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Comments:	For information purposes only--PI moved in June 2014; however, funding did not go with her. As of 6/1/14, Colorado State University, Department of Environmental and Radiological Health Sciences, 485 MRB - 1618 Campus Delivery, Fort Collins, CO 80523-1618, Office: (970) 491 7618, Email: Claudia.Wiese@colostate.edu		
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Task Description:	We have studied whether or not homologous recombination (HR) is an important pathway for the repair of spatially correlated complex DSBs such as introduced by 1 GeV/n Fe ions. Our investigation was initiated by several published reports that approached the question indirectly. First, non-homologous end-joining (NHEJ) was progressively inhibited by an increase in the structural complexity of DSBs in vitro, suggesting that secondary lesions, proximal to the DSB end and within the footprint of the eukaryotic NHEJ complex, would interfere with the end-joining process. Second, high LET radiation was shown to stimulate HR in CHO cells, and third, a defect in the ATR signaling and checkpoint protein, that decreased the efficiency of HR, was shown to increase the sensitivity of human cells to 1 GeV/n Fe ions. Interestingly, more recent studies, all carried out in rodent cells, now describe a direct role for HR in DNA repair after high LET radiation, including 1 GeV/n Fe ions. These studies suggest that bi-stranded clustered lesions are left unrepaired in G1 phase, to be processed by HR in S phase through restart of broken replication forks by strand invasion. We also had hypothesized that, for a fraction of spatially correlated breaks induced by high-energy iron ions, extensive resection of the DSB ends to generate protruding 3'-overhangs for HR would be a more feasible way to repair these lesions than NHEJ. To test the contribution of homologous recombinational repair (HR) in repairing DNA damaged sites induced by high-energy Fe ions, we used: 1) HR-deficient rodent cells carrying a deletion in the RAD51D gene and 2) syngeneic human cells impaired for HR by RAD51D or RAD51 knockdown using RNA interference. We show that in response to Fe ions, HR is essential for cell survival in rodent cells, and that HR-deficiency abrogates RAD51 foci formation. Complementation of the HR defect by human RAD51D rescues both enhanced cytotoxicity and RAD51 foci formation. For human cells irradiated with Fe ions, cell survival is decreased, and, in p53 mutant cells, the levels of mutagenesis are increased when HR is impaired. Human cells synchronized in S phase exhibit more pronounced resistance to Fe ions as compared with cells in G1 phase, and this increase in radioresistance is diminished by RAD51 knockdown. These results implicate a role for RAD51-mediated DNA repair (i.e. HR) in removing a fraction of clustered lesions induced by charge particle irradiation. Our results are the first to directly show the requirement for an intact HR pathway in human cells in ensuring DNA repair and cell survival in response to high-energy high LET radiation.
Rationale for HRP Directed Research:	
Research Impact/Earth Benefits:	Our research has led to a better understanding of the mechanisms of DNA double-strand break repair in general. Our results have shown that, in p53 wild type human cells, homologous recombination (HR) is essential to protect from both spontaneous and radiation-induced DNA damage. Our results also have shown that in p53 wild type human cells, HR cannot be easily substituted for by mutagenic DNA double-strand break repair pathways. Conversely, in permissive p53 mutant human cells deficient in HR, mutagenic DSB repair pathways can substitute for HR, promoting mutagenesis and carcinogenesis.
	The overall goal of this Research Project is to investigate whether homologous recombinational DNA repair (HR) contributes to the repair of double-strand breaks (DSBs) generated by the radiation types found in the space radiation environment. We hypothesized that, in some cases, correlated, clustered radiation damage, as induced by heavy charged (HZE) particles, requires the resection of the damaged DNA and the HR pathway, and therefore is not channeled into the non-homologous end-joining (NHEJ) pathway. Importantly, HR is a DNA repair pathway with close ties to cancer biology and crucial for maintaining genomic stability, limiting mutagenesis and preventing carcinogenesis. It is well established that conditions promoting reduced levels of HR compromise the fidelity of DSB repair and correlate with an elevated cancer risk. The risk of developing cancer is increased in individuals exposed to space radiation, and defects in HR, leading to the increased utilization of error-prone DNA repair pathways (i.e. NHEJ), are likely to contribute to this process. Therefore, it is a necessity to establish the relevance of HR to HZE radiation for better prediction of the astronaut's sensitivity to radiation carcinogenesis. We have shown that CHO cells deleted for the RAD51D gene (i.e. deficient in HR) show increased sensitivity to the cytotoxic effects of iron ions compared to wild type cells, and that ectoptic expression of human RAD51D in rad51d-deleted cells reverts their sensitivity to iron ions to close to wild type levels. In these experiments, we have chosen a RAD51D-complemented CHO clone whose protein expression level for human RAD51D is very similar to that of endogenous hamster RAD51D expressed in AA8 wild type CHO cells, as demonstrated previously (1). Our results showing full complementation for cell survival in response to graded doses of X-rays also support a role for human RAD51D in response to ionizing radiation. We obtain RBE values for iron ions (D10) of 2.4 and 2 for wild type and rad51d-deficient cells, respectively, that are virtually identical to the RBE values reported by Wang and collaborators (2) for the same high LET radiation type and AA8 and irs1SF cells. Irs1SF cells also are deficient in HR due to inactivation of the XRCC3 gene (3). Although NHEJ-deficient rodent cells were not included in our study, they were part of the investigation by Wang and collaborators (2), who obtained RBE values (D10) ~1 for ku80-deficient mouse embryonic fibroblasts (MEFs) exposed to 1 GeV/n iron ions, supporting their additional results for the specific inhibition of the NHEJ pathway after high LET radiation in rodent cells. From our data we infer that HR is active in rodent cells after exposure to 1 GeV/n iron ions, and that proper function of this high-fidelity DNA repair pathway is required to fully protect CHO cells against the cytotoxic effects of iron ions. RAD51 is the key protein in HR and forms a filament on single-stranded DNA, a filament that is essential for homology search and strand invasion. Ongoing HR can be detected as RAD51 focus formation by immunostaining and fluorescence microscopy, and rodent and human cells impaired in HR due to loss of a RAD51 paralog are impaired in RAD51 foci formation after low LET IR (4, 5). As reported here, in response to high LET iron ions rad51d-deficient CHO cells also were unable to form RAD51 foci, but ectopic expression of human RAD51D in rad51d-/- cells reverted RAD51 foci formation back to wild type levels, indicative for 1) the causal relationship between cellular sensitivity and ongoing HR, and 2) the full functionality of the heterologous human RAD51D protein in CHO cells deleted for the hamster RAD51D gene. In near-normal human cells exposed to iron ions, RAD51 foci formation is restricted to S/G2 phase and absent from G1 phase cells, further pointing to the biological relevance of the HR pathway after high LET radiation. Since RAD51 directly binds to single-stranded DNA, it also accumulates into micro-foci after high LET radiation, as demonstrated earlier after laser micro-irradiation (6). Human lymphoblast cells depleted for RAD51D also show enhanced sensitivity to iron ions. However, the effects of RAD51D protein knockdown on cell survival are more pronounced in p53 wild type TK6 cells than in p53 mutant WTK1 cells. The reason for this difference it unclear at this point, but it could be speculated that mutagenic DNA repair pathways (i.e. NHEJ, single-strand annealing (SSA) or microhomology-mediated end-joining (MMEJ)) may substitute at higher levels in p53 mutant cells, rescuing cytotoxicity in the absence of properly functional HR. Conversely, since we observe that RAD51D knockdown is more deleterious for iron ion-induced cytotoxicity in TK6 cells, these p53 wild type

Task Progress:

cells may more heavily rely on faithful HR, and may not tolerate unfaithful DNA repair events in substitution for HR. Our results obtained for high and low LET radiation-induced mutagenesis are in support of this speculation, as very different effects of RAD51D knockdown on iron ion-induced mutagenesis are observed when TK6 and WTK1 cells are assessed (see below). Although the RBE values (D10) obtained for cell death of control and DNA repair protein-depleted cell lines (both TK6 and WTK1 cells) ranged from ~1-1.5 only, it is interesting to note that for both TK6 cells and WTK1 cells the smallest RBE values (i.e. ~1) were obtained for their respective XRCC4-depleted cell populations. This observation is in accord with the recently published report on repair-deficient rodent cells, demonstrating smallest RBEs for NHEJ-deficiency due to the specific inhibition of this DNA repair pathway by 1 GeV/n iron ions (2).

The inhibition of the HR pathway via knockdown of RAD51D has different effects on the outcome of mutagenesis at the thymidine kinase (TK) locus in Fe ion-exposed TK6 and WTK1 cells. Notably, when expression of RAD51D is reduced, TK mutant fractions are enhanced significantly in iron ion- and X-irradiated WTK1 cells. In these cells approximately twice as many TK mutants were recovered from RAD51D-depleted cells than from control-transfected cells at all Fe ion and X-ray doses investigated. These results suggest that in the absence of faithful HR, unfaithful DNA repair pathways take over, leading to elevated levels of mutagenesis in permissive (i.e. p53 mutant) human cells. Conversely, no such increase in mutagenesis was observed in HR-compromised p53 wild type cells, suggesting that non-mutagenic HR is tightly regulated in TK6 cells, and cannot be substituted for by error-prone DNA damage repair pathways. However, down-regulation of NHEJ (i.e. XRCC4) in p53 wild type cells limited mutagenesis after both Fe ions and X-rays, demonstrating that NHEJ is a mutagenic DNA repair pathway active in TK6 cells after both high and low LET radiation. Unexpectedly, this was not observed for XRCC4-depleted WTK1 cells, for which the levels of iron ion- and X-ray-induced TK mutant fractions remained at those of control-transfected cells. These results indicate that NHEJ does not contribute to mutagenesis in WTK1 cells under wild type repair conditions. This finding suggests that error-prone homology-mediated events, such as SSA, MMEJ (for review see (7)), or PARP-dependent alternative end-joining (8, 9) may account for radiation-induced mutagenesis in these p53 mutant cells. It is unlikely that the levels of XRCC4-depletion were insufficient to uncover a phenotype for reduced radiation-induced mutagenesis in WTK1 cells under conditions when NHEJ was impaired, since the same cells demonstrated reduced clonogenic potential after irradiation.

HR is restricted to late S and G2 phase of the cell cycle when the sister chromatid is present, and HR-defective cells show S phase-dependent radiosensitivity (for review see (10)). In repair-proficient human cells, S phase-dependent radioresistance to high-energy Fe ions had not been investigated. To the best of our knowledge, our results for a derivative of U2OS cells are the first to show S phase-dependent radioresistance for human cells in response to high LET iron-ion irradiation, and our findings are in accord with findings from Blakeley and co-workers (11) studying synchronized human T-1 cells and 425 MeV/n neon ions. To our surprise, the difference in radioresistance between U2OS cells irradiated in G1 or S phase was almost as pronounced after iron ions as after X-rays (sensitivity factors (D10) were 1.6 and 1.8, respectively), suggesting that S phase-specific DNA repair pathways (i.e. HR and SSA) are as important after iron ion exposure as they are after X-rays. Notably, whereas knockdown of RAD51 in S phase U2OS cells reduced X-ray radioresistance to the levels of G1 phase cells, significantly less decrease in radioresistance was observed for iron-ion exposed RAD51-depleted cells in S phase. As recently discovered in rodent cells (12), we propose that SSA, a mutagenic sub-pathway of HR that is restricted to S/G2 cells, and that requires DSB end resection but is RAD51 independent, operates at higher levels after high LET radiation damage than after low LET exposure.

In summary, proper HR is required to fully protect both human and rodent cells from the cytotoxic effects of iron ions. Furthermore, in permissive human cells (i.e. cells with gain-of-function mutant p53) reduced level of HR leads to a significant increase in iron ion-induced mutant fractions. No such increase in mutagenesis is observed in HR-compromised p53 wild type cells. While HR is important for some lesion repair (i.e. helps limit cytotoxicity), recombinational repair events in p53 wild type cells are highly controlled, they are mostly faithful, and cannot easily be substituted for by other DSB repair pathways. In addition to HR, our results support a role for other S phase-specific DNA repair pathways after high LET radiation. These pathways are currently the subject of intense investigation in rodent cells (12), and, in the future, should be investigated in human cells to better understand the astronauts' risk for cancer from space radiation.

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Bibliography Type:	Description: (Last Updated: 04/11/2018)
Abstracts for Journals and Proceedings	Zafar F, Seidler SB, Kronenberg A, Schild D, Wiese C. "Homologous recombination contributes to the repair of double-strand breaks induced by Fe-ions." Presented at the 20th Annual NASA Space Radiation Investigators' Meeting, Cologne, Germany, July 6 - 10, 2009. Abstract Book. 20th Annual NASA Space Radiation Investigators' Meeting, Cologne, Germany, July 6 - 10, 2009. , Jul-2009
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