Project Summary

Project ID number (DOE EMSP): 69981

Project ID number (DOE OBER): DE-FG07-99ER62878

Project Title: Mechanisms of enhanced cell killing at low doses: Implications for radiation risk

Lead Principal Investigator: Dr Peter J. Johnston johnston@gci.ac.uk

Co-Investigator(s): Dr George D. Wilson wilson@gci.ac.uk

Address (all investigators): Gray Cancer Institute
PO Box 100, Mount Vernon Hospital, Northwood,
Middlesex HA6 2JR, United Kingdom
Tel: 011 44 1923 828611 **Fax:** 011 44 1923 835210

Additional graduate students and/or post-doctorates actively involved: 2

Specific DOE problems that are being addressed

We have shown that cell lethality actually measured after exposure to low-doses of low-LET radiation, is markedly enhanced relative to the cell lethality previously expected by extrapolation of the high-dose cell-killing response. Net cancer risk is a balance between cell transformation and cell kill and such enhanced lethality may more than compensate for transformation at low radiation doses over at least the first 10 cGy of low-LET exposure. This would lead to a non-linear, threshold, dose-risk relationship. Therefore our data imply the possibility that the adverse effects of small radiation doses (<10 cGy) could be overestimated in specific cases. It is now important to research the mechanisms underlying the phenomenon of low-dose hypersensitivity to cell killing, in order to determine whether this can be generalized to safely allow an increase in radiation exposure limits. This would have major cost-reduction implications for the whole EM program.

Research Objective

Our overall aim is to gather understanding of the mechanisms underlying low-dose hyper-radiosensitivity (HRS) and induced radioresistance (IRR). There is now some direct evidence that this dose-dependent radiosensitivity phenomenon reflects changes in the amount, rate or type of DNA repair, rather than indirect mechanisms such as modulation of cell-cycle progression, growth characteristics or apoptosis. There is also indirect evidence that cell survival-related HRS/IRR in response to single doses might be a manifestation of the same underlying mechanism that determines the well-known *adaptive response* in the two-dose case, thus HRS can be removed by prior irradiation with both high- and low-LET radiations as well as a variety of other stress-inducing agents such as hydrogen peroxide and chemotherapeutic agents.

Our goals in this project were therefore:

1. Identify which aspects of DNA repair (amount, rate and type) determine HRS/IRR,
2. Investigate the known link we have discovered between the extent of HRS/IRR and position in

the cell cycle, focusing on changes in DNA structure and conformation which may modulate DNA repair,

3. Use the results from studies in (1) and (2) to distinguish, if necessary, between HRS/IRR and the *adaptive response*. The aim is to finally determine if these are separate or interlinked phenomena.

Use the results from studies in (1), (2) and (3) to propose a mechanism to explain HRS/IRR.

Research progress and implications

This report summarizes progress as of October 2002, the end of the 36 month program activated in November 1999.

Technical Achievements

Over the course of the program a number of significant developments have been made that improve both the delivery and measurement of the effects of low dose irradiation at the cellular level. These have been made in collaboration with colleagues in the Advanced Technology Group and the Cell and Molecular Biophysics Group at the GCI

1) We have already established cell-plating techniques that improve the accuracy of clonogenic assays, as is required for examining the effects of low doses on cellular survival. However, low-dose clonogenic assays require large cell samples to maintain statistical accuracy. Manually counting the resulting colonies is a laborious task in which consistent objectivity is hard to achieve. This is true especially with some mammalian cell lines that form poorly defined or 'fuzzy' colonies typified by glioma or fibroblast cell lines. A computer-vision-based automated colony counter has been developed. This system utilizes novel imaging and image-processing methods involving a modified form of the Hough transform. The automated counter is able to identify less-discrete cell colonies. The results from the automated counts fall well within the distribution of the manual counts with respect to surviving fraction (SF) versus dose curves, SF values at 2 Gy (SF2) and total area under the SF curve (Dbar). This system also permits quantitative assessment of colony size, another potential indicator of cellular effects of low dose irradiation.

2) We have also applied improved image analysis sub-routines and automated image recognition algorithms to improve the sensitivity and accuracy of the measurement of DNA strand breaks. The assay exhibits a sensitivity of approximately 50 strand breaks per cell for both SSB and DSB. This equates to approximately 0.05 Gy or 2 Gy respectively. The primary limitation to improving the sensitivity of the SCGE assay has been sample throughput due to the heterogeneous response of cells in varying cell cycle phases (e.g. using alkaline lysis conditions. S-phase cells exhibit single-strand breaks equivalent to approximately 5 Gy while under neutral conditions S-phase cells show significant retardation of migration of damaged DNA, both phenomena due to the presence of replication forks). Therefore, at low doses, minor changes in cell cycle distribution will have a greater influence on damage detection than changes in the number of strand breaks induced by radiation. In contrast to other biophysical methods of DNA damage measurement, such as pulsed field electrophoresis and filter elution, the SCGE assay examines damage on an individual cell basis permitting analysis of heterogeneity of response within different cells of a single population and compensation for cell cycle effects. Current,

manual systems provide a sample throughput of approximately 300 comets per hour. Our fully automated microscopy system has a sample-rate of approximately 5000 comets per hour that will enable further increases in sensitivity, particularly at very low doses.

3) A novel low dose-rate irradiation system that utilizes a ^{60}Co gamma source and an attenuating water tank has been developed to carry out simultaneous irradiations over a wide range of dose rates (1 to 100 cGy/h). We have now obtained definitive data indicating a greater reduction in cell survival per unit dose of irradiation at continuous low dose rate exposures of 2, 5 or 10 cGy h^{-1} compared with 20 and 60 cGy h^{-1}. We predicted this effect from our acute-dose HRS experiments. Previous explanations of such inverse dose-rate effects have invoked putative accumulation of cells in the $_{G2}$ phase of the cell cycle as a G_1 block is lost with decreasing dose rate. However, we have shown that i) G_2 accumulation becomes less as the dose rate is reduced, ii) HRS/IRR is observed in p53 mutant cells and iii) HRS/IRR is observed in cells arrested by confluence during irradiation. Cells that do not exhibit HRS also fail to exhibit an inverse dose rate effect.

Scientific Summary

1) The DNA single strand break has been proposed as the key lesion in the initiation of increased radiation resistance. Cell survival studies using hydrogen peroxide as a priming agent indicate that the production or repair of SSBs can induce induced radiation resistance. Poly(ADP-ribose) polymerase (PARP) has been described as a co-ordinator of the cell's early response to DNA damage, in particular SSBs. Rapid binding of this abundant nuclear protein to single and, to a lesser extent, double strand breaks (DSBs) activates the poly(ADP-ribosyl)ation of a number of nuclear proteins including itself. This leads to changes in chromatin structure and modulates the activity of a number of enzymes involved in DNA damage sensing and repair. PARP is thus a prime candidate for mediating induced radiation resistance. Treatment with 3-aminobenzamide (3AB), a potent inhibitor of PARP has been reported to block the development of induced radiation resistance in V79 Chinese hamster fibroblasts. A collaboration with the Drug Development Unit at Newcastle University have allowed access to novel, more potent and specific inhibitors of PARP; 8-hydroxy-2- methylquinazolin-4-one (NU1025), 4-amino-1,8-naphthalimide(4-ANI) and PJ34. Treatment with these agents accentuated the low dose hyper-radiation sensitivity of Chinese hamster and human cell lines but had little effect on the transition radiation resistance. Only slight radio-sensitization in the high dose region was observed, consistent with published data. These results give further support to our hypothesis that PARP may be a critical component of the sensing mechanism that underlies the activation of the increased radioresistant response once sufficient damage has accrued. Additional experiments were performed that applied antisense, ribozyme and knockout technology to achieve a more specific down-regulation of PARP-1. It is apparent that depletion of one member of the PARP family is insufficient to disrupt the multi-phasic response to low doses.

To examine the hypothesis that the HRS/IRR response of cells involves alterations in the repair capacity of cells we have the examined the repair of chromosomal damage and the role played by the major DNA double strand break repair pathway, non-homologous end-joining. Cell lines deficient for the key DSB repair enzyme DNA dependent protein kinase fail to exhibit IRR. Similarly, non-toxic concentration of wortmannin, an inhibitor of DNA-PK, radiosensitized both T89G and U373 cells abrogating the HRS/IRR type response. However, no definitive evidence for alterations in repair kinetics or fidelity have been demonstrated. Changes in the activity of

DNA-PK have been reported after low doses. Here, a reduction in DNA-PK activity was observed in cells that exhibited low dose hyper-radiation sensitivity whereas an increase was seen in cell lines that did not. This is contrary to what might be expected in an induced repair mechanism. Subsequently, our own work has failed to identify any significant variation in DNA-PK transcription, protein levels or activity after low dose irradiation. Current, biophysical assays such as filter elution and electrophoretic methods do not have sufficient sensitivity to examine the induction and repair of DSBs. Surrogate markers, such as chromatid breaks and the resulting micronuclei, do enable an approximation to made of these critical lesions. Low dose hyper-radiation sensitive responses for the induction of micronuclei have been reported. Micronuclei are predominantly formed from acentric chromosomal fragments that fail to segregate properly during mitosis. Acentric fragments may arise from non- or mis-repaired DNA double strand breaks and are generally accepted as markers of lethal events. Our own work indicates that low dose hyper-radiation sensitivity is observed for both total micronucleus induction and fraction of undamaged cells. However, this is observed in both cells that do and do not exhibit clonogenic survival low dose hyper-radiation sensitivity when asynchronous populations are examined. A key consideration when interpreting micronucleus assay data is the cell cycle selectivity of the technique. Only cells that have passed through a single mitosis are scored, therefore the assay will be skewed towards cells in G_2 at the time of irradiation. Pilot experiments with chromatid breaks indicate that there is no difference in the repair kinetics after 20 cGy (in the low dose, hypersensitive region) and 60 cGy (in the high dose, resistant region) in V79 cells.

The conclusion of these studies is that while the repair of DSBs does appear to be essential for the transition to a resistant phenotype, the evidence that specific pathways are induced is lacking.

2) Distinct high dose radiation sensitivities are apparent in cell populations defined by cell cycle position. Similar variations occur at low doses with G_2 cells exhibiting marked low dose hyper-radiation sensitivity. This is also seen in G_2 population of cell lines such as CHO-K1 and U373 that do not appear hypersensitive when asynchronous population are examined. This cell cycle specificity may be reflected in the apparent low dose hyper-radiation sensitive response seen with the micronucleus assay.

The low-dose response of cells during different phases of the cell cycle is of interest because the efficiency and type of DNA repair are recognised to vary with cell-cycle phase. In the context of the induced repair model, that the G_2 cell-cycle population shows a marked multi-phasic response, this would suggest that the DNA damage recognition/repair pathways that function in G_2 are most sensitive to dose in the low-dose range. However, G_2 cells are also more radiosensitive at high doses. This suggests that, if inducible repair contributes preferentially to repair in G_2 then other repair pathways are less effective in this phase. Homologous repair is thought to function primarily in G_2 and is an inducible pathway. However, low dose hyper-radiation sensitivity is also observed in G_1 of some cell lines, e.g. T98G glioblastoma cells, though homologous repair is least effective here.

Therefore, while the repair of DSBs does appear to be essential for the transition to a resistant phenotype, the evidence that specific pathways are induced is lacking. This is in contradiction to the induced repair model but can be reconciled by taking into account the dynamics of cell cycle distribution and redistribution post-irradiation. In this respect the more dynamic sub-population model proposed by Wouters et al. reconciles more closely with the data.

Support for a dynamic cell cycle model comes from a number of dose-rate studies. At very low dose rates, inverse dose rate effects are observed whereby lowering the dose rate increases cell killing per unit dose. At dose rates down to 32 cGy hr^{-1}, this was explained by increased accumulation in the radiation sensitive G_2 phase. At even lower dose rates (2-10 cGy hr^{-1}) where an inverse dose rate is still seen, this accumulation does not occur, i.e. the cells continue to cycle normally. This indicates that it may be a failure to induce cell cycle checkpoints that is a determinant of low dose hyper-radiation sensitivity.

The lethality of a DSB will be primarily effected at mitosis. Here, DSBs compromise genomic integrity by the loss or misdistribution of chromosomal DNA as a result of acentric fragment formation. Therefore, changes in the rate of traversal through the critical G_2/M transition point (as well as other critical cell cycle points such as replication) will provide additional time for repair.

It has long been recognised that the transition from G_2 to mitosis is readily inhibited by low doses of ionising radiation. That the G_2/M checkpoint is responsive to low doses was confirmed by an analysis of the radiation-induced changes to the mitotic index of 6 cell lines. In all cell-lines the passage of cells into mitosis was inhibited by doses as low as 5 cGy. The mitotic index decreased in an exponential manner with a mean half dose value of 30 cGy. This value is similar to the transition dose to radiation resistance as measured by clonogenic survival. However, in this preliminary examination, there was no clear correlation between the inhibition of mitosis and the clonogenic response of asynchronous cell populations to low doses. Preliminary pulse labelling studies on the U373 glioblastoma cell line indicate that while low doses of radiation are sufficient to inhibit the progression of G_2 cells, higher doses (≥0.6 Gy) are required to inhibit cells in S-phase at the time of irradiation. This indicates a potential threshold for G_2/M delay.

The DNA damage induced G_2/M checkpoint may be either transient (TP53 independent) or chronic (TP53 dependent. Methylxanthines are recognized radio-sensitizers of TP53 disrupted cells lines. They cause abrogation of the radiation induced G_2/M checkpoint through modulation of cyclin B1 and $p34^{cdc2}$ levels via inhibition of the ATM/CHK2/CDC25A. When T98G glioblastoma cells (TP53 mutated) were irradiated in the presence of a variety of concentrations of caffeine, there was a significant enhancement of the low dose hyper-radiation sensitivity. At low doses of caffeine, transition to increased radiation resistance was still observed. At high, toxic, concentrations caffeine sensitized cells to high doses of radiation. This implies that while the G_2/M checkpoint may play a role at very low doses (and dose rates) the response to higher doses may involve other checkpoints or pathways. It has been demonstrated that activation of TP53 by post-translation modification occurs at doses > 0.5Gy in normal human diploid fibroblasts. These results indicate that the multi-phasic response to low doses of radiation may differ from the interaction between both ATM dependent (low doses) and TP53 dependent (high dose) cell signalling pathways.

3) In summary, we have determined a complex multi-phasic response to low doses of ionizing radiation. The current model of an induced repair mechanism for the increase in radiation resistance that occurs at doses > 0.5 Gy is proving to be less likely than one involving variations in cell cycle kinetics. Evidence is increasing for a model of increased radiation resistance through the induction of cell cycle checkpoints that provide additional time for DNA repair.