The following report (July 2008 – April 2009) represents studies undertaken as part of the collaborative programme with Francis Cucinotta (NASA, Lyndon B. Johnson Space Centre, Houston TX, USA) and Janice Pluth (DOE, Lawrence Berkeley National Laboratory, Berkeley CA, USA). The following specific aims in the Oxford project are being addressed within the overall timeframe of the project.

Specific Aim from the proposal

A: *Do the mechanisms of processing of DSB/clustered DNA damage depend on dose and thereby the extent of damage induced in any one cell? Are the mechanisms of rejoining DSB at low and high doses of gamma-rays or alpha-particles different?*

B: *Does the rejoining of DSB induced by HZE particles depend on the particle fluence and thereby the initial amount of damage induced?*

Progress reported based on the original research plan (in italics)

Introduction

Non-homologous end joining (NHEJ) predominates in the repair of DNA double strand breaks (DSB) over homologous recombination (HR). NHEJ occurs throughout the cell cycle whereas HR occurs in late S/G_2 due to the requirement of a sister chromatid (Rothkamm *et al*, Mol Cell Biol **23** 5706-15 [2003]). To date evidence obtained with DSB repair deficient cells using pulsed-field gel electrophoresis has revealed the major pathway throughout all phases of the cell cycle for processing high dose induced DSBs is NHEJ (Wang *et al*, Oncogene **20** 2212-24 (2001); Pluth *et al*, Cancer Res. **61** 2649-55 [2001]). These findings however were obtained at high doses when on average >> 20-30 DSBs are formed per cell. The contribution of the repair pathways (NHEJ and HR) induced in response to DNA damage during the various phases of the cell cycle may depend upon the dose (the level of initial DSBs) especially since low levels of DSBs are induced at low dose. To date, low dose studies using NHEJ and HR deficient mutants have not been carried out to address this important question with radiations of different quality.

Progress

3.B.1) Processing of DSB induced in mammalian cells by high-LET radiation

The complexity of DSB increases with the ionization density of the radiation and these complex DSBs are distinct from the damage induced by sparsely ionizing gamma-radiation. It has been hypothesized that complex DSBs produced by heavy ions pose problems to the DNA repair machinery. To explore this hypothesis, specifically the role of NHEJ in DSB repair, we have utilised M059J and M059K cells (originally supplied by our collaborator Janice Pluth). Although both cell lines were established from the same human glioblastoma, M059J cells have been shown to have no detectable DNA-PK activity (Lees-Miller *et al*, Science **267** 1183-85 [1995]).

Using these cell lines we have previously reported that following exposure to high-LET ^{56}Fe ions and alpha-particles and low-LET gamma-rays the subsequent repair rate of DSBs (as measured by the dynamics of formation and loss of phosphorylated histone H2AX [gamma-H2AX], as a marker of DSBs) is slower and there is greater persistence of RAD51 (a marker

of HR) in M059J cells compared with M059K cells and this difference is independent of the ionization density of the radiation.

NHEJ predominates in DSB repair in all phases of the cell cycle and HR is thought to play a role only in late S/G2 so we also examined the effect of cell cycle on the observation of tracks in M059J and M059K cells tangentially irradiated with alpha-particles and using BrdU pulse-labelling as a marker of S-phase cells. Whilst many of the cells expressing RAD51 tracks are in S-phase (assessed through the co-staining of BrdU and RAD51) co-staining was not 100%. At 24 h, at least 50% of cells containing RAD51 tracks in both cell lines are thought to be in the G2-phase of the cell cycle when HR is functional, potentially explaining this discrepancy. A greater number of M059J cells contained RAD51 foci at 24 h compared with M059K cells and a large proportion of these co-stained with BrdU. This is in line with M059J cells becoming arrested in S-phase post alpha-particle irradiation and consistent with results obtained using 10 Gy gamma-radiation.

In order to confirm these suggestions we used cyclin A as an alternative cell cycle marker and in both cell lines we observed that all of the cells with visible RAD51 tracks co-stained with cyclin A. With BrdU and cyclin A we observed an accumulation of M059J cells positive for BrdU or cyclin A over time indicating an S/G2-phase block. These results are consistent with a recent study by Stracker *et al* (Mol and Cell Biol **29** 503-514 [2009]) which suggested that DNA-PK_{cs} exerts an inhibitory effect on the DNA damage response in S and G2 by inhibiting the S-phase block induced by ATM/ATR so when DNA-PK_{cs} is absent (i.e. in M059J cells) the block can be induced.

We used a specific inhibitor of ATM in order to verify that the differences we observe between the M059J and M059K cell lines is the result of a lack of functional DNA-PK and not due to reported low levels of ATM in M059J cells. We observed a reduction in gamma-H2AX formation in both M059J and M059K cells in the presence of the ATM inhibitor compared with untreated cells. In M059K cells, gamma-H2AX foci are visible albeit with less than half the number of foci/cell 30 min post-irradiation. At later times (>2 h) gamma-H2AX foci numbers are similar to untreated cells and these foci disappear at the same rate as in un-treated cells. In M059J cells, however, at early times (<1 h) very few radiation induced foci are formed when treated with the ATM inhibitor and at later times the number of foci/cell is still significantly lower than in un-treated cells. p53 phosphorylation at the serine 15 residue is also inhibited in both M059J and M059K cells following ATM inhibition indicating that although ATM levels may be lower in M059J cells ATM is present with sufficient activity to carry out cellular signalling events such as phosphorylation of p53 and H2AX. Therefore we are confident that the different effects observed between the M059J and M059K cells is a result of inactive DNA-PK_{cs} in the M059J cell line and not due to an ATM deficiency.

3.A.1) Quantification of the number of γH2AX foci induced in mammalian cells as a function of γ-radiation dose and cell cycle?

3.A.2) Processing of DSB/non-DSB clustered DNA damage induced in mammalian cells as a function of γ-radiation dose

Having observed an increase in the recruitment of RAD51 in M059J cells following exposure to both high- and low-LET radiation we also observed that RAD51 foci were present in only S/G2 phase cells (as measured using BrdU or cyclin A). We hypothesize that radiation-induced complex non-DSB damage therefore contributes to DSB formation in cells that are already in S-phase or as they progress into S-phase. We assessed H_2O_2-induced DSB

formation using immunofluorescent staining for gamma-H2AX. In exponentially growing cells, we observed an induction of DSBs within 15 min post treatment; foci levels reached a peak at 30 min post treatment. A similar induction of gamma-H2AX foci in response to 0.08 mM H_2O_2 (a dose previously shown to yield a 10 Gy equivalent of SSBs but no DSBs [Dahm-Daphi *et al* IJRB **76** 67-75 (2000)]) was seen in human fibroblasts. We also assessed the contribution of HR to the repair of H_2O_2-induced DSBs using RAD51 as a marker. An increase in RAD51 foci numbers was observed at 30 min post treatment and levels peaked at 1 h post treatment. By 3 h post treatment both gamma-H2AX and RAD51 foci numbers approach background levels. During analysis it was noted that distinct populations of cells existed with some showing many gamma-H2AX foci and others showing very few indicating a possible cell cycle effect on H_2O_2-induced DSB formation.

In order to assess whether low levels of H_2O_2-induced DSB are formed in G1-phase cells, confluent cell populations were treated. Very few DSBs were induced above background in confluent cells, a cell population containing ~80% of cells in G1-phase and only 7% in S-phase. At 1 h post treatment with H_2O_2, mean numbers of gamma-H2AX foci were 2.7 in confluent cell populations compared with 11.5 in exponentially growing cells (numbers corrected for background levels). The small increase in gamma-H2AX foci numbers over the time course is likely due to the fact that a small proportion of cells are in S-phase and therefore replication breaks could be formed in these cells.

H_2O_2-induced DSB formation was assessed specifically in S-phase cells by pulse labelling cells with BrdU. Gamma-H2AX foci were induced in S-phase cells and followed a similar time course pattern to that observed in exponentially growing cells. Foci numbers peaked at 15-30 min post treatment and approached background levels by 3 h post treatment. In non-S-phase cells, the mean number of gamma-H2AX foci did not change significantly with time indicating that there is no induction of DSBs in response to H_2O_2 treatment in this cell population. Gamma-H2AX foci were not observed in non-BrdU labelled nuclei. However, large numbers of gamma-H2AX foci are observed in BrdU labelled nuclei. These foci are therefore associated with replication-induced DSBs.

Immediately post H_2O_2 treatment, foci are not detected above background levels suggesting that the numbers of SSBs induced are not directly leading to a significant induction of DSBs. The lag time of 15-30 min before DSBs are detected is consistent with the idea that the DSBs are induced as a result of SSBs encounter replication forks. Quantification of gamma-H2AX foci formation in treated cells showed that ~20 % of cells were negative for foci (i.e. foci numbers not significantly above background); this is consistent with the proportion of cells known to be in G1 phase of the cell cycle as determined by flow cytometric analysis suggesting that these foci are present in S-phase cells. Indeed, studies in confluent cells, where the majority of cells are in G1-phase, showed that very few gamma-H2AX foci are induced with 0.08 mM H_2O_2 further supporting the idea that the H_2O_2-induced DSBs are associated with S-phase cells. This was clarified by pulse labelling cells with BrdU allowing quantification of gamma-H2AX foci formation in S-phase and non S-phase cells. All induced gamma-H2AX foci were observed in S-phase cells while foci in non S-phase cells are close to background levels. The lack of gamma-H2AX foci formation in non S-phase cells also suggest that H_2O_2-induced DSB formation is not as a result of close proximity of SSBs since if this were the case it would be expected to occur in all phases of the cell cycle.

In order to confirm whether H_2O_2-induced DSBs are formed as a result of cells undergoing replication, we attempted to inhibit replication using aphidicolin. Aphidicolin is a specific

inhibitor of α-like polymerases and thereby blocks DNA synthesis. The assumption would be that in the presence of aphidicolin, 0.08 mM H_2O_2 would not induce DSBs in a similar way that aphidicolin pre-treatment has been shown to inhibit camptothecin-induced gamma-H2AX foci formation. However, in contrast to studies by Liu et al (Mutat Res **532 (1-2)** 215-26 2003) who saw no gamma-H2AX foci in response to aphidicolin treatment, we found that aphidicolin treatment alone induced gamma-H2AX foci at levels significantly above background thereby masking any effect on H_2O_2-induced DSBs. Indeed, it has been shown that aphidicolin induces H2AX phosphorylation which is mainly associated with early S-phase cells as detected by multiple parameter flow cytometry and also DSB formation as shown by PFGE. It was noted that gamma-H2AX foci numbers induced when cells were treated with aphidicolin together with H_2O_2 were very similar to those induced by aphidicolin alone. Since treatment of cells with H_2O_2 in the presence of aphidicolin did not induce additional DSBs compared to aphidicolin treatment alone, this indirectly indicates that aphidicolin treatment inhibited H_2O_2-induced DSB formation further confirming that H_2O_2-induced DSBs are replicative DSBs.

Using laser microbeam technnology and ultrasoft X-ray irradiation of cells through micron grids, preliminary studies indicate that a fraction of DSB recruit fluorescently tagged Ku80 and undergo repair independently of the presence of DNA-PKcs whereas a fraction of DSB require both Ku80 and DNA-PKcs for repair. The information obtain will be used to refine the non homologous end joining model we reported in Radiation, 169, 214–222 (2008) as part of the joint programme.

Summary

The work presented here leads us to suggest that HR plays a relatively minor role in the repair of radiation-induced prompt DSBs. SSBs lead to the induction of DSBs which are associated specifically with S-phase cells consistent with the idea that they are formed at stalled replication forks in which HR plays a major role in repair. That DNA-PKcs is in some way involved in the repair of the precursors to replication-induced DSB remains an open question. Persistent non-DSB oxidative damage also leads to an increase in RAD51 positive DSBs. Both simple and complex non-DSB DNA damage may therefore contribute to indirect DSBs induced by ionising radiation at replication forks.

Future directions

The focus o the remaining 2 months will be to finalise manuscripts and to ensure all required data has been obtained.

Exchange of material and collaborative visits

- Two visits (April 2008 and June 2008) have been made to BNL to use the NSRL for heavy ion irradiations.
- Several tele-conferences have been held with our collaborators during the reporting period.
- Peter O'Neill attended the Low Dose Radiation Research Investigators' Workshop held at Washington D.C. in April 2009.

Collaborations within the NASA/DOE supported consortium

- Francis A. Cucinotta (NASA, Lyndon B. Johnson Space Center, Houston, USA)
- Janice Pluth (DOE, Lawrence Berkeley National Laboratory, Berkeley CA, USA)

References

1. Francis A. Cucinotta, Janice M. Pluth, Jennifer A. Anderson, Jane V. Harper, and Peter O'Neill. Biochemical Kinetics Model of DSB Repair and γH2AX foci by Non-homologous End-Joining. Radiation Research, 169, 214–222 (2008).

2. Jane V. Harper, Pamela Reynolds, Emma L. Leatherbarrow, Stanley W. Botchway, Anthony N. Parker, Peter O'Neill. Induction of persistent double strand breaks following multi-photon irradiation of cycling and G1-arrested mammalian cells: replication-induced double strand breaks Photochemistry and Photobiology, 84, 1506-14 (2008).

3. Jennifer A. Anderson, Jane V. Harper, Francis A, Cucinotta and Peter O'Neill. Participation of DNA-PK_{cs} in DSB Repair Following Exposure to High- and Low-LET Radiation (in preparation)

Platform Presentations

1. Jennifer A. Anderson, Jane V, Harper, Francis A, Cucinotta and Peter O'Neill, The role of radiation ionization density on DSB repair by NHEJ. Invited Speaker at Radiation Research September 2008

2. Jennifer A. Anderson, Jane V, Harper, Francis A, Cucinotta and Peter O'Neill, Efficiency of repair of prompt DSB requires DNA-PK_{cs}. Poster presentation at the DoE Low Dose Investigators Workshop, Washington D.C. April 2009

3. . Jennifer A. Anderson, Jane V, Harper, Francis A, Cucinotta and Peter O'Neill, Efficiency of repair of prompt DSB requires DNA-PK_{cs}. Abstract submitted to Heavy Ions in Therapy and Space Symposium, Cologne June 2009

4. Pamela Reynolds, Jennifer Anderson, Mark Hill, Jane Harper, Stanley Botchway, Anthony Parker and Peter O'Neill, Real-time dynamics of repair proteins recruited to radiation induced DSBs: Evidence of sub-classes for DSBs. Invited Speaker at Radiation Research October 2009