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The Delayed Effects of Acute Radiation Syndrome: Evidence of long-term functional changes in the clonogenic cells of the small intestine

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Abstract

Long term or residual damage post-irradiation has been described for many tissues. In hematopoietic stem cells (HSC) this is only revealed when the HSC are stressed and required to regenerate and repopulate a myeloablated host. Such an assay cannot be used to assess the recovery potential of previously irradiated intestinal stem cells (ISC) due to their incompatibility with transplantation. The best approximation to the HSC assay is the crypt microcolony assay, also based on clonogen survival. In the current study the regenerative capacity of intestinal clonogenic cells in mice that had survived 13 Gy irradiation (with 5% bone marrow shielding to allow survival through the hematopoietic syndrome) and were then aged for 200 days, was compared to previously unirradiated age matched controls. Interestingly, at 200 days following 13 Gy there remained a statistically significant reduction in crypts present in the various small intestinal regions (illustrating that the gastrointestinal epithelium had not fully recovered despite the 200 day interval). However, upon re-irradiation on day 196, those mice previously irradiated had improved crypt survival and regeneration compared to the age matched controls. This was evident in all regions of the small intestine following 11–13 Gy re-exposure. Thus, there were either more clonogens per crypt within those previously irradiated and/or those that were present were more radioresistant (possibly because a subpopulation was more quiescent). This is contrary to the popular belief that previously irradiated animals may have an impaired/delayed regenerative response and be more radiosensitive.

Keywords

crypt stem cell; radiation damage; acute radiation exposure; gastrointestinal; residual damage

INTRODUCTION

Exposure to high-dose irradiation, either as a result of a nuclear terrorist event, accidental exposure or local radiotherapy can cause acute symptomatic effects in the exposed tissue.

Received doses below 10 Gy are typically lethal for the bone marrow, with those above 10 Gy causing acute damage and lethality as a result of gastrointestinal (GI) toxicity, termed GI-ARS (Gastrointestinal-Acute Radiation Syndrome). In the mouse small intestine one of the first effects within the epithelium is that of stem cell apoptosis, evident within a few hours of even low-dose irradiation (Potten 1977). This cell death is believed to be a form of altruistic suicide, protecting the population from the propagation of induced DNA damage. However, stem cell death induced by low-dose radiation does not cause GI-ARS. This requires death of the extended clonogenic cell population. A hierarchical response to radiation has been proposed within the clonogenic cell population, with increasing doses of radiation targeting successive tiers of clonogens within each intestinal crypt (Potten and Loeffler 1990; Hendry et al. 1992). The model also suggests that the maturing stem cell daughter experiences a gradual loss of stem cell functionality over the next two generations before ultimately becoming the fully committed transit amplifying cell. It is believed that this reserve force of potential clonogenic cells must be killed or reproductively sterilized in order to kill the crypts and therefore create the severe loss of barrier function responsible for GI-ARS. However, these cells, with increasing radiotolerance are also the cells that are recruited to fulfill their clonogenic potential and ensure crypt regeneration following a traumatic event, such as irradiation.

Following the death of such clonogenic cells, and interruption of cell proliferation in the face of continued cell migration and loss into the intestinal lumen, the mucosal area is reduced as the amplitude of the undulating epithelium from crypt base to villus tip dramatically shrinks, often referred to as villus blunting. If clonogenic cells remain in the crypt this effect is transient. However, when all the clonogenic cells are killed the crypt will be lost, and if sufficient crypts are killed an ulcer may develop. Symptoms resulting from ulceration and breakdown of the epithelial barrier include weight loss, diarrhea, sepsis and, ultimately, death (particularly if the sepsis cannot be controlled). However, if even a single clonogenic cell per crypt remains the regeneration is rapid, such that at just four days post-irradiation the surviving regenerating crypts can be easily identified and distinguished from their dying neighbors. The extent of regeneration at this point is clearly dose-dependent, which in turn reflects the number of surviving clonogens present. The clonogenic cells in the normal young adult mouse intestine have been well characterized with full dose response curves for intestinal crypt survival post-irradiation established (Hendry 1979, Hendry 1993, Potten and Hendry 1983, Potten 1998, Potten and Loeffler 1990, Cai et al. 1997a, b; Booth et al. 2012a).

Whilst the acute effects of radiation on the epithelium of the GI system have been well described, the persistence of any long-term damage to the surviving and regenerated clonogenic cell population, responsible for the acute mucosal repair and long-term tissue functionality, is not known. These irradiated clonogens may have impaired long-term functional capacity - either as a direct result of the radiation damage or as a result of reduced fidelity during their pre-irradiation lineage progression as second or third tier reserve clonogens. These cells may not contain the template or parental DNA present in the original ultimate stem cell (normally selectively retained within it by asymmetrical division) and hence carry replication induced errors (Potten et al. 1978; 2002; Buczacki et al. 2013). Further, the crypt regeneration post-irradiation from such a surviving clonogen may then

amplify numbers by symmetrical division and gradually repopulate a crypt – thereby generating a monoclonal population with propagated damage or infidelities. These processes may be common to many tissues and have been proposed to be the cause of many long-term radiation induced cancers. However, the degree of residual clonogenic cell damage is extremely difficult to prove. Residual damage post-irradiation has been described in HSC but is only revealed when the HSC are stressed and required to regenerate and repopulate a syngeneic myeloablated host. Unlike the bone marrow, it is not possible to perform serial transplants of gastrointestinal clonogenic cells in order to compare their long-term functionality. One option available is to examine the radiation dose-response (comparing the response of previously irradiated surviving clonogens to age matched controls via the use of an intestinal microcolony assay) and to determine whether the extensive mucosal repair following GI-ARS impacts this response.

We have compared the regenerative capacity of intestinal clonogenic cells in adult mice surviving a GI-ARS inducing dose of 13 Gy X-ray irradiation (with 5% bone marrow shielding to allow long-term survival through the hematopoietic syndrome) and then aged for 200 days, to that of age matched controls.

In addition to clinical scenarios, the aim is that this work begins to form a basis of understanding the natural history of the GI-ARS response and the consequential later effects (termed DEARE – Delayed Effects of Acute Radiation Exposure). It is intended that the data will inform future studies analyzing acute and long-term recovery of the GI system irradiated under similar conditions with or without administration of a GI-ARS medical countermeasure (MCM).

MATERIALS AND METHODS

Mice

All procedures were certified according to the UK Animal (Scientific Procedures) Act 1986. Male CBA/Ca mice, aged eight to ten weeks were purchased from Harlan UK and allowed to acclimatize for two weeks prior to irradiation.

Groups of six mice that survived 13 Gy irradiation at 12 weeks of age (termed day 0) were allowed to age to day 196 and then irradiated with a further high dose of radiation (11, 12 or 13 Gy) prior to euthanasia on day 200 for crypt analysis using the established microcolony assay (Withers and Elkind 1970; Withers et al. 1993; Potten and Hendry 1985). Parallel groups of animals were unirradiated at 12 weeks but allowed to age to 196 days and then subjected to 9, 10, 11, 12 or 13 Gy prior to euthanasia on day 200 for crypt analysis in order to compare the effect of age on this response. Control animals were either unirradiated at both timepoints or irradiated only at 12 weeks of age.

Husbandry

All mice were held in individually ventilated cages (IVCs) in a specific pathogen free (SPF) barrier unit. A twelve hour light:dark cycle was maintained with lights being turned on at approximately 07:00 hours and off at approximately 19:00 hours. There was a constant room temperature of 21 + or – 2°C and a mean relative humidity of 55% + or – 10%. The animals

received 2918× extruded rodent diet (Harlan) and sterile acidified water (ad libitum) from time of arrival and throughout the study. Animals were identified by ear punches in cages labeled with the appropriate information necessary to identify the study, dose, animal number and treatment groups.

Supportive Care

Ciprofloxacin (0.67 g L^{-1}), was administered in the drinking water from day four post-irradiation until day 20 [i.e. through GI-ARS and when absolute neutrophil counts (ANC) have previously been shown to drop below $500 \mu\text{L}^{-1}$ (Plett et al. 2012)] and was also used to wet the food and generate mash, supplied within the cage from days 4 to 20 to allow for easier access by mice potentially weakened from radiation exposure.

Irradiation

Animals were irradiated at $15:00 \pm$ one hour (20 animals per radiation dose). Irradiation was performed using an XStrahl RS320 X-ray set, operated at 300 kV, 10 mA. The X-ray tube has additional filtration to give a radiation quality of 2.3 mm Copper half-value layer (HVL). Mice were restrained in a compartmentalized Perspex (plexiglass) jig, positioned at a distance of 700 mm below the focus of the X-ray tube. Each rectangular box is divided to provide twelve ventilated restraints, each holding one animal. Thus, up to twelve mice were irradiated simultaneously. On day 0, Partial-Body Irradiation (PBI) was performed shielding the lower hind limbs (fibulae, tibiae and feet) using lead tubes. This is estimated to protect approximately 5% of the bone marrow (PBI BM5) (Boggs 1984; Taketa et al. 1970). Radiation was delivered at a dose rate of $0.812 \text{ Gy min}^{-1}$. Animals were anaesthetized intraperitoneally with Ketamine (Fort Dodge) and Xylazine (Bayer) to allow immobilization within the restraint and accurate lead shielding of the appropriate area.

The X-ray output was recorded for each radiation run using an ion chamber embedded into the X-ray chamber. All radiation doses delivered were within 5% of the intended dose with the mean variance being 2.36% (min -0.88% : max 4.67%).

Health Status Monitoring

All animals were weighed and their well-being inspected daily from days 0 to 20 and days 196 to 200. Between days 20 and 196 the mice were weighed twice weekly (with more frequent weighing with any change in health status). Animals were humanely euthanized if the weight loss was sustained at greater than 20% for 24 hours and mice also demonstrated signs of a moribund state (withdrawn behavior, reduced body temperature as judged by feeling cool to touch, lack of grooming and dehydration as judged by a persistent skin tent on pinching). During the peak periods of diarrhea incidence and general decline in well-being, animals were inspected several times during each 24 hour period.

Blood Counts

On the day of euthanasia (day 200), blood was taken via cardiac puncture. The blood was placed into a tube coated in EDTA. $50 \mu\text{L}$ of the blood was then added to $200 \mu\text{L}$ of Cellpack (the Sysmex analyzer diluent) and analyzed on a Sysmex XT-2000iV analyzer.

Intestinal Histopathology

Following euthanasia the small intestine was removed and divided into four (duodenum jejunum, upper ileum and lower ileum). The mid-colon was also excised and the contents flushed out. All regions were then placed into buffered formaldehyde fixative. The intestines were 'bundled' prior to embedding in order to obtain ideal orientation of the crypts (Merritt et al. 1992). From each region a series of small lengths of intestine, approximately 0.5 cm long, were placed within a loop of surgical Micropore tape, and the tape tightened to immobilize the lengths. This allows the alignment of many pieces of fixed intestine alongside each other like a series of logs, so that in every section from a mouse there are several well-orientated cross-sections. Each paraffin block generated was then sectioned to provide one slide per block, each slide containing two non-serial sections which were stained with Hematoxylin and Eosin (H & E). The number of surviving and regenerating crypts per intestinal circumference was then scored and the average per region, per mouse and per group determined. A surviving crypt was defined as one that had ten or more tightly packed strongly H & E stained cells (excluding Paneth cells). Only regions that were orientated correctly and did not contain Peyer's patches were scored (Peyer's patches influence both the number of crypts in a normal circumference and the ability of a crypt to survive insult).

Since the girth of the crypt (seen as the crypt width in cross-sections) also increases post-irradiation there is a greater probability of sectioning and scoring a crypt in these samples compared to those containing smaller crypts. A correction factor derived from the crypt cross-sectional widths was therefore applied to reduce the risk of over-scoring the number of crypts present (Potten et al. 1981). The mean crypt width of each irradiated region was divided by the mean crypt width in a similar region of unirradiated control mice. This number was then used to multiply the number of crypts scored to give a corrected crypt survival score (see formula below).

$$\text{Corrected number of crypts} = \frac{(\text{Mean crypt width of irradiated mouse}) \times (\text{number of crypts in the irradiated mouse})}{\text{Mean crypt width of the unirradiated group}}$$

Fifteen crypts per mouse had their widths measured at their widest point. The crypts chosen were preferably the larger, fully regenerated crypts.

Statistics

An ANOVA model of the number of crypts per circumference on radiation dose level at day 0 and radiation dose level at day 196 was fit for each study outcome. Radiation dose level at day 0 and at day 196 were treated as categorical variables in the models. Comparisons of crypts per circumference were performed between mice that were irradiated by 0 Gy vs. 13 Gy at day 0 with 0 Gy, 11 Gy, 12 Gy, or 13 Gy at day 196. All tests were performed using two-sided tests at the 0.05 significance level.

RESULTS

Body Weights

From the start of the study when mice were 10–12 weeks of age until the start of the next irradiations at 196 days, or 28 weeks the control mice increased their starting body weight by approximately 50% (Fig. 1). Mice that were exposed to 13 Gy with 5% bone marrow protection at 10–12 weeks of age, lost body weight as expected and then when allowed to age over this period regained their starting weight, but failed to add any additional mass over the intervening 200 days. 13 Gy with this level of bone marrow protection is an LD_{30/6-8} dose when ciprofloxacin is included (LD₅₀ without ciprofloxacin) in this strain of mouse, and hence causes major GI damage in addition to significant multi-organ injury and consequential sub-syndromes during the timecourse of this study (Booth et al. 2012a,b). This long-term failure to gain weight is consistent with our previous observations in survivors of 13 Gy GI-ARS (albeit the previous study employed higher levels of bone marrow shielding and only ran for four months post-irradiation (Booth et al. 2012b).

Mice surviving the acute radiation damage were randomized on day 196 into groups, irradiated with 11, 12 or 13 Gy, euthanized and then clonogenic responses evaluated using the crypt microcolony assay. Age matched control mice were irradiated with 9–13 Gy for comparative purposes. Due to the failure of the 10–12 week irradiated mice to gain weight there was a large difference in animal weights at the start of the second round of irradiations (the unirradiated mice being, on average, 12 g heavier). Although mice that were previously irradiated lost slightly more relative weight upon day 196 irradiation with 11 or 12 Gy compared to the previously unirradiated mice, the absolute weight loss (g) compared to the age matched controls was less, at all radiation doses (Table 1, Fig. 2).

Blood Counts

On the day of euthanasia (day 200), blood was taken via cardiac puncture. White blood cell (WBC), red blood cell (RBC) and platelet (PLT) counts were all similar in the two control groups unirradiated on day 196 (not statistically different for any of the cell types, although the WBC counts were more variable following the earlier irradiation), illustrating that blood counts in the previously irradiated animals returned to the level of the age matched control (Fig. 3). In previously unirradiated mice there was no evidence of a radiation dose response as a result of the day 196 irradiations, with all the radiation doses being higher than the LD_{100/30} bone marrow dose. Comparing to animals irradiated on day 0, the only significant ($p=0.01$, ANOVA) effect of irradiation on red cell counts was at 12 Gy, where cell numbers in the twice irradiated (13:12 Gy) were much lower than the animals that were previously unirradiated (0:12 Gy). 13 Gy exposure followed the same pattern but did not achieve significance. As may be expected, white cell counts were dramatically reduced following such high-dose irradiation, with counts in the previously unirradiated mice reduced slightly more than those previously irradiated ($p=0.04$, $p=0.005$ following 11 and 12 Gy), suggesting a slight radio-resistance at this time and radiation dose. Normalization of these WBC data to the day 196 unirradiated controls confirmed the radioresistance at 11 and 12 Gy. By day 200 the platelet counts were consistently, but not significantly, slightly reduced post-irradiation in the previously unirradiated mice, with those receiving the prior exposure having highly

depressed numbers following 12 and 13 Gy ($p=0.001$, $p=0.01$) compared to those unirradiated at 10–12 weeks of age.

Crypt number in long-term survivors of GI-ARS

Figures 4a and 4c illustrate that the number of crypts per small intestinal circumference over a 200 day aging process did not change. However, following 13 Gy irradiation on day 0 crypt number failed to return to the age matched control levels 200 days later ($p=0.04$), i.e. the number of crypts present at the time of the second radiation exposure was lower than those in the previously unirradiated animals. Interestingly crypt widths increased with age (12%, $p=0.001$, ANOVA) but were also 10% larger ($p=0.01$, ANOVA) in the previously exposed group compared to the age matched controls (Fig. 4b and 4c). H & E images illustrating the increased size of the crypts in the previously irradiated animals are shown in Figures 5a and 5b. A correlation of increased crypt girth and reduced crypt number has been reported many times, but is usually associated with regenerating mucosa, rather than the aging process and is the reason for the use of the crypt correction factor (Potten et al. 1981).

Functional competence in long-term survivors of GI-ARS

The relative number of crypts surviving and regenerating post-irradiation in the various regions of the small intestine and the mid-colon is shown in Figure 6a, with illustrative images in Figures 5c–f. The failure to return to control crypt levels 200 days post-irradiation was consistent across all regions of the intestine. There was also a clear and consistent radiation dose response from 11–13 Gy across all regions. However, following 12 and 13 Gy (doses of radiation that are associated with GI-ARS) there was increased crypt survival and regeneration in animals that had been previously exposed to 13 Gy 28 weeks previously i.e. these crypts were more radio-resistant. Given that the baseline crypt numbers were different between the 13 Gy irradiated and aged vs aged only, on day 196, the same data has been plotted relative to the day 196 crypt number in Figure 6b. The increased radio-resistance is more apparent and also evident at 11 Gy when the data is normalized in this way.

Since the girth of the crypts was also increased by the prior irradiation (Fig. 4b) there was a possibility that these increased numbers are due to the greater probability of scoring larger crypts. The crypt widths in all groups were therefore measured (Fig. 7) and crypt counts corrected for this factor (Fig. 8). Statistical analysis of these corrected crypt counts confirmed that crypt numbers were significantly lower on day 200 post-irradiation compared to the age matched controls (ANOVA $p<0.001$ for all regions of the small intestine and the mid-colon). Following 11 Gy on day 196 only the ileum achieved statistical significance: $p=0.005$ and 0.015 for the upper and lower ileum respectively (Table 2). However, following 12 and 13 Gy statistical significance was greater and throughout the intestinal regions. Thus, increased radioresistance remains apparent in all regions except the colon, which has slower regeneration kinetics than the small intestine (day 4 is at least a day too early to detect colonic changes with maximum sensitivity) (Cai et al. 1997a, b). Pooled data for the entire small intestine and the fold increase in crypt number by regions are summarized in Figure 9 and Table 3.

DISCUSSION

Male CBA/Ca mice were used in the current study, since this strain of mouse has been extensively employed in DEARE studies following similar high-dose radiation exposures to the lung. This, therefore, allows a model to be developed that examines both co-morbidities. It has been preferred to the C57BL/6 mouse in lung since it is less susceptible to the development of pleural effusion and hence more akin to the fibrotic symptoms developed in man (which do not commonly develop pleural effusion) (these strain differences have been extensively reviewed by Down 1983; Jackson et al. 2010, 2011). Whilst damage to the lung epithelium does not give rise to any acute symptoms, unlike the gastrointestinal system, the DEARE symptoms of inflammation, fibrosis and reduced functional capacity are common to both tissues over a similar timeline and support the use of this strain of mouse and a 200 day study duration.

Stem cell and crypt maintenance during normal aging

Of the approximately 250 cells in a young adult mouse small intestinal crypt, two thirds are actively dividing, with each crypt producing around 300 cells per day and hence in a three year old laboratory mouse a crypt stem cell has been estimated to divide ~1000 times (reviewed by Potten 1995). This high proliferative activity creates a replication error prone environment and is thought to increase the susceptibility to genetic and epigenetic issues, in addition to the extrinsic risk associated with the bacterial flora within the intestinal lumen. As a result of this hazardous existence the intestinal crypts have evolved a protective stem cell hierarchy, where quiescent and actively cycling stem cells co-exist with differing day to day functions, radiosensitivities and regenerative capabilities. Under normal conditions the reserve of cells with stem, or clonogenic, potential are present but do not function as stem cells becoming committed to terminal differentiation with their stem cell capabilities only called upon when required for rapid tissue regeneration, such as that triggered by irradiation. The normally limited number of 4–6 functioning stem cells per crypt as defined by Potten and Loeffler 1990 was recently elegantly confirmed by Kozar in a clonal labeling study (Kozar et al. 2013). In this context a stem cell was defined by Potten and Loeffler (1990) as being undifferentiated (relative to the other epithelial cell types, but not necessarily relative to embryonic cells), capable of proliferation and self-maintenance, producing many differentiated progeny, and regenerating the tissue after injury. In the last decade the discovery of markers of intestinal stem cells has confirmed the co-existence of different stem cell phenotypes within the crypts. In particular *Lgr5* and *Bmi1* appear to identify two distinct, but potentially overlapping or complementary, stem cell populations within the crypt, supporting the original hierarchical proposal (Barker et al. 2007; Sangiorgi and Capecchi 2008, Yan 2012). There have been many discussions regarding the exact physical location of cells expressing these markers within the crypt and which of them is the true stem cell. Given that there is an agreed stem cell pool in a highly dynamic tissue and that at any one moment in time, even in a normal adult, some may divide via symmetric or asymmetric division (and thus regulating the levels of *Lgr5* and *Bmi1* expression) it is not surprising that clear identification is difficult. This confusion is compounded by many transgenic/inducible models that include data from young mice where the stem cell population will be actively expanding to support intestinal growth. It is generally accepted

that the entire stem/clonogenic cell population results from the first two to three cell divisions and that these cells reside at the base of the crypt up to around cell position seven, with an *average* location being cell position four. The bulk of these express Lgr5. Whether a cell in the round bottomed crypt base moves up and away, sideways or remains at the mother location is intimately linked to its position and role within the hierarchy. The spatial locations and necessary re-arrangements of the stem cell tiers within an undulating Paneth cell landscape was discussed by Potten et al. (1997) and visualized real-time recently by Ritsma et al. (2014).

In conclusion it seems that as long as clonogenic cells remain within the cell position 2–7 location, and have experienced a limited number of divisions, they may realize their stemness if required. During the aging process there may be a gradual decline in stem cell fidelity and possibly homeostatic regulation of their number. In the small intestine, stem cell apoptosis and preservation of the template strand DNA is thought to assist the maintenance of stem cell fidelity in the face of such massive mitotic activity, with the expression of telomerase in the stem cells (coupled with the presence of long telomeres in mice) protecting the stem cells from intrinsic senescence as a result of aging (Potten et al. 1978; 2002; Buczacki et al. 2013; Itahana 2004, Booth and Potten 2000). Relevant to the data presented in the current study, data from Kozar et al. (2013) suggests that, in a mouse, functioning stem cell number does not change over a two year aging period i.e. there is no evidence of stem cell exhaustion. However, replication stress has been shown to be a potent driver of functional decline in old hematopoietic stem cells (Flach et al. 2014). Studies by Martin et al. (1998b; 2000) revealed that in aged animals, the number of crypt stem cells that were susceptible to low-dose radiation was increased i.e. either this stem cell subpopulation was increased or the accumulation of DNA damage over time had reduced the threshold required to trigger apoptosis. Hence replication stress may be compounded by the continuous exposure to oxidative stress causing cellular damage. This was linked to damaged mitochondrial function in the intestine with age by Potten et al. (2001), in particular to mitochondrial DNA damage and cytochrome C oxidase activity (Potten et al. 2001).

Either way the data suggested that control within the stem cell population may have drifted with age, along with the possible balance of Lgr5 and Bmi1 expression. Consistent with the observations documented in the current study, Martin also found that crypt and villus size increased with age. The observed development of larger villi, at ages older than used in the current study, lead to reduced functional absorptive surface area and villus motility, a characteristic of aging, possibly resulting from the gradual loss of stem cell number (although this contradicts the recent data from Kozar et al. (2013) or function, causing reduced day to day cell production (Martin et al. 1998a, Booth et al. 2012). The increase in crypt girth observed in the day 200 mice may be due to an extended lineage within each crypt (in an attempt to maintain functional cell output onto the villus), or a reduced rate of cell emigration onto the villus. An extended lineage may be due to an expansion of the potential clonogenic cell number linked to a delayed commitment to terminal differentiation of the transit amplifying cells. This suggests that cells higher up the normal lineage may be recruited into the clonogenic compartment in older animals, possibly indicative of a deterioration of the crypt organization. Martin et al. (1998b) suggested that aged crypts were

slower to respond to high-dose irradiation than younger adults, although the total number of cells with clonogenic potential was estimated to be higher in radiation split-dose studies.

Stem cell and crypt regeneration following injury

Upon exposure to GI-ARS inducing doses of radiation the surviving crypt clonogens are triggered to cycle rapidly in order to restore their number via symmetrical expansion. This has been proposed to be due to the response of the high Bmi1 expressing cells (the majority of the Bmi1 negative, Lgr5+ cells being killed), which clonally expand post-irradiation (Yan et al. 2012). These cells will then restore the crypt structure and generate further crypts via fission, with each new crypt also developing a new stem cell hierarchy and a new population of Lgr5+ cells (Tian et al. 2011, Yan et al. 2012). Thus, sustained stem cell expansion is required in order to restore the tissue, followed by a return to normal daily production of differentiated progeny and general tissue maintenance. In many ways this process of clonogen regeneration and crypt turnover may therefore be equated to an accelerated aging process, compressing the events of several years into months, and contributing to the further changes in crypt number and size seen in aged irradiated mice in the current study. Crypt number was not completely restored, at least within the 200 day window, but the fewer, larger crypts are able to provide a functional villus cell population. The crypts were larger than their unirradiated age matched counterparts, suggesting a further extension of the cell lineage. The day 200 timepoint was a pragmatic cut off point for this study – a time later than which we have previously seen animals decline i.e. a time at which any surviving mice may have sustained long term or delayed effects. It is also consistent with the lung DEARE timeframe in mice following similar high doses, where animals become moribund by day 100–180 depending on their strain but deaths have typically plateaued by day 200.

Delayed effects of acute radiation exposure (DEARE) – residual GI damage (RGID)

Given that the functional competence of the stem cell populations within these regenerated or prematurely aged crypts may well be compromised, their regenerative capacity following 11–13 Gy irradiation was therefore tested in a microcolony assay. Contrary to expectation, which might suggest impaired regenerative capacity post-irradiation, as is observed in the hematopoietic system (Chua et al. 2012, Shao et al. 2014) the number of surviving regenerating crypts in the small intestine was increased in a dose-dependent manner, with over 3× more crypts present following 13 Gy irradiation. Thus, consistent with the further increased crypt size at the time of irradiation, there were either more/resistant (possibly quiescent) clonogens present or the regeneration was faster than in the previously unirradiated animals.

The fact that a high-dose irradiation event 28 weeks prior to a crypt microcolony assay has a sustained impact, suggests that there is residual damage to the tissue, albeit that the mice are asymptomatic apart from their failure to gain weight. Further, given that the epithelial lining of the small intestine is replaced every 5–7 days, with the only long-term residents over 28 weeks being the stem cells, this damage must reside in this population.

Residual damage in the other acutely radiation damaged tissue, the bone marrow, and reduced long stem cell function following serial transplantation has been described (Chua et

al. 2012, Shao et al. 2014). It is important to note that residual damage effects reported in relation to these H-ARS/GI-ARS exposures employ much higher initial doses of radiation than the low potentially protective exposures described as the 'Adaptive Response' or 'Yonezawa Effect' which only exposed mice to doses of 0.5 Gy and lower, although changes in DNA repair capacity may be common to both (Olivieri et al. 1984; Yonezawa 1990). There is also a commonality in that doses of less than 1 Gy have been demonstrated to induce apoptosis in a subpopulation of small intestinal stem cells, thereby triggering a small regenerative response or shift in function in the remaining clonogens (Potten 1977). Transplantation methods which would enable one to distinguish between stem cell versus niche effects are not currently available to the GI. Ex vivo stem cell culture models with engraftment capability are starting to be described but the impact of the manipulations on normal stem cell function is unknown and likely to be dramatic since the culture process itself is a regenerative model (Sato et al. 2009, Li and Clevers 2012).

A potential intrinsic molecular candidate for the cause of RGID includes a sustained increase in Wnt signaling, which can lead to increased crypt size and reduced crypt death. Wnt family signaling in crypt stem cells is normally maintained in check by Notch, which is also responsible for the balance of stem cell maintenance and daughter cell proliferation and differentiation (Tian et al. 2015). Thus, reduced Notch signaling in the stem cell may enable rapid Wnt driven regeneration. Further, Bmi1 regulates murine intestinal stem cell proliferation and self-renewal downstream of Notch, linking to the stem cell phenotype responsible for post-irradiation regeneration (López-Arribillaga et al. 2015). Crucially, however, if Wnt signaling is increased and remains elevated it may protect clonogenic cells that accumulate DNA damage.

Residual extrinsic damage, affecting the stem cell niche may also be crucial. Radiation induced endothelial damage may create a hypoxic stem cell niche, which in turn may increase radioresistance. Hypoxia has also been shown to reduce checkpoint arrest and sustain increased expression of TGFβ – which is known to be associated with GI-DEARE and the associated fibrotic pathologies (Richter et al. 1997).

Finally, whilst the ciprofloxacin treatment was for less than 3 of the 28 weeks between irradiations, antibiotic exposure will also induce changes in the balance of bacterial species within the gastrointestinal lumen. Given that changes in the enteric bacterial population have been shown to affect radiosensitivity, one cannot completely exclude contributing effects on RGID, albeit it is more likely to be relevant to the more heavily populated large intestine (Ciorba et al. 2012; Duran-Struuck et al. 2008).

Co-incident interacting multi-organ damage

Published observations relating to DEARE in the GI have previously referred to fractionated low dose or focal abdominal / intestinal irradiations (Dubois et al. 1988, Hauer-Jensen 1990; Langberg et al. 1996; Booth 2012b) due to the bone marrow lethality induced by GI-ARS doses of irradiation. Thus, in these models any co-incident damage to other organs is the result of abscopal effects. The current use of a minimal bone marrow sparing model allows the bone marrow to regenerate following lethal exposure to the unshielded bones, whilst inducing total organ high-dose irradiation to other major organs. This is a reality multi-

syndrome model for an accidental exposure or terrorist attack and allows multiple long-term sequelae to be investigated simultaneously. However, it also illustrates the complexity of understanding the interactions of such multiple sequelae. The influence of mobilized hematopoietic progenitors on the epithelial tissues of the GI, poor tissue oxygenation due to lung or vascular damage, impaired liver function etc. may also affect tissue and stem cell behavior in other organs, such as the GI. Even restricting the investigation of interacting syndromes to those within the GI system, such as the stomach, liver and pancreas and those that influence it such as the brain or the enteric bacteria is extremely complex. However, the use of the minimal shielding model allows some preliminary descriptions of RGID. As part of our remit to develop MCMs to mitigate the lethal effects of GI-ARS it is crucial that the natural history of the GI damage response is thoroughly understood and can be used as a baseline from which MCM efficacy and long term effects can be evaluated. Ultimately MCM efficacy on delayed pathologies will also need to be defined.

CONCLUSION

These data suggest that 28 weeks following a GI-ARS inducing dose of radiation there are sustained irregularities within the intestinal epithelium. The increased level of crypt survival and regeneration compared to aged matched controls suggests that more clonogens were present in the GI-ARS surviving crypts and/or those that were present were more radioresistant (possibly because there is a quiescent subpopulation present, as a result of a shifted balance of Lgr5/Bmi1 cells). This observation is contrary to the initial hypothesis and popular belief that previously irradiated animals may have an impaired/delayed regenerative response and appear more radiosensitive. The changes within the clonogens and effects on GI function, including the ability to produce the differentiated lineages is unknown. Future analysis may reveal the changes in the stem cell phenotypes present.

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a.

b.

Fig. 1.

Animal weights following 13 Gy irradiation, compared to controls (all groups pooled – controls n=36; irradiated n=24). a: Group Means b: Group Means + Std Dev. Following 13 Gy irradiation there is a dramatic loss of body weight within the first week of GI-ARS, followed by a gradual gain with inflection points reflecting the passage through the hematological radiation syndrome (H-ARS) and mild transient lung associated DEARE, in the presence of minimal bone marrow protection. Animals are then asymptomatic but remain at the original starting weight.

Fig. 2.

Mean group weights (g) from time of the 2nd irradiation on day 196. The table shows the mean weight per day, the plots the relative weight change. Groups of 6 mice received either no irradiation or 13 Gy when 10–12 weeks old, and then on day 196 a range of radiation doses. At the higher radiation doses the previously unirradiated mice lost more weight than those that had survived the prior irradiation, although since the starting weight was greater, the relative weight loss was less.

Fig. 3.

Mean blood counts + Std Dev in the irradiated animals. Blue bars indicate mice that were not irradiated when 10–12 weeks old, red bars are mice irradiated twice, initially with 13 Gy and then on day 196 (28 weeks later) with 11, 12 or 13 Gy (the top row of the X-axis indicates the day 0 treatment, the second row the day 196 treatment).

A

B

C

Treatment	Number of crypts / circumference	Std Dev
d0 0Gy	105.9	7.3
d200 0Gy, 0Gy	104.4	7.0
d200 13Gy, 0Gy	93.7	4.2
	Crypt width (um)	Std Dev
d0 0Gy	36.8	1.8
d200 0Gy, 0Gy	41.2	1.7
d200 13Gy, 0Gy	45.5	1.6

(Editor: table also submitted as separate Word file)

wTreatment		Number of crypts / circumference	Std Dev
d0	0Gy	105.9	7.3
d200	0Gy, 0Gy	104.4	7.0
d200	13Gy, 0Gy	93.7	4.2
		Crypt width (μm)	Std Dev
d0	0Gy	36.8	1.8
d200	0Gy, 0Gy	41.2	1.7
d200	13Gy, 0Gy	45.5	1.6

C

Fig. 4.

Comparison of crypt number and width on day 200 post-irradiation (38–40 weeks of age) to control 10–12 week old mice. d0 0 Gy = control 10–12 week mice; d200 0 Gy, 0 Gy = control 38–40 week mice; d200 13 Gy, 0 Gy = 38–40 week old mice previously irradiated with 13 Gy at 10–12 weeks. Illustration is data from the ileum (other regions follow the same pattern – data not shown).

Fig. 5.

Example H & E images of the ileum ($\times 10$ objective). a: control, day 200 (38–40 weeks old). b: 38–40 weeks old, irradiated with 13 Gy at 10–12 weeks and then aged 200 days. c: 4 days following 11 Gy irradiation on day 196, mice previously unirradiated. d: 4 days following 11 Gy irradiation on day 196, mice previously irradiated with 13 Gy at 10–12 weeks. e: 4 days following 12 Gy irradiation on day 196, mice previously unirradiated. f: 4 days following 12 Gy irradiation on day 196, mice previously irradiated with 13 Gy at 10–12 weeks. g: 4 days following 13 Gy irradiation on day 196, mice previously unirradiated. h:

4 days following 13 Gy irradiation on day 196, mice previously irradiated with 13 Gy at 10–12 weeks.

a.

b.

Fig. 6.

The mean number of crypts per intestinal circumference + Std Dev in the various regions of the gastrointestinal tract as a percentage of the day 0 (a) and day 196 (b) unirradiated controls. Blue bars indicate mice that were not irradiated when 10–12 weeks old, red bars are mice irradiated twice, initially with 13 Gy and then on day 196 with 11, 12 or 13 Gy. D=Duodenum, J=Jejunum, U=Upper Ileum, L=Lower Ileum and C=Midcolon.

a.

b.

Fig. 7.

Mean crypt width in all regions + Std Dev as a percentage of both the unirradiated control (a) and the day 196 unirradiated control (b). Blue bars indicate mice that were not irradiated when 10–12 weeks old, red bars are mice irradiated twice, initially with 13 Gy and then on day 196 with 11, 12 or 13 Gy. D=Duodenum, J=Jejunum, U=Upper Ileum, L=Lower Ileum and C=Midcolon.

a.

b.

Fig. 8.

Mean corrected crypt survival counts in all regions + Std Dev as a percentage of both the unirradiated control (a) and the day 196 unirradiated control (b). Blue bars indicate mice that were not irradiated when 10–12 weeks old, red bars are mice irradiated twice, initially with 13 Gy and then on day 196 with 11, 12 or 13 Gy. D=Duodenum, J=Jejunum, U=Upper Ileum, L=Lower Ileum and C=Midcolon.

a.

b.

Fig. 9.

Mean corrected crypt survival counts in whole small intestine + Std Dev as a percentage of both the unirradiated control (a) and the day 196 unirradiated control (b). Blue bars indicate mice that were not irradiated when 10–12 weeks old, red bars are mice irradiated twice, initially with 13 Gy and then on day 196 with 11, 12 or 13 Gy.

Table 1

Mean group weights (g) from time of the 2nd irradiation on day 196. The table shows the mean weight per day, the plots the relative weight change. Groups of 6 mice received either no irradiation or 13 Gy when 10–12 weeks old, and then on day 196 a range of radiation doses. At the higher radiation doses the previously unirradiated mice lost more weight than those that had survived the prior irradiation, although since the starting weight was greater, the relative weight loss was less.

Radiation Doses	Day				Weight lost (g)
	196	197	198	199	200
0Gy, 0Gy	38.8	38.6	38.6	38.5	38.4
0Gy, 9Gy	41.1	40.6	39.7	38.6	37.2
0Gy, 10Gy	41.3	41.2	40.5	38.4	37.0
0Gy, 11Gy	40.8	40.3	39.3	37.5	35.7
0Gy, 12Gy	39.0	37.9	36.9	35.3	33.5
0Gy, 13Gy	39.6	38.6	36.4	35.0	32.7
13Gy, 0Gy	26.6	26.3	26.6	26.5	26.9
13Gy, 11Gy	28.5	27.4	26.2	25.5	24.3
13Gy, 12Gy	28.2	26.7	25.7	24.7	23.3
13Gy, 13Gy	26.2	25.3	24.5	23.6	22.2

Table 2

ANOVA summary for the different small intestinal regions comparing the responses following 0, 11, 12 or 13 Gy on day 196 with and without 13 Gy irradiation on day 0.

Comparison Day 0:0Gy vs. 13Gy. Unirradiated on Day 196	Difference Estimate	95% Lower CI	95% Upper CI	P value
Duodenum	37.8	29.5	46.1	<0.0001
Jejunum	19.4	11.5	27.3	<0.0001
Upper Ileum	19.4	11.3	27.6	<0.0001
Lower Ileum	17.6	9.5	25.7	<0.0001
Comparison Day 0:0Gy vs. 13Gy. Day 196:11Gy	Difference Estimate	95% Lower CI	95% Upper CI	P value
Duodenum	1.03	-6.1	8.14	0.7730
Jejunum	-0.86	-7.6	5.87	0.7977
Upper Ileum	-10	-17	-3.2	0.0053
Lower Ileum	-8.8	-16	-1.8	0.0146
Comparison Day 0:0Gy vs. 13Gy. Day 196:12Gy	Difference Estimate	95% Lower CI	95% Upper CI	P value
Duodenum	-6.2	-13	0.58	0.0723
Jejunum	-12	-19	-5.7	0.0004
Upper Ileum	-15	-21	-8.1	<0.0001
Lower Ileum	-5.8	-12	0.87	0.0870
Comparison Day 0:0Gy vs. 13Gy. Day 196:13Gy	Difference Estimate	95% Lower CI	95% Upper CI	P value
Duodenum	-5.7	-12	1.11	0.0992
Jejunum	-13	-20	-6.8	0.0002
Upper Ileum	-14	-21	-7.5	<0.0001
Lower Ileum	-8.0	-15	-1.0	0.0253

Table 3

Fold increase in corrected crypt number in each intestinal region relative to the day 196 control groups.

Radiation Dose (Gy)	Fold increase in crypt number compared to d196 control			
	Duodenum	Jejunum	Upper Ileum	Midcolon
11	1.5	1.2	1.6	1.7
12	2.1	2.2	2.4	1.8
13	3.0	3.5	3.5	3.0
				1.6