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

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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 are 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, and planned activities

This report summarizes progress as of July 2001, which is 20 months into a three-year programme activated in November 1999.

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. In collaboration with Paul Barber and Boris Vojnovic of the Advanced Technology Group at the GCI, a computer-vision-based automated colony counter has been developed. This system utilises 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. A second-generation system is currently under construction that will provide additional versatility.

2) A novel low dose-rate irradiation system that utilises 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.

3) To examine the hypothesis that the HRS/IRR response of cells involves alterations in the repair capacity of cells we are currently developing non-clonogenic assays. Micronuclei are readily quantifiable lesions that correlate with the induction and repair of DSBs, chromosomal breaks and lethal lesions. Results with cytochalasin-B block micronucleus assay indicate that cells exhibiting an HRS/IRR clonogenic response also exhibit similar hypersensitivity to the formation of acentric chromosomal fragments. This implies the DNA double strand break in the HRS/IRR response. Current DSB assays do not provide the sensitivity to examine the repair of DSBs at low doses, nor do they provide qualitative information of DNA repair processes. Construction is underway of a high-speed automated epifluorescent cytometer that will be

employed to measure low levels of DNA damage by use of the single-cell gel electrophoresis ("comet"). By applying novel algorithms to image capture and analysis we have increased the accuracy and robustness of comet analysis. DSB induction, repair and misrepair are also being examined at low doses by the premature chromosome condensation technique. This system is also employed in identifying the lesions responsible for triggering the IRR response.

4) Direct and circumstantial evidence has indicated that DNA double strand break repair via non-homologous end joining (NHEJ) is probably the process most closely connected with IRR. We have therefore examined the role of DNA dependent protein kinase in HRS/IRR. 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. It has been reported that a significant correlation exists between the relative change in DNA dependent protein kinase activity in response to irradiation and the extent of IRR was observed. Considerable effort has been made to confirm these observations. However, no significant changes in DNA-PK activity were observed in HRS/IRR positive/negative cells over the 0.05-0.8Gy dose range. It appears that the kinase assay employed is not sufficiently sensitive to detect very small changes in DNA-PK activity with reproducibility. Similarly, there is no apparent correlation between the presence of HRS/IRR and levels of DNA-PK and associated proteins. Work has now commenced using time resolved multi-photon microscopy analysis to determine the role of DNA-PK and chromatin organisation and distribution in HRS/IRR.

5) We have demonstrated HRS/IRR was also modified by 3-aminobenazmide, an inhibitor of poly (ADP-ribosyl) transferase (PARP) prevented the expression of IRR. We have extended these observations by examining the effect of novel inhibitors of poly (ADP-ribosyl) transferase in the T98G and U373 human glioma cells. A collaboration with the Drug Development Unit at Newcastle University have allowed access to novel, more potent and specific inhibitors of PARP. Initial observations with non-toxic doses of the inhibitor 8-hydroxy-2- methylquinazolin-4-one (NU1025) have shown the inhibition of IRR in T98G cells (HRS/IRR positive) while no significant modification of clonogenic survival was evident in U373 cells (HRS/IRR negative). Interestingly however, we did observe a paradoxical increase in plating efficiency in U373 cells after exposure to NU1025. The reason for the increase in PE has yet to be determined. 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. To this end, we are applying antisense and ribozyme technology to achieve a more specific down-regulation of PARP-1 at the mRNA level. A panel of six hammerhead ribozymes directed at specific sequences of the PARP-1 mRNA has been designed, targeting splice sites in the DNA-binding and catalytic domains. Four of these have been successfully cloned into a ribozyme expression vector which also encodes a reporter EGFP gene. Transfection of one of these constructs into T98G glioma cells and its effect on PARP-1 protein levels in cells selected for EGFP expression has been assessed by Western blot and immunoblot techniques. After 48, 72 and 96 hour incubations, a modest down-regulation of PARP-1 (c. 20%) has been reproducibly achieved. We have recently acquired a PARP-1 antisense vector from the Smulson group in Washington DC. Our ribozymes will be tested against this dexamethasone-inducible antisense construct. An effective anti-PARP-1 ribozyme will be used as an investigative tool in low-dose survival experiments on T98G glioma cells which demonstrate

HRS/IRR and U373 glioma cells which do not. The differential effects of PARP-1 depletion in these and other cell lines will elucidate its role in the low-dose hypersensitivity phenomenon.

Information Access: key recent publications

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2. **Joiner MC**, Lambin P and **Marples B**, 1999, Adaptive response and induced resistance. *Comptes Rendus de l'Académie des Sciences Série III*, **322**, 167-175.
3. Robson T, **Joiner MC, Wilson GD**, McCullough W, Price ME, Logan I, Jones H, McKeown, SR, and Hirst DG, 1999, A novel human stress response-related gene with a potential role in induced radioresistance. *Radiation Research*, **152**, 451-461.
4. Vaganay-Juéry S, Muller C, Abdulkarim B, Deutsch E, Marangoni E, Lambin P, Calsou P, Salles B, **Joiner M,** and Bourhis J, 2000, Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation. *British Journal of Cancer*, **83**, 514-518.
5. Olive PL, Durand RE, Banath JP, **Johnston PJ**. 2001. Analysis of DNA damage in individual cells. *Methods Cell Biol.* **64**, 235-49.
6. **Joiner MC, Marples B**, Lambin P, Short SC, Turesson I., 2001, Low-dose hypersensitivity: current status and possible mechanisms.. *Int J Radiat Oncol Biol Phys.*, **49**, 379-89.
7. Barber, P.R., Vojnovic, B., Kelly, J., Boulton, P., Mayes, C., Woodcock, M. and **Joiner, M.C**., 2001, Automated counting of mammalian cell colonies. *Physics in Medicine and Biology*, **46**, 63-76
8. Short, S.C., Kelly, J., Mayes, C.R., Woodcock, M. and **Joiner, M.C.**, 2001, Low-dose hypersensitivity after fractionated low-dose irradiation *in vitro*. *International Journal of Radiation Oncology Biology Physics*, **6**, 655-664