

Altered stem cell regeneration in irradiated intestinal crypts of senescent mice

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SUMMARY

Ageing is associated with a progressive deterioration in the functions of many organs within the body. In tissue with high cell turnover, the maintenance of the stem cells is of particular importance. Any accumulation of damage in stem cells may affect their function and hence threaten the homeostasis and regenerative capacity of the tissue. The small intestine represents a good model for the study of stem cells because of its spatial and hierarchical organisation. We have examined the effect of age on stem cell regenerative capacity after irradiation, using the microcolony assay. Crypt survival levels, the growth rate of surviving crypts, and the number of cells able to repopulate a crypt have been investigated by irradiating groups of 6-7 month old and 28-30 month old ICRFa male mice. After

high doses of irradiation, the surviving crypts in old mice were both smaller and fewer in number than in young mice. The growth rate of surviving crypts was determined by measuring the crypt area and the number of cells/crypt at various times after 14 Gy irradiation. There was a growth delay of between about one half and one day in the older mice. Surprisingly, the number of clonogenic cells per crypt was estimated to be greater in the older mice. These studies indicate important age-related alterations in the capacity to regenerate the crypts after radiation damage.

Key words: Ageing, Stem cell, Small intestine, Regeneration, Radiation

INTRODUCTION

The functional capacity of many organs within the body declines with age, probably due to intrinsic changes at the cellular level. Cells are continually exposed to various endogenous and exogenous stressors, such as oxygen radicals and heat, any of which can cause damage. To protect against damage, organisms have evolved potent cellular defence and repair mechanisms but these mechanisms are not perfect. Over time, damage can accumulate in cells and such damage is thought to underlie the ageing process (Kirkwood and Franceschi, 1992). On this hypothesis, understanding the cellular basis of ageing and longevity means understanding how the functional homeostasis of tissues is maintained during the life time of an organism and how these systems eventually break down.

We are interested in the extent to which tissue stem cells undergo functional alteration with age. Stem cells can be defined as undifferentiated cells capable of self-maintenance, which have the capacity to produce a large number of differentiated cells and to regenerate the tissue after injury (Potten and Loeffler, 1990; Potten et al., 1997). Regeneration is secured by a process of clonal expansion and hence these cells are sometimes called clonogenic stem cells. Stem cells

have a crucial role in the maintenance of functional homeostasis within a tissue, and any decline in their integrity will therefore have important long-term consequences. The extent to which ageing affects tissue stem cells is largely unknown. We hypothesise that ageing has important effects on the functions of tissue stem cells and that these effects are responsible for tissue alterations in old age.

The small intestinal epithelium is a good model for the investigation of stem cell functions because this tissue undergoes continuous renewal and the stem cells can be readily investigated because of their specific location. The epithelium is organised into hierarchical cell lineages derived from small numbers of undifferentiated stem cells, which are located around the fourth or fifth cell position as measured from the base of flask-shaped structures known as the crypts of Lieberkühn. The immediate daughter cells formed by stem cell division may acquire stem cell function under certain conditions, and are called potential stem cells or clonogenic cells (Potten and Loeffler, 1990; Potten et al., 1997). These produce cells that undergo rapid clonal expansion, migrating outwards and differentiating into mature cells on the surface of the villi (finger-like projections from the intestinal wall), where they are eventually shed into the lumen of the gut. The stem

cells are therefore fundamental to maintenance of the cell population of the intestinal wall, and it is significant that a small but steady level of apoptosis is seen at the stem cell position within the crypts. The spontaneous level of apoptosis can be markedly elevated by exposure to radiation or cytotoxic agents, which suggests that cell suicide may be an important system to maintain integrity of the stem cell population (Potten, 1992). We recently showed (Martin et al., 1998) that in old mice (29 months) the level of apoptosis following low-dose irradiation was increased twofold compared with young (5 months) and middle-aged (15-18 months) mice, suggesting that stem cells in the oldest mice may have accumulated some damage in spite of the protection afforded by stringent 'quality assurance' mechanisms. We also obtained striking changes in the histology of the distal ileum in old mice (Martin et al., 1998).

An important function of the intestinal stem cells is to regenerate the tissue after injury. Following high-dose ionising radiation, cells located at the base of the crypt either die rapidly via apoptosis or cease dividing temporarily or permanently. The fate of a crypt is determined by whether any stem cells or clonogenic cells repair any damage and survive, survival being inversely dose-dependent. Sterilised crypts (no surviving clonogenic cells) shrink in size due to cell death and migration of remaining cells onto the villi. Paneth cells at the base of a crypt are radioresistant and hence frequently mark the site of a sterilised crypt for several days. Crypts containing surviving clonogenic cells reform a structure resembling a crypt, with a lumen, Paneth cells at the base, and numerous dividing cells which first repopulate the crypt and then migrate onto the villus. The survival of the animal depends on the rate of crypt depopulation and the efficiency and number of surviving clonogenic cells capable of regenerating a crypt (Potten and Hendry, 1995).

The crypt regeneration process can be studied using a microcolony assay originally described by Withers and Elkind (1970). The technique involves detecting and counting surviving crypts on the fourth day after irradiation. At this time, surviving crypts can be readily detected, whereas sterilised crypts will have disappeared. Survival curves can then be generated by plotting crypt survival against dose. These curves are characterised on a semi-logarithmic plot by a shoulder up to around 9 Gy, beyond which the crypt survival fraction decreases exponentially with increasing dose (Potten and Hendry, 1985). The extent of the shoulder depends on two parameters; the number of clonogenic cells per crypt and their sensitivity to irradiation (intracellular repair capacity). Above 9 Gy, each colony can be regarded as a clone derived from a single cell. The number of clonogenic cells can be estimated by irradiating animals with either one or two equal doses separated by 4-5 hours, i.e. the time required to allow repair of the sublethal damage caused by the first irradiation (Hendry et al., 1992; Roberts et al., 1995). Using this split-dose approach, three stem cell populations have been suggested, characterised by their sensitivity to stress and their ability to repair damage. The first population comprises about 6 highly sensitive cells which are the actual stem cells. The second population comprises a further 6 clonogenic cells with lower sensitivity. The third comprises about 20 clonogenic cells with even greater resistance. If crypt survival is to occur, cells that are

normally destined to differentiate but which still possess some stem cell function must regenerate the crypt. The number of cells amenable to such recruitment decreases with the dose.

In the present study, we first investigated the ability of clonogenic cells from the small intestine to regenerate crypts after different doses of gamma- or X-irradiation in young and old mice. Surviving crypts were counted and measured in sections cut through the distal and proximal regions of the small bowel. Crypt survival curves were determined. We then determined the rates of growth of surviving crypts in young and old animals during the first few days following high dose irradiation. Lastly, the number of clonogenic cells was investigated using the split-dose technique.

MATERIALS AND METHODS

Experimental animals

Male C57/BL (ICRFa) mice were obtained from a well-characterised ageing mouse colony (Davies and Shofield, 1980). The colony was housed under conventional laboratory conditions with a 12 hours light/dark cycle (lights on at 8.00 am). Young mice were aged 6-7 months and old ones 29-30 months.

Irradiation procedure

Crypt survival curves

Unanaesthetized animals were exposed to whole-body irradiation with air being pumped into the chamber during exposure. A ^{137}Cs γ -ray irradiator was used with a dose-rate of 3.8 Gy per minute. All irradiation treatments were begun at between 09.00 and 10.00. Doses were 7.5, 10, 10.5, 12, 13, 14, 15 or 16 Gy and animals were killed 4 days after irradiation. 40 minutes prior to sacrifice, animals were injected with tritiated thymidine intra-peritoneally (^3H]TdR ip). Each crypt survival curve was determined in a single experiment using groups of 4-6 animals per dose.

Growth of surviving crypts

Mice were irradiated with 14 Gy X-irradiation at a dose rate of 0.52 Gy per minute. All irradiation treatments were begun at between 09.00 and 10.00. Unanaesthetized animals were held in individual Perspex tubes with head and thorax shielded (partial body irradiation). Groups of 4-6 animals were killed at 3, 3.5, 4, 4.5, and 5 days after irradiation. 6 hours 40 minutes and 40 minutes prior to sacrifice, the animals were injected with ^3H]TdR ip.

Split-dose procedure

Animals were exposed to whole-body irradiation ^{137}Cs γ -rays, as above. All irradiation treatments were begun at between 09.00 and 10.00. Groups of 4 animals were given either no irradiation, or a single dose of x Gy, or two doses of x Gy separated by 5 hours. Doses (x) were 7.5, 8, 10, 10.5 and 12 Gy. 5 days after irradiation, the animals were killed. 40 minutes before being killed, they were injected with ^3H]TdR ip.

Tissue preparation, sectioning, and histological procedures

Following sacrifice, the entire small intestine was removed and fixed in 10% formal saline overnight. A length of 25 cm from the appendix was removed for analysis, which corresponds to the ileum. This was then cut into 4 equal parts, of which the two middle parts were discarded, and the two end parts kept (proximal and distal ileum). The end parts were each cut again into five approximately 1 cm long segments. Each set of segments was bundled together in 3M micropore tape prior to paraffin embedding. After processing, the

segments were sectioned transversely at 3–5 μm and the sections were stained with haematoxylin and eosin. Autoradiographs were also prepared for measurement of labelling index (i.e. S phase cells that had taken up [^3H]TdR).

Scoring procedure

The parameters measured in each part of the ileum were the number of surviving crypts and the size of the crypts (width, height, area). A surviving crypt was defined as containing 10 or more adjacent, healthy-looking, chromophilic, non-Paneth cells, some Paneth cells, and a lumen. The circumference of a transverse cross section of the intestine was used as a unit of length. The number of crypts was counted in each circumference. Usually, there were 5 circumferences per mouse (proximal and distal) and 4 mice per experimental group. The width, height and area of the crypts were measured using a Zeiss AxioHOME computer driven microscope system. Fifteen crypts were measured for each mouse. The width was determined at the middle of each longitudinal crypt section. Height of crypts was measured from the bottom of the crypt to the crypt-villus junction. Area of the crypts was determined by tracing the outline of the crypts using the computer mouse which displays the outline on a VDU and superimposes it down the microscope. To determine the rate of growth of surviving crypts, the area of the surviving crypts and the number of labelled (three or more grains) and unlabelled cells per crypt section were determined. In both the dose-response and split-dose experiments, the width and numbers of surviving crypts were determined.

Data analysis

For the generation of the crypt survival curve, all data were corrected for crypt size changes following irradiation using previously established procedures (Potten et al., 1981; Potten and Hendry, 1985). Measured numbers of surviving crypts were multiplied by a factor t_0/t , where t_0 is the mean crypt width measured in longitudinal crypt sections in the control group, and t is the mean crypt width in the treated group. This allowed for the fact that smaller surviving crypts would be less likely to be detected in a given tissue section.

Standard multi-target functions (see Roberts, 1990) were fitted to each data set giving estimates of the conventional parameters D_0 (reciprocal of the slope of the exponential portion of the line, a measure of radiosensitivity) and N (the extrapolation number, obtained

by extrapolating the straight line portion of the curve to the survival axis at zero dose). The multi-target function has the form

$$\text{SF} = 1 - (1 - \exp(-D/D_0))^N,$$

where SF denotes the fraction of crypts surviving. Estimates of D_0 and N were obtained for each set of data using the DRFIT program (Roberts, 1990). Statistical comparisons between data sets were performed in DRFIT using a variance-ratio F-test.

Data on crypts per circumference and crypt size (area, width, height) were averaged to give a single figure for each measure in each region (proximal/distal) of the small intestine within each animal. Means and standard errors of the means for groups of animals were calculated on the basis of these figures. Significance was tested by Student's t -test.

Least squares linear regression analysis was applied to the log-transformed data to determine the growth rates (slopes) of surviving crypts. Appropriate terms were added to the model to estimate the magnitude of the apparent time delay in the growth of surviving crypts in old mice as compared with young mice (intercepts), and to test for differences in the slopes of the two groups (interaction term). Where the slopes were found to be significantly different, the time delay is not well-defined and the values computed represent an average over the time range studied. Growth rates were expressed in terms of the doubling time, T_d .

The numbers of clonogens were estimated from the crypt survival data as described previously (Hendry et al., 1992; Potten et al., 1987; Roberts et al., 1995). Mean clonogen numbers were computed on a logarithmic scale. Confidence intervals were computed and a matched-pair Wilcoxon test was used to demonstrate the overall difference in clonogen number between old and young mice, matching on dose.

RESULTS

Effects of high dose irradiation on crypt survival

In young animals (6–7 months), large expanding foci of regeneration could be seen in the small intestine. These foci possessed Paneth cells at the base and a lumen, like normal crypts. A strikingly different picture was seen in old (29 months) animals, where the regenerative crypts were smaller

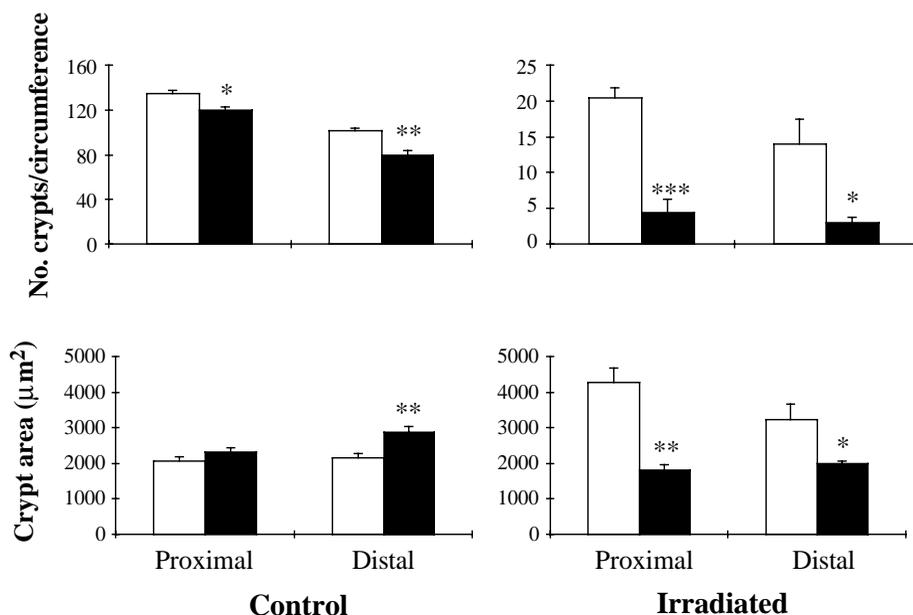


Fig. 1. Number of crypts per circumference in transverse cross-sections of the intestine and the area of the crypts in the distal and proximal ileum in young (white bars) and old (black bars). The standard errors of the mean are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

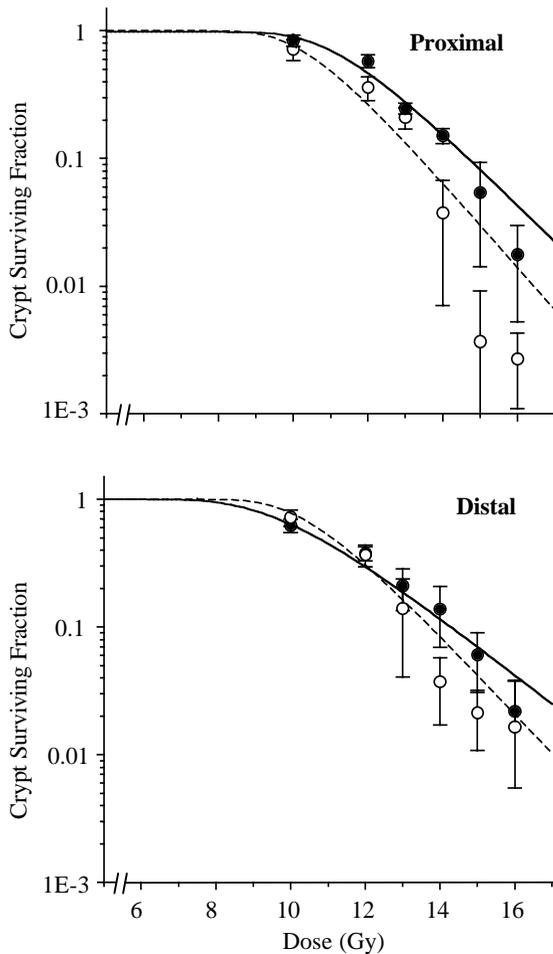


Fig. 2. Crypt survival curves obtained for the proximal and distal ileum from young (filled circles, full line) and old (open circles, broken line) mice 4 days after irradiation. The standard errors of the means are indicated.

and less chromophilic. This was a general observation (an example of which can be seen in Fig. 3). To quantify these differences, the sizes (area, width and height) and the numbers of the regenerative foci were measured within distal and proximal regions of control and irradiated (14 Gy) young and old animals. These data are plotted in Fig. 1.

Table 1. Estimates of parameters defining the survival curves

	No. of mice	Parameter	Proximal	Distal
Young	42	D_0 (Gy)	1.5±0.1	1.3±0.2
		N	1,928±896	194±113
Old	38	D_0 (Gy)	1.9±0.1	1.4±0.1
		N	3,133±2,019	1,924±1,445
Young-old		P	<0.001	0.014

D_0 (a measure of radiosensitivity) is the reciprocal of the slope of the exponential portion of the line. N (extrapolation number) is the zero-dose intercept on the crypt survival fraction axis. The standard errors of the estimates are indicated. The last row shows the difference (young-old) and its statistical significance (P).

Table 2. Estimates of growth rate of the surviving crypts in young and old mice and growth delay (old-young) after 14 Gy X-irradiation

		Crypt area	Labelled cells	Total cells
Growth rate	Young	0.94±0.06	1.41±0.11	1.29±0.09
	Old	0.91±0.10	1.50±0.18	1.70±0.19*
Growth delay (days)	Old-young	0.42±0.08**	0.74±0.10**	0.68±0.09**

Estimates are based on three measures: crypt area, number of labelled cells, and total number of cells. The standard errors of the estimates are indicated.
* $P<0.05$; ** $P<0.001$.

In control animals, an increase was seen with age in crypt size in both proximal and distal regions (statistically significant, $P=0.009$, in the distal region). The numbers of crypts per circumference decreased with age ($P=0.011$, proximal; $P<0.001$, distal). These observations confirmed previous results (Martin et al., 1998).

Four days after irradiation (14 Gy), the crypts in young mice were bigger than in controls ($P=0.02$, proximal; $P=0.056$, distal), whereas in old irradiated mice they were smaller than in controls ($P=0.04$, proximal; $P=0.01$, distal). Crypt areas after irradiation were significantly bigger in young than in old mice ($P<0.001$, proximal; $P=0.031$, distal).

The numbers of surviving crypts following irradiation were lower in the old mice than in the young in both regions ($P<0.001$, proximal; $P=0.022$, distal).

Fig. 2 shows crypt survival curves (crypt surviving fraction) as a function of radiation dose in the proximal and distal small intestine measured on day 4 after whole body γ -irradiation. The fitted D_0 and N values are summarised in Table 1. The differences between young and old animals were highly significant ($P=0.014$, distal; $P<0.001$, proximal).

Growth of surviving crypts

To investigate the growth rate of the surviving crypts, groups of mice were sacrificed at different times after 14 Gy partial body irradiation. Fig. 3 shows sections from the proximal ileum from young and old mice taken at various times after irradiation. When young and old mice were compared, a difference in the size of the surviving crypts was observed (see above). Old mice had smaller surviving crypts than young animals at all of the observed times after irradiation.

The crypt area and the total number of labelled and unlabelled cells were measured. The growth rates are expressed as rates of increase in crypt area, in the total number of cells

Table 3. Estimates of clonogen number for the proximal and distal region of the ileum

	No. of mice	Low doses	No. of mice	High doses
Young	10	7.9±2.2	15	19.1±2.3
Old	6	11.7±3.3	14	164±51

Means were computed on a logarithmic scale. The low doses ranged from 7.5 to 8 Gy, and the high doses ranged from 10 to 12 Gy. The standard errors of the estimates are indicated.

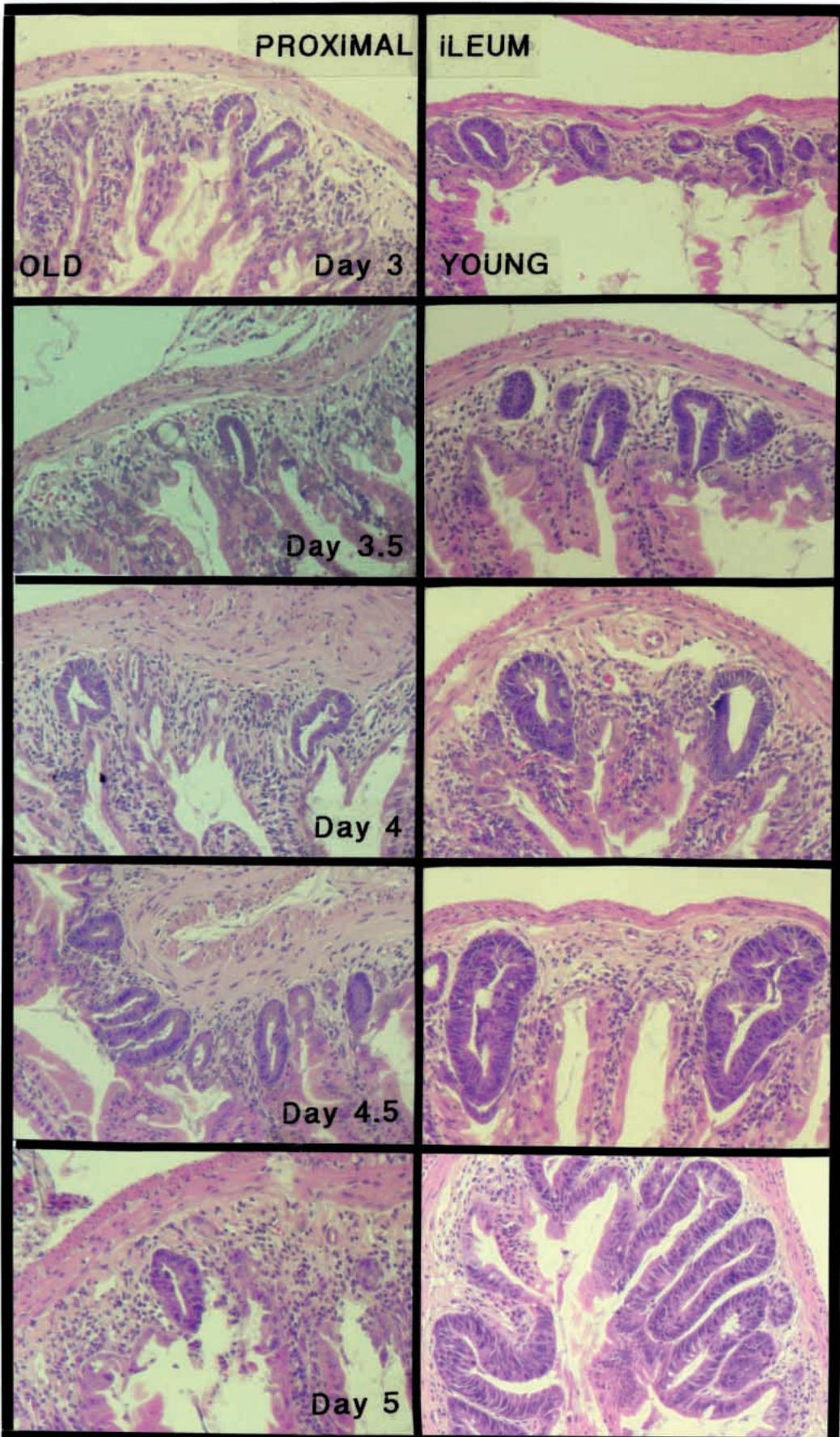


Fig. 3. Photomicrographs from the proximal small intestine of young and old mice at various times (3-5 days) after a 14 Gy partial body X-ray irradiation. Young mice (right hand panels) and old mice (left hand panels). Surviving crypts are the intensely-stained bodies. $\times 700$.

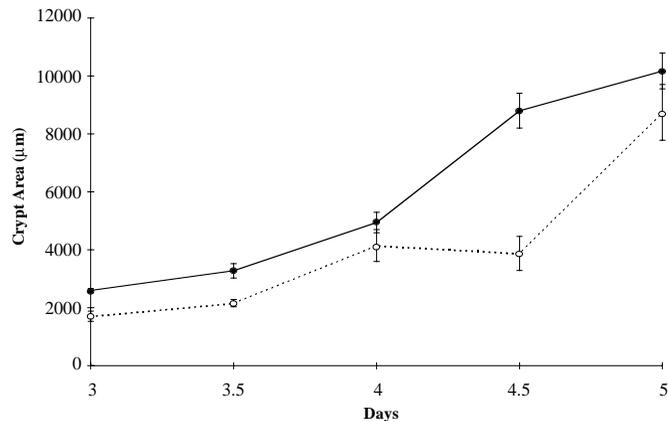


Fig. 4. Crypt growth curves obtained from pooled data of the proximal and distal ileum young (filled circles, full line) and old (open circles, broken line) mice 3-5 days after irradiation. The standard errors of the estimates are indicated.

per crypt, and in the number of labelled cells (Table 2). Data from both regions (proximal and distal) of the small intestine were similar and have been pooled. Growth rate did not differ significantly between old and young animals, except for total cells ($P=0.029$). However, for all three measures there was a highly significant ($P<0.001$) growth delay of between 0.4 and 0.7 days in the old mice compared to the young. This growth delay is evident in the growth curve shown in Fig. 4.

Number of clonogens

Table 3 shows the estimated number of clonogenic cells for low and high irradiation doses obtained by pooling data into two dose groups: low (<9 Gy) and high (>9 Gy). Data from the distal and proximal regions of the small intestine were similar and have been pooled. The estimated number of clonogens in young mice are consistent with previous data obtained in young (10-12 weeks) mice of another strain (BDF1) (Hendry et al., 1992; Roberts and Potten, 1994). In both young and old mice, the estimated number of clonogens increased with radiation dose. The estimated clonogen number was also higher in old mice than in young mice. This effect was most pronounced in old mice after high dose irradiation ($P<0.001$).

DISCUSSION

Regenerative foci could be easily detected four days after irradiation in the small intestine of young (6-7 months) mice. These foci were larger than the crypts in unirradiated tissue. The number of surviving crypts remained roughly constant as dose increased until about 9 Gy, and then decreased in a dose-dependent manner. Above 9 Gy, which defines a shoulder in the crypt survival curve, it is believed that each surviving crypt is usually regenerated from only a single clonogenic cell, and is therefore a clone (Potten and Hendry, 1995). As radiation dose increases, the number of surviving crypts declines because more of the clonogenic cells are killed.

We found that the crypt survival curve differed markedly between young and old mice in terms of both the slope ($1/D_0$) and the extrapolation number (N). The extrapolation number

was greater in old mice, compared to young mice, in both proximal and distal regions of the small intestine which is compatible with the increase in the estimated number of clonogenic cells (Table 3). This difference in the shape of the crypt survival curve indicates an age-related alteration in the radiosensitivity of crypt cells. Following high doses of irradiation (>12 Gy), the surviving crypts in old mice were not only fewer but also smaller than in young mice, suggesting an alteration in the proliferative capacity (regenerative potential) of the clonogenic cells with age.

The growth rate of surviving crypts was examined after partial body X-irradiation of the abdomen. Soon after irradiation, crypts shrink due to cell death and migration onto the villi. With further passage of time, surviving crypts grow and enlarge while sterilised crypts disappear. The advantage of partial-body irradiation for these studies was the enhanced survival of the animals, compared to whole-body irradiation. Survival for several days was necessary in order to observe the time course of regeneration. Previously it has been shown that γ - and X-irradiation result in similar crypt survival curves (Cai et al., 1997a), allowing valid comparison of data from the two phases of the current work.

We examined crypt regeneration from day 3 to day 5 after irradiation. Before day 3, it was impossible to distinguish between surviving and sterilised crypts, especially in old mice, but by day 3, small surviving crypts could be detected and measured. Beyond day 6, the animals did not survive. Three measures of the growth of surviving crypts were used: crypt area, number of labelled cells, and total number of cells. There was no marked difference in growth rate of the surviving crypts in young and old mice. However, growth was consistently delayed by about half to one day in the old mice.

Our observations suggest that the altered crypt regeneration is at the level of the triggering of the regenerative response rather than in the growth itself in old mice. Alterations in the control of proliferation in both small and large bowel have been reported by some authors (Fry et al., 1961, 1962; Leshner et al., 1961; Leshner, 1966). These alterations are likely to be the result of progressive accumulation of damage occurring with age which may affect regulation of proliferation, migration and differentiation of crypt cells and their interactions with neighbouring cells (junctional proteins) and the cellular microenvironment. This prediction is supported by the increase in chromosomal abnormalities in crypt cells of aged rats (Ellsworth and Schimke, 1990).

Our estimates of clonogen numbers in young mice showed a dependence on the magnitude of the radiation dose (see Table 3), consistent with previous data (Cai et al., 1997b; Hendry et al., 1992; Roberts et al., 1995; Roberts and Potten, 1994). The results found here using 5-7 months old C57/BL (ICRFa) mice were similar to those obtained previously using 10-12 weeks old BDF1 mice (see references above). However, a striking effect of age was seen in the estimates of clonogen numbers. In old mice at high doses, the estimated clonogen numbers were some eightfold higher than in young mice at similar doses, in fact the number of clonogens may be approximately equal to the total number of proliferative cells per crypt. However, these numbers should be viewed with some caution since the microcolony assay is subject to certain assumptions and the clonogen estimates have large error limits.

These data suggest that in old mice cells from higher up the

crypt lineage may be recruited into the clonogenic compartment following damage by ionising radiation, possibly signifying some age-related deterioration in the cellular organisation of the crypts. These extra recruited cells may, however, be less efficient clonogens, thus explaining the delayed regenerative response which we have observed. An expanded proliferative zone has been observed in colonic crypts in aged rats (Holt and Yeh, 1989) and in humans (Deshner et al., 1988; Paganelli et al., 1990; Roncucci et al., 1988) which is compatible with our hypothesis. In the small intestine, the refeeding of starved rats resulted in similar cellular responses in young and senescent rats but showed a broadening of the proliferative zone within the crypts in old animals (Holt et al., 1988).

Cell death (apoptosis) might be an important signal for the recruitment of clonogens (Potten, 1992). Altered levels of apoptosis in old mice might explain the elevated number of clonogens. Experiments using p53 knockout ($-/-$) mice showed increased resistance to radiation-induced apoptosis (Merritt et al., 1994) and a reduced value of clonogens per crypt compared to controls (J. H. Hendry et al., personal communication). In contrast, old mice show increased levels of low dose radiation-induced apoptosis (Martin et al., 1998) and elevated numbers of clonogens. This process may be amplified if successive waves of apoptosis occur after irradiation. Shortly after irradiation, a first wave of apoptosis occurs which may trigger surviving clonogens to start proliferation. However, several of these will already be damaged and may undergo apoptosis rather than continuing to proliferate. The second wave of cell death may provoke further activation of potential clonogens.

In conclusion, our study has shown that marked age-related changes occur in the capacity of the stem cells to regenerate the intestinal epithelium after injury. The clonogenic cells in old mice appear to be more radiosensitive than in young mice, resulting in a decreasing number of surviving crypts. The growth of the surviving crypts after high doses of irradiation was delayed in old mice compared to young, in spite of the fact that many more clonogenic cells were susceptible to recruitment in the older animals. These data indicate the suitability of intestinal stem cells as a model system for investigating the complex changes that underlie the ageing phenotype, and they suggest that further detailed studies on the time course and mechanisms (including the genetic regulation) of cell proliferation and death in old and young animals following damage by irradiation will be revealing. Experiments are planned or underway to investigate whether the damage recognition and response mechanisms may be defective (i.e. alterations in the competence of p53/p21 gene action) and the relative importance of oxidative damage and mitochondrial defects in the deterioration of intestinal stem cell function.

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