the mathematical model. Other details are as in Fig. 2.

92% of unperturbed value (Fig. 3). The rise in r_{G2M} commenced after 14 h and reached 33% of the unperturbed value by 96 h, suggesting a recovery fraction of approximately 8%. Cell number measured 96 h after radiation at 9 Gy was 75% of that expected, suggesting that some cell loss was occurring as a result of radiation. Clonogenic survival data were not available for this line.

Discussion

The technique of fitting data of a series of flow cytometric profiles from a time series to a dynamic mathematical model of the cell division cycle has been applied here to measurement of cell cycle perturbations induced by radia-

tion, in five human melanoma cell lines. Amount of available cytokinetic information has been increased by analysing effects of radiation in combination with paclitaxel, a drug that acts on mitosis without causing interphase cycle arrest (19). Cell cycle distribution data have been combined with total cell recovery data and, for four of the five cell lines, with previously published clonogenic survival data. Recovery of NZM6 and NZM13 cells, measured 96 h after irradiation was 30% and 75% respectively consistent with induction of cell loss, while recovery of cells from the other lines suggested minimal cell loss. Results support the hypothesis that induction of irreversible cell cycle arrest in G_2/M -phase contributes a major component of reduction in clonogenic survival following irradiation of these cell lines at a dose of 9 Gy. It should be noted that cells that are not arrested following irradiation and pass through cell division are assumed in the model to continue proliferating. This assumption may lead to underestimation of overall radiation response, particularly at lower radiation doses where, a higher fraction of cells divide.

One of the aims of this analysis was to investigate the role of the *TP53* status in response of cells to radiation. All lines except NZM4 had wild-type *TP53* status (Table 1) and it was expected that all except NZM4 might exhibit induction of p53-dependent cycle arrest and apoptosis. G_1 -phase arrest was observed for NZM6 and NZM13 cell lines and it was noteworthy for these lines that reversal of G_1 -phase arrest was rapid, consistent with a transient effect. A term for radiation-induced G_1 -phase arrest in the NZM3 and NZM5 lines was not included significantly in the overall model, even though flow cytometry profiles, 16 h after irradiation at 9 Gy in the presence of paclitaxel, did indicate a small amount of G_1 -phase arrest (13). It is possible that the p53 pathway is attenuated in these lines, as reported for other melanoma cell lines (20), and that this attenuation leads to only transient G_1 -phase arrest and to minimal effects on the cell cycle over a time scale of 4 days. The NZM4 cell line, which has mutant p53 status, showed a small non-reversible decrease in the r_{G1S} transition, but the cause for this remains unknown. It is of interest that application of a different mathematical model to behaviour of HCT-116 colon carcinoma cells to the drug cisplatin also led to the discovery of a p53-dependent extended cell cycle arrest (9).

Previous studies on the effects of mitotic poison paclitaxel on these cell lines indicated that drug exposure resulted in mitotic arrest followed by mitotic cell death in which dying cells progressively lost DNA with time (7,11). A transition probability term (r_{MD}) was included in the modelling of these effects to account for this induced cell death, but this term was not necessary for analysis of radiation effects, even in the presence of paclitaxel. A

likely interpretation is that G₂-phase arrest induced by radiation prevents a significant proportion of cells from progressing from G₂-phase to mitotic arrest and thus to mitotic cell death.

Data for clonogenic cell survival following irradiation were available for four of the cell lines and it was of interest to determine whether analysis of flow cytometry data supported results of clonogenic assays. Radiation at 9 Gy decreased r_{G_2M} transition rates by 99.7%, 93% and 97.5% respectively for the NZM3, NZM4 and NZM5 lines (Table 2) compared to 97%, 90% and 96% respectively in clonogenic survival. The comparison was more complex for NZM6 because of simultaneous decreases in the r_{G_1S} (96.3%) and r_{G_2M} (91.4%) transition rates (Table 2) compared to 97% decrease in clonogenic survival. Thus, effects of radiation on r_{G_1S} and r_{G_2M} transition rates could be interpreted as indicating transition of cells to a senescent state that could not form a colony.

A further feature of the results was that the r_{G_2M} transition rates rose with time following attainment of minimum values (Fig. 3) and this was interpreted as indicative of growth of recovering cells against a background of arrested (senescent) cells. The calculated recovering fraction based on this analysis was usually higher than the expected value based on the surviving fraction, but this might be explained by presence of cells that resumed cell cycling, but were unable to produce colonies (that is, clusters of 50 or more cells).

A further aim of this analysis was to develop a model that would predict effects of radiation on tumour growth in a clinical setting; effects of radiation at 2 Gy, a standard daily clinical dose, would be of particular interest. Clonogenic survival responses of the NZM3, NZM4, NZM5 and NZM6 cell lines in response to radiation at 2 Gy are 91%, 89%, 36% and 15% respectively (12). While we have collected flow cytometric data for cells irradiated at 2 Gy, the relatively small induced perturbations of the cell cycle preclude accurate application of the current model and further refinements will be required to approach this problem. However, clinical treatment involves daily doses, the cumulative effect of which may be more comparable to the 9 Gy dose analysed here.

In conclusion, the analysis of a series of flow cytometry profiles for five human melanoma cell lines at various times following irradiation at 9 Gy provides a surprising overall picture in which long-term cell cycle arrest, rather than apoptosis, accounts for much of the loss of viability observed in clonogenicity assays. Such arrest is likely to reflect induction of senescence, a reported response to ionizing radiation (21,22). Results demonstrate the potential of this mathematical modelling procedure to improve our understanding of dynamic effects of radiation on cells and

also provide a basis for similar studies on effects of chemotherapeutic drugs.

Acknowledgements

This study was supported by the Auckland Cancer Society, the Genesis Oncology Trust and the Hepburn Cancer Research and Mary Manning Memorial Fellowship.

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