Intestinal stem cells protect their genome by selective segregation of template DNA strands

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Summary

The stem cells in the crypts of the small intestinal mucosa divide about a thousand times during the lifespan of a laboratory mouse, and yet they show little evidence of any decline in proliferative potential and rarely develop carcinogenic mutations, suggesting that their genome is extremely well protected. Protection against DNA-replication-induced errors can be achieved by the selective sorting of old (template) and new DNA strands with all template strands retained in the stem cell line. The template strands in the stem cells can be labelled during development or during tissue regeneration using tritiated thymidine (^{3}HTdR). Labelling newly synthesised strands with a different marker (bromodeoxyuridine, BrdUrd) allows segregation of the two markers to be studied. Template strand label is retained (^{3}HTdR), whereas label in the newly synthesised strands (BrdUrd) is lost following the second division of the stem cell. Random errors may occur in the template strands owing to environmental elements. These are protected against by the altruistic cell suicide (apoptosis) of the cells incurring such errors. A final level of protection for the tissue compensates for excessive deletion of stem cells via the apoptosis pathway. This is achieved by a hierarchical age structure in the stem cell compartment, with some cells being able to efficiently repair DNA damage and hence being more radioresistant. The presence of these protective mechanisms ensures that the small intestine rarely develops cancer and that stem cells can sustain the extensive cell proliferation needed during life.

Key words: Stem cells, DNA strand segregation, Genome protection, Intestine

Introduction

It is generally accepted that stem cells in the basal layer of interfollicular epidermis and some in the hair follicles can be marked by tritiated thymidine (^{3}HTdR) administered by multiple applications during a critical stage in the development of skin (Bickenbach, 1981; Morris et al., 1985; Cotsarelis et al., 1990). Specifically, ^{3}HTdR given as a series of injections shortly after birth in mice results in the labelling of the DNA of most of the cells in the basal layer of the epidermis. Skin samples taken from these mice when they are between 8 and 10 weeks old (young adults) contain a few basal cells in the epidermis and some cells in the upper outer root sheath of the hair follicles that retained the ^{3}HTdR label (as seen in autoradiographs). In the epidermis, these label-retaining cells (LRC) are located in the centre of the epidermal proliferative units (EPUs) (Morris et al., 1985), the location of the putative stem cells (Potten, 1974; Potten, 1981). Cells at the same position also retain radiolabelled skin carcinogens (Morris et al., 1986) and can be stimulated into division by skin injury, and in short term cultures they divide to form small colonies (Morris and Potten, 1994). These results are consistent with the hypothesis that the LRCs are the interfollicular EPU stem cells and, in the hair follicle outer root sheath or bulge region, are either the hair follicle stem cells and/or a potent reserve skin stem cell population. Surprisingly, the mechanisms underlying the establishment of LRCs has never been adequately explained. The conventional explanation is that the cells retain label because they are dividing significantly slower than the dividing transit population in the epidermis, which rapidly dilutes its DNA label by random DNA strand segregation. However, the evidence would suggest that in adult mice, the stem cell population has a cycle time of about 8 days (Potten et al., 1984), a cycle time about twice as long as that for the dividing transit population. Thus, in an 8 week old mouse these cells should have divided at least seven times; probably considerably more since their cycle time during early skin development would be expected to be considerably shorter than 8 days. Since each time a cell divides the radioactivity (label) is assumed to halve, any labelling will be reduced to background levels within four divisions. As a result, other explanations for the label retention must apply and one of the simplest explanations is that these cells do not randomly segregate their DNA but selectively retain some radiolabelled DNA strands. A hypothesis to explain this was presented by Cairns, who suggested that the selective repeated retention of the ‘old’ or template strands in the stem cell population would be an effective means of protecting against DNA-replication-induced errors in somatic stem cells (Cairns, 1975). This hypothesis has major implications for mutagenesis and cancer risk (J. Cairns, personal communication). The template strands could be labelled during development or during tissue regeneration as new stem cells are made (Cairns, 1975; Potten et al., 1978). At subsequent divisions of the stem cells, the newly synthesised strands, with any potential replication-

induced errors, would then be passed to the daughter cell destined to enter the dividing transit population and be lost from the tissue within a few days. This has turned out to be a difficult hypothesis to prove or reject convincingly.

In the label-retaining experiments in skin, stem cell template strands would become labelled if 3HTdR was administered over a time span that covers the penultimate symmetric stem cell expansionary cell divisions associated with skin development. At this time, new stem cells and new template strands would be formed and are, therefore, capable of becoming permanently labelled. In experiments performed in our laboratory in 1978, we demonstrated that, following radiation injury that stimulates stem cell amplification, in the small intestinal crypts, where new template strands would also be synthesised, some LRCs could be observed at the specific position in the crypts for the stem cells (Potten et al., 1978).

In the same paper (Potten et al., 1978), we produced evidence that in the dorsal epithelium of the tongue of the mouse, a tissue where the stem cells are located at a specific position beneath tongue proliferative units, the stem cells could be pulse labelled with ^{3}HTdR with a high frequency if several doses were given around 3 am, because of the extremely strong circadian rhythm (Hume and Potten, 1976). These stem cells were believed to have a cycle time of 24 hours, and after 48 hours (two divisions) some cells at the stem cell location apparently became completely unlabelled (non-radioactive). This observation is consistent with the stem cells having their new strands of DNA labelled, and these newly synthesised strands would then pass to the dividing transit daughter. Two cell divisions would be required for the stem cells to ‘clean themselves’ of radioactivity (Fig. 1).

The crypts of the small intestine of the mouse have been well studied and characterised in terms of cell proliferation and cellular organisation. Each crypt in the steady state adult contains about 250 cells and is composed of between four and six cell lineages, each with a lineage ancestral stem cell. These cells are believed to divide once a day and produce lineages containing six to seven dividing transit generations, and during the course of this amplification up to about four distinct differentiated cell phenotypes can be produced. It is believed that the ultimate commitment to differentiation only occurs at the level of about the third generation in the lineage. Thus the crypt contains a hierarchy of stem cells with four to six ultimate stem cells that are responsible for all the day to day cell replacement and a population of reserve potential stem cells in the first, second and third transit generation that are capable of repopulating the crypt if the ultimate stem cells are damaged or deleted (Potten et al., 1997; Potten, 1998). No marker exists for these early generation cells, but the crypt represents an elegant cell biological model system since the stem cells have a strict spatial distribution along the crypt-villus axis, with the ultimate stem cells being located in an annulus of cells about four cell positions from the base of the crypt, immediately above one of the differentiated lineages, the Paneth cells, which are located at the crypt base (Potten et al., 1997; Potten, 1998) (Fig. 2). The ultimate stem cells appear to have an exquisite radiosensitivity such that a single hit anywhere in their DNA molecule can trigger an altruistic apoptotic cell deletion (Potten, 1997; Hendry et al., 1982; Potten and Grant, 1998; Potten et al., 1992). These cells do not appear to have the option to repair DNA damage but rather commit suicide, which

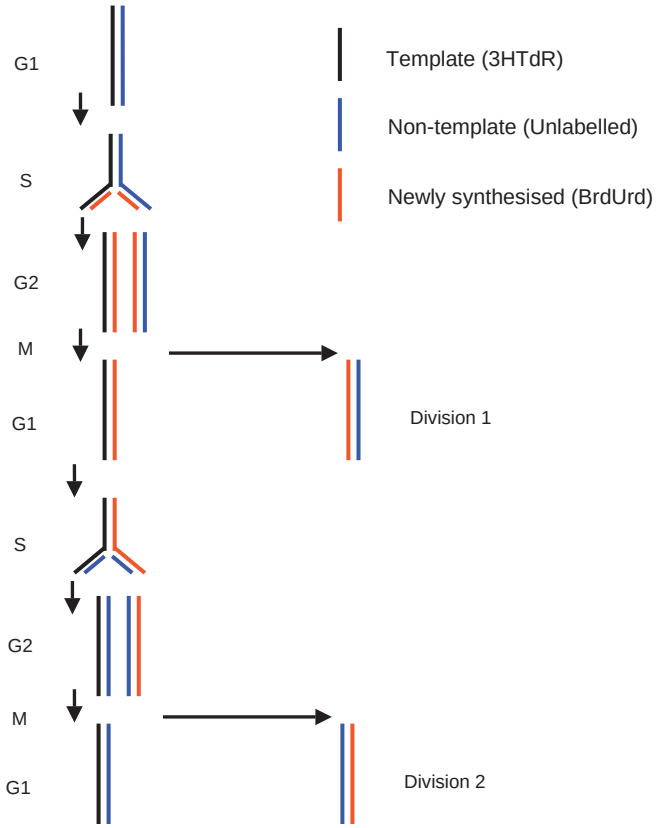

Fig. 1. A diagram showing the segregation of template and newly synthesised DNA strands in one chromosome, according to the Cairns’ hypothesis (1975), which proposes that all the chromosomes would behave in this way. The template strands are selectively retained by the stem cell daughter of a cell division, whereas the newly synthesised strands are segregated to the daughter cell destined to enter the dividing transit compartment and be shed from the tissue after a few days, thus removing any replication-induced errors. Label introduced into the newly synthesised strands takes two divisions to be removed from the stem cells. Label in the template strand would persist in the stem cell line.

can be easily compensated for by division of either undamaged neighbouring ultimate stem cells or cells in the much more resistant and repair-efficient potential stem cell compartment (Fig. 2). Although stem-cell-specific markers have not been described as yet, the possible ability of these cells to retain ^{3}HTdR label, if given under appropriate conditions, does suggest one way in which stem cells could be marked. Other possible approaches are also being developed (e.g. using an antibody (C.S.P and H. Okano, unpublished) to the RNA-binding protein Musashi-1 (Nakamura et al., 1994), which will greatly facilitate stem cell studies).

Attempts have been made previously (Potten et al., 1978) to label template strands in the ultimate stem cells of the small intestinal crypt (i.e. create LRCs as in the skin). This can be achieved at the times when new stem cells and new template strands are being produced, either during gut development or following injury and destruction of the ultimate stem cell population (e.g. by radiation) and the re-establishment of the crypt lineage from the potential stem cell compartment. A dose of 8 Gy of Cs 137 γ radiation to an adult (10 to 12 week old)

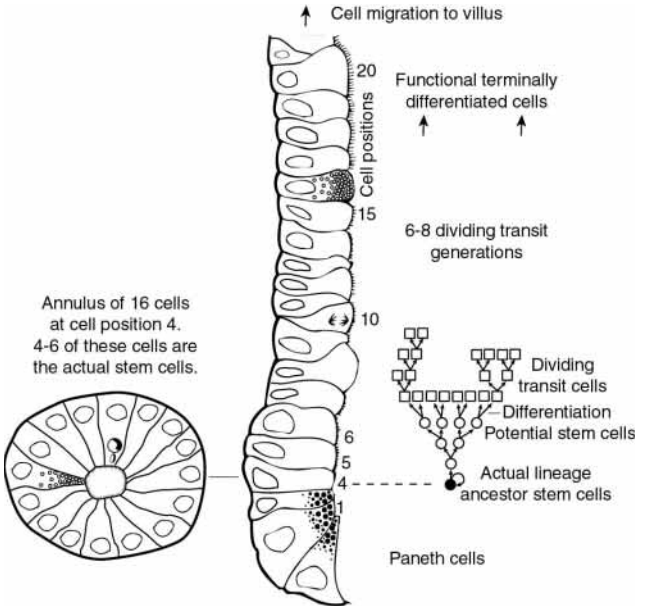

Fig. 2. A diagram showing the histological appearance of a longitudinal section through a crypt of the small intestine. Paneth cells (with apical granules) are located at the base of the flask-shaped structure, and there are between 15 and 20 proliferating cells arranged along the long axis. Circumferentially, at each position above about the fourth position from the bottom, there are about 16 cells per annulus. Also shown is the current model (on the right) for the proliferative organisation in the crypt. This consists of between four and six cell lineages, each with a self-maintaining lineage ancestor stem cell (closed circle) located within the annulus of 16 cells at about cell position four from the base. It is upon these actual stem cells that the entire cell production process is ultimately dependent in steady state conditions, and in the absence of damage or injury to the tissue these cells maintain cell production for the lifetime of the animal. If, however, some or all of these actual stem cells are killed, the next two to three generations within the cell lineage (open circles) retain the ability to repopulate the stem cell compartment and the entire cell lineage, that is, they retain the ability to function as stem cells (indicated as circles) and are hence potential stem cells, which are normally within the transit population in steady state conditions but are as yet uncommitted to differentiation. Once the commitment to differentiation has been made, the cells retain no stem cell capabilities, but do retain the ability to divide and differentiate further, that is, they are part of the dividing transit population (open squares). A further four to six divisions occur within the dividing transit population, during which differentiation into the enteroendocrine, Paneth, goblet and enterocyte lineages may occur. The other cells shown are: a goblet cell at position 16 and a mitotic cell at cell position 10. An apoptotic fragment in the crypt is shown in cross section.

BDF1 mouse results in the killing or reproductive sterilisation of all but about one of the potential stem cells (Potten and Hendry, 1985). This single surviving stem cell repopulates the crypt via a clonal expansion and hence is sometimes referred to as a clonogenic cell. During the course of this clonal expansion, it re-establishes the entire stem cell lineage. This process takes 2 to 3 days (Potten and Hendry, 1985). Our earlier work (Potten et al., 1978) showed that ^{3}HTdR administered repeatedly (every 6 hours) over the first 48 hours of this post-irradiation period resulted in regenerated crypts with all the cells labelled by the 48 hour time point. If, however, time was allowed to elapse, for example, for a further week beyond the 2 day labelling time, a total of about four to eight LRCs per crypt were observed to be distributed around the fourth position from the bottom of the crypt. During this week after labelling, the stem cells would have divided at least eight times, but probably more like 12 times, since their cycle time soon after irradiation is shorter. Thus, the stem cells should have diluted their label to sub-threshold levels, but they in fact retained abundant label (Potten et al., 1978). This observation is consistent with, but does not prove, the Cairns' selective DNA segregation hypothesis.

Materials and Methods

BDF1 mice housed under conventional conditions and of differing ages, depending on the experiment, were used for these experiments. All experiments were performed according to the Animals (Scientific Procedures) Act 1986 UK. Tritiated thymidine (^{3}HTdR) was used to label template strands of DNA, whereas Bromodeoxyuridine (BrdUrd) was used to label newly synthesised strands (see below).

In order to generate template strand labelling, the principles that have been applied for label-retention studies in the skin have been adopted and modified (Bickenbach, 1981; Morris et al., 1985; Cotsarelis et al., 1990). Two distinct protocols were adopted in order to label DNA strands in stem cells at a time when new stem cells are being made, either during development or during post-irradiation regeneration of the tissue. The protocols are outlined in Fig. 3.

Labelling during gut development (template labelling 1)

The first of the labellings (i.e. during development) involved labelling juvenile animals with twice daily injections of ^{3}HTdR (at 9 am and 9 pm) for three consecutive days. The doses of ^{3}HTdR varied depending on the age (and hence, size) of the animals. Each dose was 15 μC for animals aged between 11 and 21 days post-natum, 25 μC per injection for mice 21 to 37 days of age and 50 μC for mice older than 37 days. All animals were sacrificed at 11 weeks of age, when the small intestinal tissue was fixed (Carnoy's), sections prepared (haematoxylin and eosin staining) and autoradiography undertaken (K5 emulsion). The exposure time varied depending on the nature of the specific experiment but was generally 14 days. LRCs were counted in transverse sections of the intestine and expressed as the number of LRCs per intestinal circumference (Potten and Hendry, 1985), having excluded from the counts any labelled nuclei associated with the differentiated Paneth cell population. The threshold for detecting LRCs was set at five or more grains per nucleus. The experiment was repeated more than once, and four to six animals were used per experimental group, and up to 10 intestinal circumferences were counted per animal.

Labelling during post-irradiation crypt regeneration (template labelling 2)

The second labelling protocol for LRCs (i.e. during regeneration) involved irradiating 12 week old BDF1 mice with a dose of 8 Gy of Cs^{137} γ rays to the whole body (3.5 Gy/minute). Then during the crypt regeneration phase, which takes 2-3 days, ^{3}HTdR at a dose of 25 μC per injection was given every 6 hours for the first 48 hours post-irradiation. The animals were then left for a period of 8 days, during which the label present in the majority of the proliferating cells diluted to sub-threshold levels, leaving only LRCs present in the crypt.

Double labelling of LRCs (new strand labelling)

A second series of experiments was then conducted that was designed to label newly synthesised strands with BrdUrd in LRCs present at 11

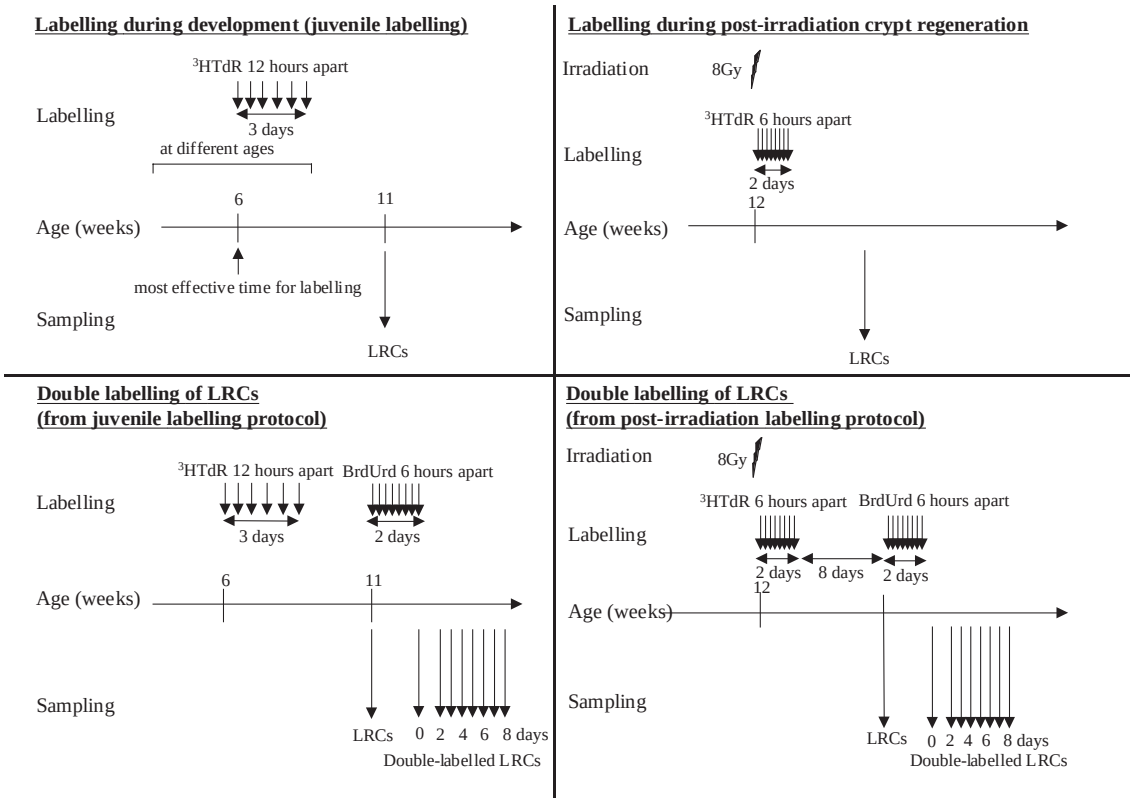

Fig. 3. A schematic outline of the experimental procedures.

weeks of age using the juvenile labelling protocol and at 8 days post-irradiation using the post-irradiation protocol. The BrdUrd labelling schedule involved injections of 1 mg in 0.1 ml saline, with injections being delivered every 6 hours for 48 hours. Samples were then taken immediately after the end of the BrdUrd labelling (40 minutes) and 2 to 10 days post-BrdUrd labelling (Fig 3). In these experiments the proportion of double-labelled LRCs was determined on a cell positional basis (see below) over time to determine whether the ^{3}HTdR and BrdUrd labels behaved in the same fashion (diluted at the same rates).

Sections were dewaxed, rehydrated and the endogenous peroxidase was inactivated with 1% hydrogen peroxide in methanol for 30 minutes. DNA was denatured to single strands by immersing sections in 1M HCl at 60°C for 8 minutes and then neutralising in boric acid buffer for 6 minutes at room temperature. Sections were blocked using normal rabbit serum 1/20 for 30 minutes prior to the application of the anti-bromodeoxyuridine antibody (MAS 250b; Sera Labs, Crawley Down, Sussex, UK) diluted 1/5 for 1 hour. After washing in PBS, a peroxidase-conjugated rabbit anti-rat secondary antibody was applied at a dilution of 1/100 in 10% normal mouse seum for 1 hour. Sections were again washed in PBS and developed using 3,3 Diaminobenzidine (DAB). The sections were washed in deionised water overnight prior to autoradiography being performed. All the sections were counterstained with thionin prior to examination.

Scoring

All LRC scoring at the level of the crypts and all double-labelled LRC scores were made on a cell positional basis, along the long axis of longitudinally sectioned crypts. In this way a frequency plot of the labelling pattern against cell position could be generated, and the labelling characteristics at the stem cell position (cell position four) could be assessed. Details of the gut bundling procedure that ensures good transverse sections of the intestine and hence longitudinal sections of crypts have been previously published (Potten and Hendry, 1985). Details of cell positional scoring have also been published previously (Potten et al., 1997; Potten, 1998; Potten and Grant, 1998).

Results and Discussion

We have undertaken further experiments labelling juvenile mice to determine the optimum time during gut development for the establishment of LRCs (Fig. 4). ^{3}HTdR delivered over a period of 11-21 days post-natum results in about 1.5 LRCs per intestinal circumference [see (Potten and Hendry, 1985) for intestinal circumference techniques], having excluded from these counts labelled Paneth cells. Paneth cells have a slow turnover of 2-3 weeks and, hence, a few become labelled and retain label for longer than the bulk of the crypt population. For injections given between 21 and 37 days after birth, a rapid increase in the number of non-Paneth LRCs per circumference is observed, with a peak when the tritiated thymidine is given at about 23 days after birth. This peak may be associated with the establishment of a changed physiology in the gut associated with weaning. If the labelling protocol of ^{3}HTdR given twice daily for three days is initiated over a period from about 37 to 57 days post-natum, a constant value of about 5.5 non-Paneth LRCs per circumference is obtained in 11 week old animals, 3.5 times more than was obtained by labelling at the earlier times. This represents about 6.5% of the crypts in an intestinal circumference (about 80) containing one or more LRCs. These cells are clearly retaining label for 3 to 5 weeks in spite of steady state cell replacement and the expected 20 to 35 rounds of cell division (on the assumption of a daily cell cycle).

Fig. 5 shows that the peak of the labelling index, about 4%, occurs at cell position four for the LRCs. The fourth cell position from the base represents the presumed positioned of

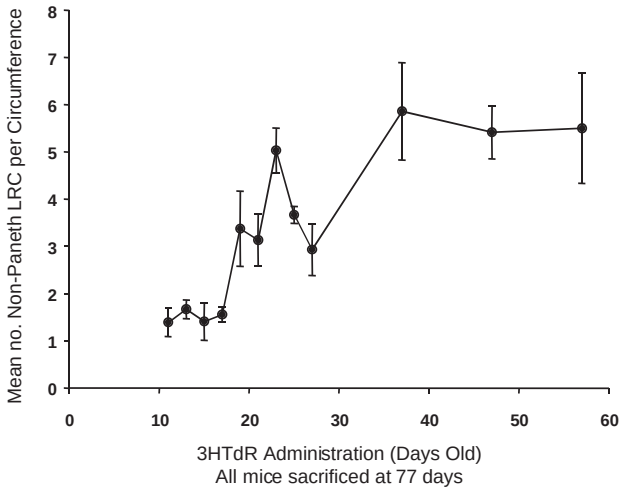

Fig. 4. The results of an experiment where twice daily (9 am and 9 pm) injections of tritiated thymidine were given for three consecutive days (Fig. 3). All mice were then sacrificed in groups of four to six when they were 11 weeks old. The number of non-Paneth LRCs per small intestinal transverse section (circumference) was determined per mouse, and the mean values from the four to six mice per group ± standard errors are shown. For mice less than 20 days old, the number of non-Paneth LRC per circumference in the 11 week old animals was about 1.5 per circumference. If ^{3}HTdR doses are given between about 37 and 57 days, a fairly constant number (about 5.5) of non-Paneth LRCs per circumference was obtained. There were about 84 crypts per circumference in these adult animals and, hence, there are about 6.5% of the crypts in a circumference that contain one or more non-Paneth LRCs. The threshold for detecting a labelled cell was set at five or more grains.

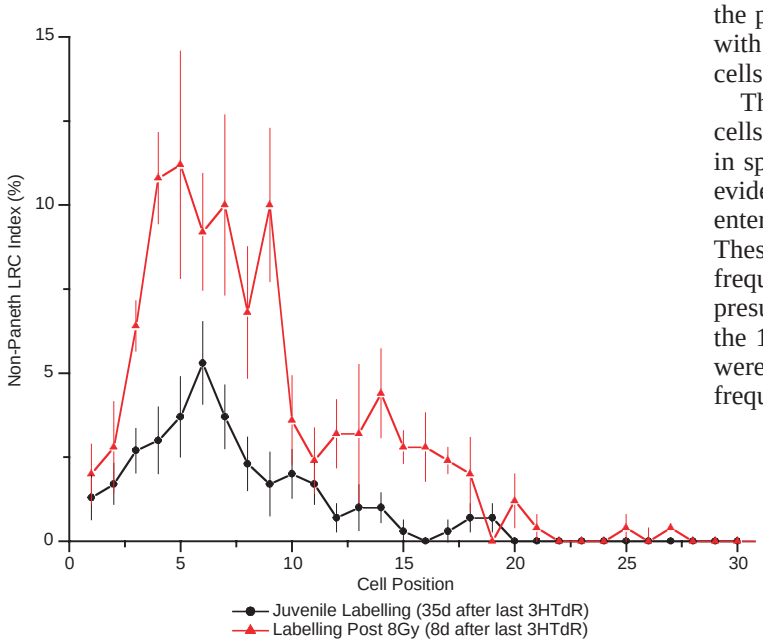

Fig. 5. The distribution of non-Paneth LRCs along the crypt axis [a cell positional analysis; see (Potten et al., 1997; Potten et al., 1992; Potten, 1998; Potten and Grant, 1998) for details] is shown for animals labelled with ^{3}HTdR at 42 days (solid line) and for animals labelled during the crypt regenerative phase following a dose of 8 Gy of gamma rays (red line). Both cell position distributions show peak values at around cell position four-five, with a fairly broad distribution and a long tail to the right. Standard error limits are shown.

the stem cells (Fig. 2) [see (Potten et al., 1997; Potten, 1998] for the cell positional scoring technique]. The labelling index distribution is quite broad, with a long tail to the right. The low percentage of LRCs at cell position four, the stem cell position, is a consequence of (a) the low frequency of ultimate stem cells per crypt (about four to six are distributed between cell positions three and seven), (b) probable sub-optimal labelling for these LRCs possibly because of highly variable cell cycle times and/or (c) the possibility that in the 3-5 week interval that some stem cells still retain a higher than 50% self-maintenance probability, that is, they are still making new stem cells, randomly segregating their DNA and making new template strands and, hence, diluting their label. The actual data are presented with standard error limits rather than our usual presentation of a smoothed cell positional frequency plot.

An alternative strategy for generating LRCs in the crypt is to use the post-irradiation regeneration process described for experiments conducted in 1978 (Potten et al., 1978). Such experiments have been repeated here using a dose of 8 Gy γ-radiation delivered to 12 week old BDF-1 mice and a labelling protocol of 25 μCi of ^{3}HTdR delivered every 6 hours between 6 and 48 hours post-irradiation. Since cell cycle times in this situation are greatly shortened compared with the steady state, this protocol labels virtually all the cells in the crypt, including the regenerated ultimate stem cell population. Eight days following the last ^{3}HTdR injections, clearly identified LRCs are apparent in the crypt. The autoradiographic background is higher than usual for pulse labelling experiments because virtually all cells contain some residual radioactivity resulting from the grain dilution process following cell division. Nevertheless the LRCs are clearly discernible (Fig. 6). The cell positional distribution for these LRCs is shown in Fig. 5, and the peak yield of about 10% is also seen at cell position four with the same long tail to the right. Label is retained in these cells in spite of up to 12 rounds of cell division.

Thus, it is relatively easy to perform experiments to generate cells in the small intestinal crypt that retain the ^{3}HTdR label in spite of numerous assumed rounds of division. To provide evidence that these cells progress through the cell cycle and enter mitosis is something of a ‘needle in a haystack’ task. These LRCs are distributed in the crypt with their highest frequency at the positions along the crypt axis, which is presumed to be the location for the stem cells. If four out of the 16 cells in the crypt annulus of cells at cell position four were labelled stem cells, a cell position four labelling frequency (LI) of 25% might be expected. However, since cell position four is the average position for the stem cells and their actual location may be spread between cell positions three and seven, a lower frequency and a tail to the right of the LI distribution would be expected.

Stem cells can currently only be studied using functional tests; however there are data that suggest that one property of the ultimate stem cells is an exquisite sensitivity to DNA damage and, hence, sensitivity to radiation (Potten, 1977; Potten and Grant, 1998). A low dose of 1 Gy of γ radiation induces a rapid (within 3-6 hours) apoptotic response in four to six cells at cell position four, the stem cell position, and these apoptotic cells have a cell-positional distribution similar to the theoretical stem cell distribution and also to the distribution of LRCs.

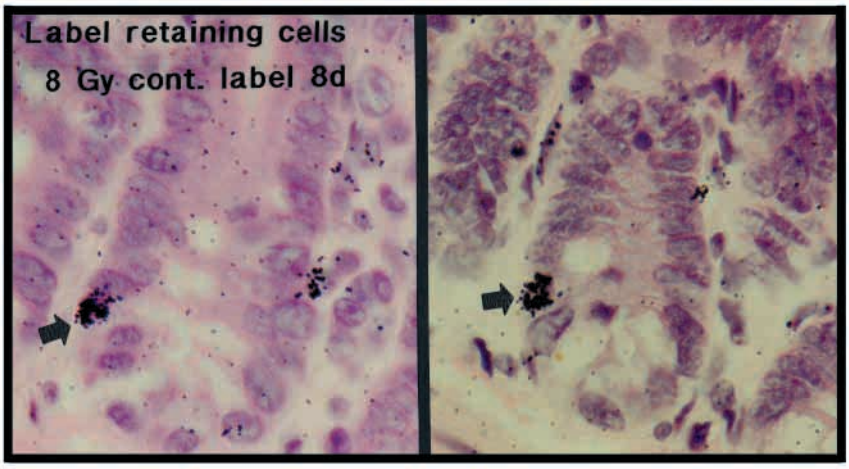

Fig. 6. Photomicrographs of two longitudinally sectioned crypts showing a single heavily labelled LRC situated just above the Paneth cells at about cell position four (arrow). These pictures were taken from animals subjected to the post-irradiation regeneration labelling protocol (8 Gy followed by 2 days of labelling with analysis and photography 8 days after labelling). Similar pictures were obtained in the juvenile labelling experiment. There are occasional labelled cells in the pericryptal stroma.

Thus an additional 'needle-in-the-haystack' experiment would be to determine whether LRCs can be induced into apoptosis by a dose of 1 Gy. Owing to the rarity of these events, quantitative data are difficult to obtain, but Fig. 7 illustrates that LRCs can clearly be labelled by an S phase marker such as bromodeoxyuridine (BrdUrd) and that LRCs in mitosis can occasionally be identified. Indeed images consistent with an asymmetric distribution of ^{3}HTdR autoradiographic grains at anaphase in LRCs can even be obtained. Although difficult to photograph, 1 Gy of radiation delivered 16 days after LRCs, generated by the 8 Gy regeneration protocol, also results in LRCs in apoptosis at 4.5 hours (Fig. 7).

A final experiment was undertaken using BrdUrd to label the newly synthesised strands in LRCs, with samples taken at various times thereafter to determine the segregation pattern of the tritium and the BrdUrd labels. Both protocols for generating LRCs (i.e. labelling of 6 week old mice and labelling following 8 Gy of radiation) were used, and these were followed by a labelling protocol of 1 mg of BrdUrd every 6 hours for 48 hours, starting at 12 weeks of age for the 6 week LRC labelling protocol and at 8 days for the 8 Gy LRC labelling protocol. Each of these two labelling protocols has been repeated more than once, and representative data are shown in Table 1. In the 6 week old labelling protocol, about 100% of the ^{3}HTdR-LRCs at cell position four are labelled by the BrdUrd protocol, confirming that these cells are entering the S phase of the cell cycle. Somewhat similar double-labelling studies have been performed on LRCs in the bulge region of the hair follicles but with different experimental objectives (Taylor et al., 2000). These studies involved administering BrdUrd before ^{3}HTdR and again illustrated that LRCs do pass through the cell cycle. For the irradiated LRC

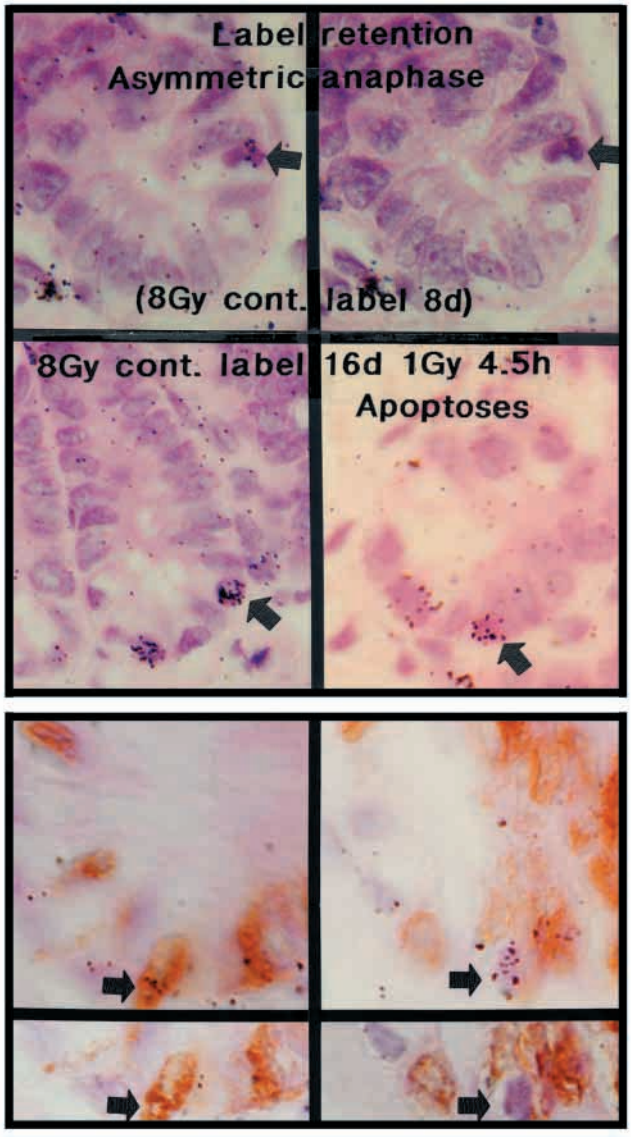

Fig. 7. The upper two panels show a mitotic anaphase figure in two different planes of focus with an asymmetric distribution of grains (same labelling and analysis as in Fig. 6). The middle two panels (arrows) show LRCs undergoing apoptosis 4.5 hours after a dose of 1 Gy γ rays delivered 16 days after the post-irradiation regeneration labelling protocol described for Fig. 6. The true morphlogical features of apoptosis in these autoradiographs are difficult to photograph and can only be determined by careful optical focusing. The next lower two panels and their different focal plane inserts (bottom panels) show a label-retaining nucleus undergoing DNA synthesis, which is labelled with BrdUrd (left hand panel), and a LRC undergoing mitosis (lower panel right), indicating that the LRCs do indeed progress through the cell cycle and divide (see also data in Table 1). (The same protocol was followed as in Fig. 6.)

Table 1. BrdUrd labelling of LRCS

(a) 6 week old ^{3}HTdR labelled animals (BrdUrd at 12 weeks)

Time after BrdUrd	^{3}HTdR LRC peak LI%	BrdUrd peak LI%	Double-labelled Peak LI%
40 minutes	16.0±0.7	96.8±1.5	11.8±4.0
2 days	11.8±1.7	90.3±6.6	6.3±2.4
3 days	16.7†	95.8±4.1	0.0
4 days	14.6±2.1	48.6±14.7	0.0
5 days	12.5†	47.9±12.9	0.0
6 days	16.7†	31.9±8.9	0.0
7 days	16.7†	12.5*	0.0
8 days	11.1±1.7	2.8±2.8	0.0

(b) 8Gy ^{3}HTdR-labelled animals (BrdUrd at 8 days)

Time after BrdUrd	^{3}HTdR LRC peak LI%	BrdUrd peak LI%	Double-labelled Peak LI%
40 minutes	11.7±2.7	99.4±0.6	11.1±3.2
2 days	11.5±2.9	93.1±0.8	2.8±2.8
3 days	14.6±2.1	93.8±6.3	0.0
4 days	22.2±5.6	57.4±9.3	0.0
6 days	16.7†	13.9±2.8	0.0
8 days	13.9±2.8	6.9±5.0	0.0
10 days	16.7†	0.0	0.0

Changing pattern of ^{3}HTdR LRCs (first column), BrdUrd-labelled cells (second column) and double-labelled LRCs (third column). The data shown here are values for the peak labelling indices taken from cell positional distributions. Data are shown for both the juvenile animal labelling protocol (upper table) and the post-irradiation regeneration labelling protocol (lower table). The number of ^{3}HTdR LRCs remains broadly constant for 8 to 10 days in spite of eight to ten cell divisions. The BrdUrd labelling remains constant for the first 3 days (during which time there would be four to six divisions in the transit cells). Thus BrdUrd labelling can be detected after several rounds of division. In contrast the percentage of double-labelled cells begins at high levels but by the third day falls to zero, that is, all the BrdUrd label is lost from these LRCs (see Fig. 1) after about two cell divisions of the LRCs. This is one representative set of data for each of the two labelling protocols. †Less than three mice per group.

protocol, about 90% of the ^{3}HTdR-LRCs are labelled by the BrdUrd protocol. As can be seen in Table 1, the percentage of ^{3}HTdR- LRCs remains roughly constant for 8 to 10 days post-BrdUrd labelling, whereas the BrdUrd labelling percentage is between 80% and 94% for the first three days, indicating that BrdUrd label can be detected even after three to six cell divisions. Thereafter, the BrdUrd detection threshold appears to fall and, therefore, these later samples were not analysed for BrdUrd labelling. The data in Table 1 show that the BrdUrd label is rapidly removed from the double-labelled LRCs over the first 2 days, whereas the ^{3}HTdR label persists. By the third day, no LRCs contain BrdUrd label. The timing of this loss of BrdUrd label (new strand labelling) is consistent with the loss occurring at a time equivalent to the second division of the stem cells after the BrdUrd labelling (Fig. 1).

The data presented in this paper are consistent with LRCs retaining ^{3}HTdR label, because they contain tritium-labelled template strands of DNA that are selectively segregated and retained in the ultimate stem cell population of the crypt in spite of many rounds of cell division. If the newly synthesised strands in such cells are subsequently labelled with BrdUrd, the tritium and BrdUrd DNA markers in the LRCs behave with the passage of time in totally different ways. The tritium assumed to be in the template strands is retained, and the BrdUrd in the newly synthesised DNA strands is selectively removed from the nucleus of the actual stem cells at, or around, the time of their second division after labelling, consistent with the model proposed by Cairns (Cairns, 1975) and the preliminary data presented from our laboratory (Potten et al., 1978). The mechanisms used by the cells to selectively retain the template strands remain unclear.

The observations on strand segregation presented here, together with other observations related to cells at the stem cell position, provide an explanation for the rarity of cancers in the small bowel of mice and men. The selective retention of template strands in the ultimate stem cells ensures that replication-induced errors are effectively avoided. One possible associated consequence of the evolution of this stem-cell-protective mechanism is a prohibition of DNA excision repair processes, since these pose the risk of sister chromatid exchanges. Exchange and recombination events would involve a mixing of old and new strands and would thus compromise the protective mechanism. The inability to undergo repair processes might be expected to render these cells highly sensitive to genotoxic damage, since some enzymes would be common to both processes. Indeed, it appears that cells at the stem cell position do have an exquisite radiosensitivity. They are so sensitive that doses as low as 1 to 5 cGy induce an apoptotic suicide in cells assumed to be the ultimate stem cells, thus removing the potential genotoxic damage by removing the cell (Potten, 1977; Hendry et al., 1982; Potten and Grant, 1998; Potten et al., 1992). This represents a second highly effective protective mechanism that would deal with random errors induced in the template strands by background radiation or genotoxic chemicals.

The removal of an occasional stem cell as a consequence of incurring DNA damage is easily compensated for by either a symmetric division of a neighbouring ultimate stem cell or the recruitment of a potential stem cell from generation 1, 2 or 3 (depending on the level of cytotoxicity) transit cells (see circles in Fig. 2). In the former case, there is a risk associated with the regenerative division and the elevation of newly synthesised strands with potential errors to form new templates. However, the risk here is only that associated with a single round of DNA replication. In the latter case (the recruitment of a potential stem cell), the risk again is one associated with the elevation of newly synthesised strands to template status, but again only a minimum number of rounds of DNA synthesis (one to a maximum of three) would be involved. It is interesting that p53 plays a critical role in regulating the altruistic cell suicide following genotoxic damage and may also be playing an important role in regulating the asymmetric strand segregation (J. Cairns, personal communication). This hierarchy within the stem cell compartment (ultimate and potential stem cells) ensures that if many, or all, of the ultimate stem cells incur template errors and commit suicide, the crypt survives and is repopulated from the repair-efficient potential stem cells – a fail-safe ultimate protective mechanism. It is, however, unlikely that in nature all ultimate stem cells die at one time, but this can be induced in the laboratory.

One final consideration related to the selective DNA strand segregation hypothesis in stem cells concerns the telomere-telomerase hypothesis for ageing. If the ultimate stem cells have a mechanism for selectively sorting the DNA template strands that they retain, it would be predicted that the telomeres on the template strands would not be subject to the same telomere strand-end replication problems that would be

expected for the newly synthesized strand. This may help to account for the fact that there is little evidence for a decline in the proliferative capacity of the 4×10^6 small intestinal ultimate stem cells over the 1000 divisions estimated to occur during the lifetime of a laboratory mouse (possibly 5000-6000 divisions in each of approximately 2×10^8 ultimate stem cells in man).

The mechanisms involved in the selective sorting of all old and new DNA strands at mitosis remain to be elucidated. Possibilities here range from the existence of some linker molecule joining the ends of all old or new strands, such that moving one would pull them all (like a string of sausages), to an association of all old or new strands with the spindle proteins associated with old and new centrioles.

It is also unclear what mode of DNA segregation (random or selective) occurs in stem cells that are actively making extra stem cells (during development or tissue regeneration).

The question of what happens in terms of these protective mechanisms in other tissues remains unanswered. However it does look as if selective segregation must be occurring in the interfollicular and hair follicle bulge stem cells to account for the label retention that is seen in those sites (Bickenbach, 1981; Morris et al., 1985; Cotsarelis et al., 1990). There is also some evidence in support of this concept for dorsal tongue epithelium (Potten et al., 1978). It is possible that in other tissues different networks of genes operate to regulate strand segregation (symmetric versus asymmetric divisions), DNA repair and apoptosis. Certainly in the adjacent intestinal epithelium of the large bowel, where data are not available at present for strand segregation and label retention, the protective altruistic suicide in the ultimate stem cells is compromised by the active expression of the bcl-2 protein (Merritt et al., 1995). Thus in this region of the gut the evolutionary pressures have selected for survival and repair of DNA damage in the crucial stem cells rather than apoptotic suicide and replacement (regeneration) as a means of correcting the DNA damage. This may be because many more genotoxic molecules are encountered in the large bowel, and if the altruistic cell suicide mechanism were adopted, repeated stem cell regeneration or replacement with the elevation of (error prone) newly synthesised DNA strands to template status would be repeatedly required. This may carry a greater risk than the possibility of errors associated with the excision repair process. However, the abandonment of the apoptosis protective mechanism, which in conjunction with the selective strand segregation process provides such an effective protection against the genetic defects leading to cancer in the small bowel, raises the risk of such events occurring more frequently in the large bowel (Potten et al., 1992; Merritt et al., 1995).

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