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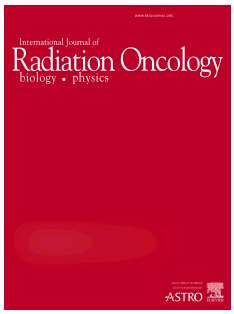

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Summary

Radiation is a known risk factor for colorectal cancer (CRC). However, much uncertainty exists over estimates of CRC risk after energetic heavy ion radiation exposures. Relative biological effectiveness (RBE) of intestinal tumor frequency for energetic ^{12}C, ^{56}Fe, and ^{28}Si ions relative to γ radiation was assessed in a mouse model ($APC^{1638N/+}$) of human CRC. Research into energetic heavy ion exposure-associated risk of CRC has implications for safe space exploration.

Abstract

Purpose: There are uncertainties associated with the prediction of colorectal cancer (CRC) risk from highly energetic heavy ion (HZE) radiation. We undertook a comprehensive assessment of intestinal and colonic tumorigenesis induced after exposure to high linear energy transfer (high-LET) HZE radiation spanning a range of dose and LET in a CRC mouse model and compared the results to low-LET γ radiation.

Methods and Materials: Male and female $APC^{1638N/+}$ mice (n=20 mice per group) were whole-body exposed to sham-radiation, γ-rays, ^{12}C, ^{28}Si, or ^{56}Fe radiation. For the >1 Gy HZE dose, we used γ-ray equitoxic doses calculated using relative biological effectiveness (RBE) determined previously. Mice were sacrificed 150 days after irradiation, and intestinal and colon tumor frequency was scored.

Results: The highest number of tumors was observed after ^{28}Si followed by ^{56}Fe and ^{12}C radiation, and tumorigenesis showed a male preponderance especially after ^{28}Si. Analysis showed greater tumorigenesis per unit of radiation (per cGy) at lower doses suggesting either radiation-induced elimination of target cells or tumorigenesis reaching a saturation point at higher doses. Calculation of RBE for intestinal and colon tumorigenesis showed the highest value with ^{28}Si and lower doses showed greater RBE relative to higher doses.

Conclusions: We have demonstrated that the RBE of heavy ion radiation-induced intestinal and colon tumorigenesis is related to ion energy, LET, and gender, and peak RBE is observed at an LET of 69 keV/μm. Our study has implications for understanding risk to astronauts undertaking long duration space missions ~~as well as to patients undergoing heavy ion radiotherapy~~.

Introduction

Increased risk of colorectal cancer (CRC) after exposure to low linear energy transfer (low-LET) radiation such as γ-rays has been widely reported in epidemiological as well as animal model studies (1-3). While on earth low-LET radiation is predominant, astronauts traveling into outer space are exposed to high-LET energetic heavy ions (HZE) such as ^{12}C, ^{56}Fe and ^{28}Si, and the risk of CRC from HZE radiation exposure remains to be established. Energetic heavy ions contribute significantly towards dose equivalent of galactic cosmic radiation (GCR), and it has been predicted that during a Mars mission about 30% of the astronauts' cell will be hit by either the primary or the secondary tracts of heavy ions (4-6). Considering that CRC is still a major form of cancer in the USA and low-LET radiation is a CRC risk factor, high-LET radiation exposure could potentially pose a greater risk of developing CRC. Therefore, assessing CRC risks associated with energetic heavy ion exposures is important for health of astronauts undertaking long-duration space missions and safe exploration of outer space.

Currently, we are unable to accurately predict CRC risk from exposure to HZE ions mostly due to insufficient *in vivo* tumorigenesis data. However, with limitations in obtaining *in vivo* human data on energetic heavy ions-associated CRC, there is an urgent need to accrue animal data necessary to predict CRC risk from long duration space missions. The adenomatous polyposis coli (APC) mutant mouse models have been extensively used to study the molecular pathogenesis of CRC (12-15). It has been previously shown that exposure to 1.6 and 4 Gy of ^{56}Fe caused higher intestinal tumorigenesis in $APC^{Min/+}$ relative to γ radiation (3). Increased intestinal tumorigenesis was also observed in $APC^{1638N/+}$ mice after ^{56}Fe radiation (16). Considering that spontaneous intestinal tumor frequency is markedly lower in control $APC^{1638N/+}$ (0 to 5 tumors) relative to $APC^{Min/+}$ (30 to 50 tumors) mice, radiation-induced tumorigenesis has a better signal-

to-noise ratio in the former relative to the later mouse model (3, 16). Therefore, we utilized $APC^{1638N/+}$ to undertake a comprehensive study for a number of HZE ions (^{12}C, ^{56}Fe, ^{28}Si) spanning a range of doses and LETs with the aim to determine calculated RBE values relative to γ-rays for intestinal and colonic tumorigenesis. The current study demonstrated a male preponderance for the RBE of intestinal and colonic tumorigenesis and the RBE of tumorigenesis peaked at an LET of 69 keV/μm, which is the similar LET for the highest RBE for survival reported earlier (17-22).

Methods and Materials

Mice. The $APC^{1638N/+}$ mice on a C57BL/6J background were bred, genotyped, and maintained as described previously (3, 23). Six to eight week old male and female $APC^{1638N/+}$ mice were used. Animal procedures were performed as per protocol approved by the Institutional Animal Care and Use Committee (IACUC) at XXXX and at XXXX and we followed the Guide for the Care and Use of Laboratory Animals for our studies.

Irradiation. Mice, transportation, irradiation procedures, and dosimetry have been described previously (3). Briefly, mice are shipped to XXXX and exposed to different doses of ^{12}C (0.1. 0.5, 2.0 Gy; energy: 290 MeV/n; LET: 13 keV/μm;), ^{56}Fe (0.1, 0.5, 1.6 Gy; energy: 1000 MeV/n; LET: 148 keV/μm), and ^{28}Si (0.1, 0.5, 1.4 Gy; energy: 300 MeV/n; LET: 69 keV/μm;) at XXXX. Mice for sham- and γ-irradiation were also shipped to XXXX and shipped back to XXXX with the aim to expose all mice to similar transportation stressors and γ irradiation was performed on the same day of heavy ion irradiation at XXXX using a ^{137}Cs source. For doses below 1 Gy (0.1

and 0.5 Gy) we used γ-irradiation doses equal to those of the heavy ions. For 2 Gy γ-rays, we used equitoxic doses of ^{12}C (2 Gy), ^{56}Fe (1.6 Gy), and ^{28}Si (1.4 Gy) radiation determined using RBE factors of survival ($LD_{50/30}$ studies) calculated earlier, which were 0.99, 1.25, and 1.40 respectively (21, 22).

Tumor count. Mice were sacrificed using CO_2 asphyxiation 150 d after radiation. The small intestine and colon was surgically dissected out, cleaned, and tumors counted under a dissection scope as previously described (3). Considering heavy ion radiation exposures are available three times a year for a specified time period, and logistical issues such as mice breeding and genotyping as well as beam size (area with uniform dose), we irradiated mice in smaller groups. Subsequently, data from multiple radiation exposures were pooled for statistical significance analysis. Tumor frequency and RBE data from male and female mice in intestine and colon are analyzed, plotted, and presented separately. The words intestine and intestinal represent small intestine.

Statistical analysis and RBE calculation. Radiation-induced tumor frequency was normalized by subtracting spontaneous tumor frequency. Normality of data distribution in each irradiated group was tested using Shapiro-Wilk's test (24). The p-values (<0.05), histograms, and skewness and kurtosis measures with standard errors revealed that the tumor data were not approximately normally distributed. Therefore, equality of variances were tested using a non-parametric Levene's test (25), which reported a p-value of <0.05 showing inequality of variances in the tumor data set. Given that data showed non-normal distribution and unequal variance but we have equal sample size (n=20 mice per study group), Welch's one-way ANOVA with Games-

Howell post-hoc test (26) was performed to determine significance ($p<0.05$ was considered as significant) among different types of radiation-induced tumorigenesis. Statistical comparison between male and female tumor frequency for a given radiation type and dose was performed using Wilcoxon matched pairs test and $p<0.05$ was considered as significance. All statistical analysis was performed using IBM SPSS Statistics for Macintosh, Version 22.0 (IBM Corp., Armonk, NY). Error bars represent mean ± standard error of mean (SEM). In each dose, intestinal and colonic tumor frequency scale (y-axis) is kept the same in male and female mice for comparison. For a calculated RBE of heavy ion tumorigenesis relative to γ-rays, radiation-induced tumor frequency was first normalized by subtracting spontaneous tumor frequency [(radiation tumor frequency-spontaneous tumor frequency)]. Subsequently, due to different doses for different radiation types at the highest doses (2 Gy and equitoxic), normalized tumor frequency for each dose (Gy) was converted to number of tumors per cGy [(radiation tumor frequency-spontaneous tumor frequency)/radiation dose in cGy]. Calculated RBE of heavy ion radiation-induced tumorigenesis is expressed as a ratio of heavy ion and γ radiation-induced tumor frequencies (heavy ion radiation-induced tumor frequency per cGy/γ radiation-induced tumor frequency per cGy).

Results

Increased frequency of intestinal tumors in APC$^{1638N/+}$ mice after HZE radiation. All doses of heavy ion radiation showed increased intestinal tumorigenesis (**Fig. 1, Suppl. Fig. 1, and Suppl. Table 1**). In male mice, all doses of ^{12}C, ^{56}Fe, and ^{28}Si showed higher intestinal tumor incidence relative to the corresponding doses of γ radiation (**Fig. 1A, B, and C**). Tumorigenesis

in female mice was also significantly higher after exposure to all doses of ^{12}C, ^{56}Fe, and ^{28}Si except after 0.1 and 0.5 Gy of ^{12}C relative to respective γ radiation doses (**Fig. 1D, E, and F**). Highest intestinal tumor frequency was observed after ^{28}Si relative to other radiation types used. Additionally, intestinal tumorigenesis was significantly higher after 0.5 and 1.4 Gy of ^{28}Si relative to ^{12}C and ^{56}Fe radiation in males (**Fig. 1B and C**).

Colonic tumor frequency is increased after heavy ion radiation exposures. Overall, colonic tumorigenesis was also increased after exposure to three types of heavy ions (**Fig. 2, Suppl. Fig. 2, and Suppl. Table 2**). Compared to γ radiation, tumorigenesis after all doses of ^{12}C, ^{56}Fe, and ^{28}Si radiation was significantly higher except after 2.0 Gy ^{12}C in male mice (**Fig. 2A, B, and C**). Female mice did not develop tumors after 0.1 Gy ^{12}C radiation (**Fig. 2D**) and the small increase in tumorigenesis after 0.5 Gy ^{12}C was not statistically significant (**Fig. 2E**). Although we observed higher colon tumorigenesis after 2.0 Gy ^{12}C, it was not statistically significant relative to γ radiation (**Fig. 2F**). In female mice, colonic tumorigenesis was significantly higher after all doses of ^{56}Fe and ^{28}Si relative to the corresponding doses of γ radiation (**Fig. 2D to F**). There was no statistically significant difference in tumorigenesis among comparable doses of ^{12}C, ^{56}Fe, and ^{28}Si radiation except for 0.1 Gy where tumor frequency was higher after ^{56}Fe and ^{28}Si relative to ^{12}C. Since there was no spontaneous tumor in the colon, we calculated percent of mice bearing radiation-induced colonic tumors. In male mice, 5 to 15% had colonic tumors after 0.1 to 2 Gy γ radiation, 20 to 30% had tumors after 0.1 to 2 Gy ^{12}C, 25 to 40% had tumors after 0.1 to 1.6 Gy ^{56}Fe, and 30 to 100% had tumors after 0.1 to 1.4 Gy ^{28}Si (**Fig. 2G, H, and I**).

Male mice showed higher tumorigenesis relative to female mice after ^{28}Si irradiation. For each radiation dose, tumorigenesis was compared between male and female mice. Intestinal tumorigenesis was significantly higher in male relative to female mice at all doses of ^{28}Si radiation (**Fig. 3A, B, and C**). However, we did not observe any significant difference in intestinal tumorigenesis between male and female mice after ^{12}C and ^{56}Fe radiation (**Fig. 3A, B, and C**). While colonic tumorigenesis was higher in male compared to female mice at all doses of ^{28}Si, we also observed higher colonic tumor frequency in male mice after 0.1 and 0.5 Gy of ^{12}C radiation (**Fig. 3D, E, and F**). Differences in colonic tumorigenesis in male and female mice after ^{56}Fe and 2 Gy ^{12}C radiation was not statistically significant (**Fig. 3D, E, and F**).

Calculated RBE values for intestinal and colonic tumorigenesis relative to γ radiation. The RBE for intestine and colon tumorigenesis in male and female mice after different doses of ^{12}C, ^{56}Fe, and ^{28}Si were calculated relative to γ radiation (**Suppl. Table 3**). Calculated RBE for intestinal tumorigenesis after 0.1 Gy ^{12}C, ^{56}Fe, and ^{28}Si were 3.7, 5.6, and 8.0 in male mice and 1.6, 2.7, and 3.2 in female mice respectively (**Fig. 4A**). For 0.5 Gy, our results showed RBE of 2.1, 3.0, and 5.3 in male and 1.4, 2.1, and 2.0 in female mice after ^{12}C, ^{56}Fe, and ^{28}Si radiation respectively (**Fig. 4B**). For 2 Gy and equitoxic doses, we calculated intestinal tumorigenesis RBE of 1.5, 2.3, and 4.8 in male and 1.6, 2.4, and 3.1 in female mice after ^{12}C, ^{56}Fe, and ^{28}Si radiation respectively (**Fig. 4C**). We calculated the RBE of colon tumorigenesis for 0.1 Gy (8, 10, and 13 for ^{12}C, ^{56}Fe, and ^{28}Si respectively) and 0.5 Gy (8, 10, and 14 for ^{12}C, ^{56}Fe, and ^{28}Si respectively) doses in male mice (**Fig 4D and E**). Considering that there was no γ radiation-induced colon tumor after 0.1 and 0.5 Gy doses, the RBE for these doses was not calculated in female mice. For 2 Gy and equitoxic doses, the RBE for colonic tumorigenesis were 3.5, 5.6, and 9.3 for ^{12}C, ^{56}Fe,

and ^{28}Si respectively in male mice and 3.3, 5.0, and 8.1 for ^{12}C, ^{56}Fe, and ^{28}Si respectively in female mice (**Fig. 4F**).

Discussion

Since we do not have sufficient human data, an important approach toward space travel-associated CRC risk estimation is to determine relative biological effectiveness (RBE) of intestinal and colonic tumorigenesis in appropriate animal models for HZE radiation compared to γ radiation. The RBE scaling factor from animal studies can then be used along with the low-LET human data on CRC to develop risk prediction models for HZE ions. While our previous studies focused on ^{56}Fe at relatively higher doses (3, 16, 23), the current study is expanded to include three HZE ions (^{12}C, ^{28}Si, and ^{56}Fe) and three doses spanning a range of energies and LETs to determine RBE ratio of intestinal and colonic tumor frequency in APC$^{1638N/+}$ mice relative to γ-rays.

Studies have shown that radiation-induced tumorigenesis is contingent upon a number of factors including radiation quality and dose, and high-LET radiation has been reported to induce higher number of solid tumors relative to low-LET radiation (27-30). In this study, all three HZE ions induced higher intestinal and colonic tumorigenesis in mice relative to γ radiation with ^{28}Si showing the highest response. It is possible that differential tumorigenesis after different irradiation results from varied physical properties such as dose distribution, and energy deposition among these radiation types leading to increased transformation due to genetic and epigenetic changes in key tumor suppressive/oncogenic pathways (31-37). However, differences

in physical properties alone may not fully explain differential tumorigenesis observed among three HZE ions and it is possible that there is involvement of a component of non-targeted effects, which could vary depending on the characteristics of the incident radiation (30, 38, 39).

In both male and female mice, although tumors in excess of control mice after 0.5 and 2.0 Gy dose was more than 0.1 Gy, we observed that tumorigenesis did not increase proportionately relative to radiation dose. For example, the fold increase of intestinal tumor was lower (4.25/2.8=1.5 and 9.45/2.8=3.3 fold) relative to fold increase of radiation dose from 0.1 to 0.5 and 2.0 Gy (0.5/0.1=5 and 2.0/0.1=20 fold) in ^{12}C-irradiated male mice suggesting disproportionately lower increase at higher doses. Indeed, tumor incidence per unit of ^{12}C radiation (cGy) showed greater increase after the lower doses compared to the higher doses (2.8/10 = 0.28 tumors per cGy after 0.1 Gy and 4.25/50=0.08 after 0.5 Gy compared to 9.45/200=0.05 per cGy after 2 Gy). These results could be interpreted to suggest that saturation effects is at play which could be due to futile multiple hits of the same cell as well as due to demise of potential tumorigenic cells after high dose exposures (40, 41).

Age-adjusted CRC incidence, grade, and mortality is higher for men relative to women and is attributed to sex-specific differential exposure to environmental risk factors and endogenous/exogenous protective factors such as estrogen (41-46). Importantly, epidemiological data from the atom bomb survivor Life Span Study (LSS) cohort (47) as well as from occupational radiation exposure cohort (48) demonstrated that excess relative risk for CRC is higher in men compared to women and thus male preponderance is also maintained in radiation exposure associated CRC. Our earlier study in APC$^{1638N/+}$ mice, which was a life span study,

showed higher intestinal tumorigenesis in male relative to female mice specifically after a high dose of γ radiation (49). In the current study, although we observed increasing trend of tumorigenesis in male mice after γ radiation, it was not statistically significant. This could be due to the lower doses as well as due to the 'time-dependent' design (50) of this study, which had a fixed ending time point at 150 d post-exposure. Conversely, frequency of tumorigenesis was significantly higher in male relative to female mice after ^{28}Si and to some extent after ^{12}C radiation. Notably, tumorigenesis was not statistically significant between male and female mice exposed to ^{56}Fe and is consistent with our previous results (16).

Analysis of RBE values support the notion that particle energy, LET, and RBE are interlinked. When we compared ^{28}Si with ^{56}Fe, we observed higher RBE for ^{28}Si than ^{56}Fe at all doses and could be due to differences in LET (53, 54). Additionally, comparing results in female APC$^{1638N/+}$ and APC$^{Min/+}$ (3) from our current and previous studies respectively after 2 Gy γ-rays and equitoxic 1.6 Gy ^{56}Fe, showed that the calculated RBEs of intestinal tumorigenesis were similar (2.42 in APC$^{1638N/+}$ and 2.80 in APC$^{Min/+}$) suggesting consistency of results across two models. Our study showing differential RBE after ^{12}C and ^{28}Si at similar energies can be partly explained since LET is linked to the Z-values of the particles and two particles at similar energies have differing LET and thus RBEs (20). In contrast, ^{28}Si with a lower Z-value compared to ^{56}Fe showed higher RBE and may be due to differences in energy, and hence LET and is consistent with earlier reports (55). Evidence in the literature suggests that RBE shows an upsurge up to an LET of ~100 keV/μm and above this the RBE declines (54, 55), which has been attributed to beam and particle characteristics described earlier (40). The RBE of heavy ions is related to the LET, Z-value, and importantly to their energy deposition pattern. Overall, our data

showed that lower radiation doses have greater RBE relative to higher doses for intestinal and colonic tumorigenesis and is similar to the reports on heavy ion radiation-induced hepatocellular carcinoma development (30). Developing risk estimates for CRC following energetic heavy ion is a priority for future space missions and therefore, it is essential that we determine gastrointestinal tissue specific biological effects for heavy ions using surrogate endpoints relevant to known human disease processes. Also, our relative comparison of tumorigenesis between γ-rays and heavy ion radiation could be further utilized to model in-depth RBE values at much lower doses for extended and mixed heavy ion radiation filed exposures expected during space missions. Finally, our *in vivo* tumorigenesis data and RBE in a mouse model of human CRC is an important step towards developing heavy ion exposure-associated CRC risk prediction models as well as preventive strategies for human.

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Figure legends

Figure 1. Increased intestinal tumor frequency after exposure to different types of radiation. Results are presented as average radiogenic tumor incidence per mouse, with spontaneous background due to model subtracted. A to C) Higher intestinal tumorigenesis was noted after

different doses of ^{12}C, ^{56}Fe, and ^{28}Si relative to γ radiation in male mice. D to F) Intestinal tumor frequency was higher after different doses of ^{12}C, ^{56}Fe and ^{28}Si relative to γ radiation in female mice. Significance ($p<0.05$) symbols - *compared to γ radiation, #compared to ^{12}C, $compared to ^{56}Fe.

Figure 2. Increased colon tumor frequency after exposure to different radiation types is presented as average radiogenic tumor incidence per mouse, with spontaneous background subtracted . A to C) Higher tumor frequency in colon was noted after different doses of ^{12}C, ^{56}Fe, and ^{28}Si relative to γ radiation in male mice. D to F) Colon tumorigenesis in female mice after different doses of ^{12}C, ^{56}Fe and ^{28}Si relative to γ radiation. G to I) Since there was no spontaneous tumor in control mice, colon tumorigenesis is expressed as percent of male and female mice bearing colon tumors after different doses of γ, ^{12}C, ^{56}Fe, and ^{28}Si radiation. Considering that data is presented as percent of mice bearing tumor relative to total number of mice in each group (total 20 mice/study group), no statistical analysis is shown. Percent of mice bearing colon tumor = (number of mice with tumor/total number of mice which is 20 in this study) x 100. Significance ($p<0.05$) symbols - *compared to γ radiation, #compared to ^{12}C.

Figure 3. Sex differences of intestinal and colonic tumorigenesis are presented as average radiogenic tumor incidence per mouse, with spontaneous background subtracted. A to C) Intestinal tumorigenesis in male and female mice after different radiation exposure. Scale in all the doses is kept same for comparison. D to F) Colonic tumorigenesis in male and female mice after different irradiation. Scale in all the doses is kept same for comparison. Significance ($p<0.05$) symbol * - compared to female.

Figure 4. RBE as a ratio of heavy ion vs. γ radiation-induced intestine and colon tumor frequency are presented using smooth fitting curve assuming a single (ion-independent) relationship between RBE and LET. A to C) RBE of intestinal tumorigenesis in male and female mice. D to F) RBE of colon tumorigenesis in male and female mice. Considering that there was no colonic tumor in female mice after 0.1 and 0.5 Gy γ radiation, RBEs for heavy ions are not shown for these doses.