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RADIOSENSITIVITY TO HIGH ENERGY IRON IONS IS INFLUENCED BY HETEROZYGOSITY for *ATM*, *RAD9* and *BRCA1*

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Abstract

Loss of function of DNA repair genes has been implicated in the development of many types of cancer. In the last several years, heterozygosity leading to haploinsufficiency for proteins involved in DNA repair was shown to play a role in genomic instability and carcinogenesis after DNA damage is induced, for example by ionizing radiation. Since the effect of heterozygosity for one gene is relatively small, we hypothesize that predisposition to cancer could be a result of the additive effect of heterozygosity for two or more genes critical to pathways that control DNA damage signaling, repair or apoptosis. We investigated the role of heterozygosity for *Atm*, *Rad9* and *Brcal* on cell oncogenic transformation and cell survival induced by 1GeV/n ⁵⁶Fe ions. Our results show that cells heterozygous for both *Atm* and *Rad9* or *Atm* and *Brcal* have high survival rates and are more sensitive to transformation by high energy Iron ions when compared with wild-type controls or cells haploinsufficient for only one of these proteins. Since mutations or polymorphisms for similar genes exist in a small percentage of the human population, we have identified a radiosensitive sub-population. This finding has several implications. First, the existence of a radiosensitive sub-population may distort the shape of the dose-response relationship. Second, it would not be ethical to put exceptionally radiosensitive individuals into a setting where they may potentially be exposed to substantial doses of radiation.

Introduction

There are a number of areas of human endeavor where the implicit assumption is made that the human population is uniform in terms of radiosensitivity, except for a few individuals such as AT homozygotes who are exquisitely sensitive to radiation, but readily recognized by their clinical symptoms (Shiloh, 2001). This is important for radiation protection standards in general, but in particular for space flight. In the context of Space Radiation Risk Assessment, the existence of an unidentified radiosensitive sub-population would have two consequences. First, it might be considered unethical to put a radiosensitive individual into a situation where they are likely to receive a substantial dose of radiation because of the possibility of a severe response. This assumes greater importance with the developing plans to return to the moon, or even to plan a trip to Mars, which would inevitably involve

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exposure to even larger doses of radiation, including high energy heavy ions, than those received on short duration shuttle missions. Second, the existence of a radiosensitive sub-population in an epidemiological study would tend to distort the shape of the dose-response relationship, thereby rendering a linear extrapolation from high to low doses invalid.

There are a number of hints from human studies that the assumption of uniform radiosensitivity is incorrect, including the fact that only a few percent of radiotherapy patients suffer late effects (Hall et al., 1998). The problem is to discover and identify the genetic basis for this radiosensitivity, which in the end would allow those individuals to be spared from the deleterious health effects that could ensue after exposure.

There are data from mouse models and human studies suggesting that heterozygosity for genes involved in DNA repair may play a central role in genomic instability and carcinogenesis (Kastan & Bartek, 2004; Largaespada, 2001; Santarosa & Ashworth, 2004; Smilenov, 2006; Vladutiu, 2001; Yan et al., 2002). In addition, several examples from mouse models indicate that double heterozygosity for functionally related genes might have an additive effect on tumor development (Cheo et al., 2000; Kucherlapati et al., 2002; Umesako et al., 2005). The number of *ATM* heterozygous humans is accepted to be 1.7-3% of the general population (Khanna, 2000), which is high enough to be recognized as an important societal issue. More difficult is to establish the number of individuals heterozygous for more than one gene. An example is a study of *BRCA1* and *BRCA2* heterozygous individuals in a diagnostic setting (Barwell et al., 2007), where double heterozygosity occurs in 0.09-0.36% of the indexed cases. This corresponds to 0.22–0.87% of proven *BRCA* mutation carriers, which rises to 1.8% in Ashkenazi Jews.

These considerations lead us to investigate the role of several genes in controlling radiosensitivity. We have shown that cells heterozygous for *Atm* and *Rad9*, singly or in pairs, exhibit a higher incidence of x-ray induced oncogenic transformation and a lower level of x-ray induced apoptotic death when compared to wild type control cells (Smilenov, Brenner, & Hall, 2001; Smilenov et al., 2005). In addition, we have also demonstrated that x-ray induced ocular cataracts appear earlier in mice heterozygous for *Atm* or *Atm* and *Rad9*, relative to controls (Kleiman et al., 2007; Worgul, Smilenov, Brenner, Vazquez, & Hall, 2005). We report herein the effects of these same genes on the response to high energy ^{56}Fe (1GeV/n ions), since it is predicted that the efficiency of DNA repair gene activity might be less biologically significant when much more intense DNA damage characteristic of a exposure to very high LET radiation is induced. Our results indicate that, with respect to transformation, *Atm*, *Rad9* and *Brcal* function is still important after cells are irradiated with ^{56}Fe ions.

Materials and Methods

Mice

Atm, *Mrad9* and *Brcal* heterozygous mice, as well as the preparation of mouse embryo fibroblasts (MEF) were described previously (Elson et al., 1996; Hopkins et al., 2004; Ludwig, Chapman, Papaioannou, & Efstratiadis, 1997). Genotypes were determined by PCR. Mice were housed and maintained in compliance with the U.S. Department of Health and Human Services Guide for the “Care and Use of Laboratory Animals” and institutional IACUCs.

Irradiation

Cells and animals were exposed respectively to the indicted doses of 1GeV/n ^{56}Fe ions in the experimental beam line of the NASA Space Radiation Laboratory (NSRL) facility at

Brookhaven National Laboratory (Upton, NY). This is an accelerator-based facility that provides charged particles for space radiation research (Lowenstein & Rusek, 2007).

Oncogenic transformation

MEFs were irradiated with a dose of 0.5Gy 1GeV/n ^{56}Fe ions. Twenty-four hours later they were replated in 100-mm dishes at a density of 6,000 cells/dish over a feeder layer of 70,000 cells prepared from the same embryo but irradiated with a supralethal dose. After 2 weeks of growth, cells were fixed, stained, and yields of transformed clones were scored. The scoring criteria were developed and examined by preliminary experiments, where embryo cells were irradiated and plated with the same density. Clones that appeared dense and had stellate-shaped piled-up cells were isolated with cloning cylinders. These clones were expanded and injected into nude mice. Those that caused the development of tumors were designated “transformed”. Clones that matched their shape and dimensions were scored as transformed in subsequent experiments.

Cell survival assay

Mice bearing each genotype of interest were irradiated with 1Gy ^{65}Fe 1000 MeV/n. Twenty-four hours later, thymuses from irradiated and unirradiated control mice were isolated and homogenized to single thymocyte cell suspension. Thymocytes were labeled with CD4 and CD8 specific antibodies. Samples (1.10^5 cells) were analyzed by flow cytometry. Counts for the surviving $\text{CD4}^+/\text{CD8}^+$ subset were estimated by examining the flow cytometry profiles.

Statistical analysis

Data are shown as means $\pm$ SEM. Statistical analysis was performed using the Student's t-test for unpaired samples.

Results

Role of single and double heterozygosity for *Atm* and *Rad9* in cell transformation and cell survival

To establish the impact of heterozygosity for *ATM* and *Rad9* on cell transformation, we estimated the transformation frequency for cells derived from wild-type animals compared with those heterozygous for *Atm*, *Rad9* or both. A total of 24 embryos were used (6 for each genotype). Yields of transformed clones were measured for unexposed controls and after exposure to a dose of 0.5Gy ^{56}Fe ions 1GeV/n. The results shown in Table 1 and Table 2 indicate a statistically significant higher transformation frequency for the *Atm*h_z/*Rad9*wt cells and for the *Atm*h_z/*Rad9*h_z cells relative to the wild-type controls. Transformation frequency for the double heterozygous cells was almost twice that for the *Atm*h_z/*Rad9*wt cells, while *Atm*h_z/*Rad9*wt cells showed a slight increase in comparison to the *Atm*wt/*Rad9*wt cells. The *Rad9* heterozygous *Atm*wt/*Rad9*h_z cells showed no difference in transformation relative to the wild type population.

We examined survival of $\text{CD4}^+/\text{CD8}^+$ thymocytes, the most numerous and radiosensitive cell population in the thymus (more than 80% of all cells in the thymus) 24 hours after *in vivo* irradiation with 1Gy ^{56}Fe 1GeV/n. The results (Fig.1) showed that the percentage of surviving cells is very different for cells with each genotype, although similar damage was induced in all animals. Remarkably, the double heterozygous cells survived better than those with other genotypes, which points to inefficient detection of DNA damage and subsequent lack of activation of apoptosis in these cells. As a result, more cells with this genotype will survive with mutations, which could lead to an increase in the probability of tumor

development. These results complement the cell transformation data, such that as survival increases, transformation also increases.

Role of single heterozygosity and double heterozygosity for *Atm* and *Brca1* in cell transformation and cell survival

The same experimental scheme used for *Atm/Rad9* mice was applied to examine the role of *Atm* and *Brca1* heterozygosity in cell transformation and cell survival. Table 3 and Table 4 show the data for radiation-induced oncogenic transformation of MEFs from mice heterozygous for *Atm* and *Brca1*. The transformation frequency of the heterozygous cells was compared with the wild type control. The incidence of oncogenic transformation increased significantly in *Atm*^h*z*/*Brca1*^{wt}, *Atm*^{wt}/*Brca1*^h*z* and *Atm*^h*z*/*Brca1*^h*z* cells in comparison to the wild type. There was no statistically significant difference between the transformation efficiency of *Atm*^h*z*/*Brca1*^{wt} and *Atm*^h*z*/*Brca1*^h*z* cells. Thymocyte survival data (Fig.2) show the same trend as observed for the *Atm/Rad9* cells. Cells representing all heterozygous genotypes show increased survival in comparison to the wild type population, and the double heterozygous *Atm/Brca1* show a statistically significant increase in cell survival when compared with all other genotype groups.

Discussion

The main goal of this study was to show the degree heterozygosity for *Atm*, *Rad9* and *Brca1* influences cell survival and cell transformation - two processes correlated with tumor development. These three proteins are key DNA damage response elements and interact as part of DNA repair complexes. ATM can phosphorylate Rad9, which is part of the Rad9-Hus1-Rad1 (9-1-1) protein complex (Lieberman, 2006). Rad9 plays an important role in prostate cancer development (Zhu, Zhang, & Lieberman, 2008). ATM and BRCA1 are involved in both NHEJ and HR where ATM phosphorylates BRCA1. Both BRCA1 and ATM mutations confer a high risk for developing breast cancer (Campeau, Foulkes, & Tischkowitz, 2008). Regarding the endpoints we used in this study, cell transformation was shown in MEFs after γ -ray irradiation. We demonstrated previously that transformation frequency depends on the abundance of *Atm* and *Rad9* proteins (Smilenov et al., 2005). Cell survival after radiation was studied in CD4⁺/CD8⁺ thymocytes where expression of ATM was shown to be very important for this process (Bebb et al., 2001; Xu & Baltimore, 1996). Logically we expected that the roles of *Rad9* and *BRCA1* in transformation could be tested in these same cell systems since ATM is functionally related to both of these proteins and does impact on the transformation process.

Our results demonstrate that the gene status corresponding to any of these proteins was not of great biological significance when cells are not challenged with radiation. The frequency of cell transformation was the same for MEFs of all genotypes tested, and the same was true for the relative numbers of CD4⁺/CD8⁺ thymocytes. However, both processes develop very differently after irradiation. Transformation frequency was higher for MEFs with heterozygous genotypes when compared to wild type cells, with the exception of those which were *Atm*^{wt}/*Rad9*^h*z*. Transformation frequency was also noticeably higher for the double heterozygous *Atm/Rad9* MEFs when compared to cells with all the other genotypes examined. Similarly, there was a marked difference in cell survival among cells with different genotypes. Survival was increased in heterozygotes in comparison to wild type cells, showing that the former become partially radioresistant as a result of the heterozygous genetic state. Survival was the highest for the double heterozygous cells, those with *Atm/Rad9* or *Atm/Brca1* genotypes. This demonstrates an additive effect of compound heterozygosity relative to single gene heterozygote status. The data for cell survival provides a direct link to the efficiency of DNA dsb detection and repair. Similar damage results in

different levels of cell survival, indicating that DNA damage detection depends on genotype. Increased cell survival for cells with the heterozygous genotypes shows that more cells with unrepaired DNA survived, and that can induce increased transformation. These results imply that under stress the repair capacity for the heterozygous cells is less than that of the wild type. The lower capacity might lead DNA misrepair or mutation, manifested in our test systems as increased cell transformation

Our findings support the conclusion that the cellular stress response depends on genotype and that combined heterozygosity can have an additive, negative impact. The results reported herein show that there might be at least three low frequency high penetrance genes, mutations within which confer cellular sensitivity to high energy heavy ions. It is likely that the few percent of individuals in the human population that carry similar mutations in comparable genes might constitute a radiosensitive subpopulation. Such a population would be important for two reasons. First, it could distort the shape of the dose-response relationship for radiation induced carcinogenesis. Second, it would thus be unethical to deploy radiosensitive individuals to a situation where they are at a higher than normal risk of being exposed to substantial doses of radiation, including heavy ions, and would have an increased chance of developing deleterious health issues. While the data presented here all involve cells cultured *in vitro*, it has been shown that heterozygosity for the same genes also results in sensitivity to ocular cataract formation following exposure to low LET radiation. (Kleiman et al., 2007; Worgul et al., 2005).

The data presented above also suggest that haploinsufficiency for various proteins could lead to defects in local network assembly (Hopkins et al., 2004). The role of protein haploinsufficiency could be explained with network models. It was shown that biological networks are scale-free networks where few highly interconnected nodes communicate with the remaining low interconnected nodes. Biological networks in many cases are self assembly/disassembly networks. The requirement for assembly in response to an event at an unknown point in a relatively large (on a molecular scale) area, introduces spatial and quantitative limitations on the process. DNA double-strand breaks, for example, are a local event that might appear at any place in the nucleus. The proteins, potential members of the local networks, have to be in close proximity to the break or to be able to translocate quickly to the critical site. Low expression levels of highly interconnected proteins like ATM and Rad9 could lead to defective assembly of local DNA repair networks and subsequent deficiencies in signaling and repair.

Overall, based on the results shown above, the following concept emerges: cells have DNA repair capacity that depends on the levels of DNA repair proteins. Lower abundance of some DNA repair proteins reduces the DNA repair capacity of the corresponding cells and individuals. Additionally, heterozygosity for two key repair proteins has additive negative effects, lowering this capacity even further. In a normal environment, basic DNA repair capacity is sufficient to maintain the status of the genome. However, low levels of some DNA repair proteins results in excessive residual DNA damage, especially when exogenous genotoxic agents are applied, with consequences that could foster tumor development.

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Fig.1.

Survival of CD4⁺/CD8⁺ thymocytes 24 hrs after mice are irradiated *in vivo* with 1Gy of ⁵⁶Fe 1GeV/n. The number of animals and the genotypes used were respectively: *Atmwt/Rad9wt* (five), *Atmhzt/Rad9hz* (six), *Atmwt/Radhz* (five), *Atmhzt/Rad9wt* (seven). Mice were from six different litters. Asterisks show statistically significant differences between cells bearing each genotype and the wild type controls. All data shown as means $\pm$ SEM.

*p<0.02 compared to the controls.

Fig.2.

Survival of CD4+/CD8+ thymocytes 24 hrs after irradiation of mice *in vivo* with a 1Gy dose of ^{56}Fe 1GeV/n. The number of animals and the genotypes used were respectively: *Atmwt/Rad9wt* (four), *Atmhz/Rad9hz* (four), *Atmwt/Radhz* (five), *Atmhz/Rad9wt* (six). Mice were from five different litters. Asterisks show statistically significant differences between each genotype group and the wild type control. All data shown as means $\pm$ SEM. * $p < 0.02$ compared to the controls.

Table 1

Transformation frequencies of cells with different ATM/Rad9 genotypes after exposure to 0.5 Gy of 1GeV ^{56}Fe .

Genotype	Dose (Gy) ^{56}Fe 1GeV/n	Number of clones scored	Number of transformed clones
<i>Atmwt/Rad9wt</i>	0	15342	3
	0.5	12876	15
<i>Atmwt/Rad9hz</i>	0	16524	4
	0.5	11823	11
<i>Atmhz/Rad9wt</i>	0	15761	5
	0.5	13798	27
<i>Atmhz/Rad9hz</i>	0	11567	6
	0.5	9164	34

Table 2

Comparisons of radiation-induced transformation between MEFs with different genotypes versus wild-type MEF controls. Relative transformation is defined as the ratio of the number of transformed clones per surviving heterozygous cells relative to the number of transformed clones per surviving wild-type cells. The statistical significance of differences in transformation frequency between the various cells with heterozygous genotypes and wild-type cells was analyzed by the Student's t-test.

	<i>Atmhz/Rad9wt</i>	<i>Atmwt/Rad9hz</i>	<i>Atmhz/Rad9hz</i>
Relative transformation (0.5Gy 56Fe)	1.680	1.252	3.185
p-values	0.034	0.571	0.005

Table 3

Transformation frequencies of cells with different ATM/BRCA1 genotypes after exposure to 0.5 Gy of 1GeV ^{56}Fe .

Genotype	Dose (Gy) 0.5Gy of ^{56}Fe 1GeV/n	Total number of clones scored	Number of transformed clones
<i>Atmwt/BRCA1wt</i>	0	14645	5
	0.5	13897	12
<i>Atmwt/BRCA1hz</i>	0	15098	7
	0.5	14235	24
<i>Atmhz/BRCA1wt</i>	0	15761	6
	0.5	11567	27
<i>Atmhz/BRCA1hz</i>	0	14765	11
	0.5	10231	31

Table 4

Comparisons of radiation-induced transformation between MEFs bearing different genotypes versus wild-type MEF controls. Relative transformation is defined as the ratio of the number of transformed clones per surviving heterozygous cells relative to the number of transformed clones per surviving wild-type cells. The statistical significance of differences in transformation frequency between the various cells with heterozygous genotypes and wild-type cells was analyzed by the Student's t-test.

	<i>Atmhz/BRCA1wt</i>	<i>Atmwt/BRCA1hz</i>	<i>Atmhz/BRCA1hz</i>
Relative transformation (0.5Gy 56Fe)	2.703	1.953	3.509
p-values	0.003	0.054	0.000083