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Phil. Trans. R. Soc. Lond. B 1998 **353**, 821-830
doi: 10.1098/rstb.1998.0246

The Royal Society

Stem cells in gastrointestinal epithelium: numbers, characteristics and death

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The mammalian intestinal mucosa, with its distinctive polarity, high rate of proliferation and rapid cell migration, is an excellent model system to study proliferative hierarchies and the regulation of cell division, differentiation and cell death. Each crypt contains a few lineage ancestral stem cells (the 'ultimate stem cells'). However, there are other potential stem cells within the early lineage, and many rapidly proliferating transit cells with no stem cell capabilities. Apoptosis under two circumstances has a specificity for the ultimate stem cells in the small intestine and this represents, in one case, part of the stem cell homeostatic process and, in another case, a protective mechanism against DNA damage.

Apoptosis occurs with a lower frequency in the large intestine owing to the expression of the *bcl-2* gene in this region, and this probably contributes to the causes for the low cancer risk in the small bowel and the high risk in the large bowel.

Current studies are beginning to unravel the complex interaction of growth factors and regulatory genes that determine whether a cell divides, differentiates or dies.

Keywords: stem cells; intestine; clonogenic cells; apoptosis; *p53*; *bcl-2*

1. STEM CELLS

The definition of stem cells tends to be strongly context dependent and hence varies depending on the research interests of the investigator and the system under analysis. For adult steady-state renewing tissues, such as the small intestine, a definition was formulated by Potten & Loeffler (1990) which considered most of the observations and conclusions of, at least, gastrointestinal researchers. The stem cells were defined as relatively undifferentiated, proliferative cells that maintain their numbers, while at the same time producing a range of differentiated progeny that may continue to divide. The stem cells reserve the potential to regenerate the stem cell population and the tissue after cytotoxic exposure. This they do via a process of clonal growth, and hence stem cells have been called 'clonogenic cells' when expressing this potential. Stem cells were further described as cells that maintain a flexibility to vary their options within this definition depending on the circumstances.

The small intestine, which in the mouse has been extensively studied, is characterized by rapid cell cycle progression, a high level of cell production per unit time and a strong polarity both at the level of individual cells and the tissue. These properties make it well suited to the study of proliferative hierarchies and their lineage ancestor cells, the stem cells, regulatory mechanisms and the determinants for cell death (apoptosis).

Associated with the strong tissue polarity are clear migration pathways for cells, which can be studied in a variety of ways. In simple terms this is illustrated in figure 1 where cells marked in the crypt and studied at various times can be seen to move inexorably up the villus at a velocity of between one and two cell diameters per hour (Kaur & Potten 1986). *Dolichos biflorus* lectin staining in appropriate mice demonstrated that the cells move in straight vertical columns (Winton *et al.* 1988). The information shown in figure 1 indicates that cells produced by mitosis in the crypt live for only 2–3 days before they reach the tip of the villus from which they are extruded. The number of cell divisions a cell has undergone can be estimated from the reduction in intensity of the tritiated thymidine-derived silver grains in the autoradiographs. These show that the labelled cells in the upper regions of the crypt only divide once because the silver grains are approximately halved. They are the cells that reach the villus tip first. Cells slightly lower down the crypt appear to divide twice and cells further down three times and so on. Proliferation studies show that the crypt is replaced approximately every two days and that the stem cells divide about once a day. This implies that during the three-year life of a laboratory mouse the stem cells must have a large division potential (about 1000 cell divisions) and a high self-maintenance efficiency. However, figure 1 demonstrates that most crypt cells have neither a large division potential nor a significant self-maintenance probability. Various approaches have been used to measure the cell migration velocity at each position in the crypt and to determine the point of origin for all this movement, the source from which everything comes. This will be the position in the crypt at which the stem cells reside. This is illustrated in the graphs in figure 1, which show that in the small intestine the stem cells are located at about the fourth position from the bottom of the crypt: individual experiments tend to give values between three and five. In the large intestine, at least in the mid colon region, the stem cells appear to be located at the very base of the crypt.

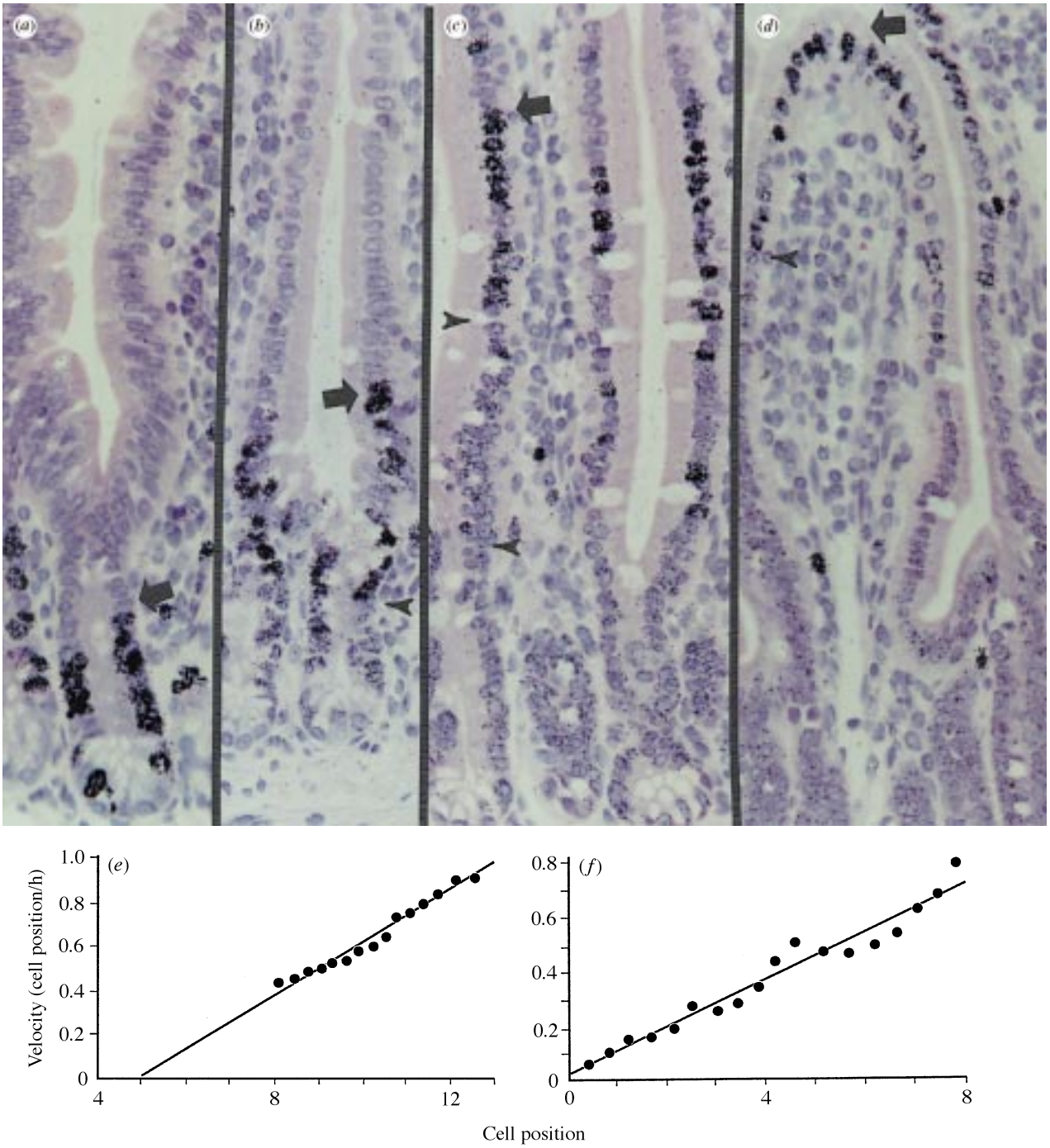

Figure 1. Autoradiographs showing the small intestine crypt–villus axis prepared (*a*) 40 min, (*b*) 1 day, (*c*) 2 days and (*d*) 3 days after injecting tritiated thymidine into mice. Movement of labelled cells and grain dilution due to cell division is evident. Such preparations can be used to measure cell migration velocities. The graphs show the changing velocities with increasing position up the crypt as determined by the successive labelling-index percentiles comparing 40 min and 3 h samples: (*e*) small intestine and (*f*) large intestine. The stem cells are assumed to be located at the origin of the cell movement at cell position 4.9 ± 0.2 (*e*) and -0.2 ± 0.2 (*f*) (in addition, see Qiu *et al.* (1994)).

2. CLONOGENIC STEM CELLS

The ability of stem cells to clonally regenerate tissue after injury provides an approach whereby the functional potential of stem cells can be assessed experimentally. This is the basis of the spleen colony assay for bone marrow stem cells and a similar assay at the microscopic level has been developed for the intestine (Withers & Elkind 1970; Potten & Hendry 1985). The approach is illustrated in figure 2 where the regenerating crypts or colonies observed three to four days after exposure to various doses of radiation can be observed easily and counted. In this way, dose–response curves (survival curves) for the regenerating crypt-like foci can be generated, which have a very characteristic shape. These curves and crypt-survival levels can be interpreted to provide estimates for the number of cells that possess this clonal regeneration capacity per crypt, i.e. the number of clonogenic stem cells. The data suggest that the number of clonogenic cells in a crypt depends on the level of damage that has been

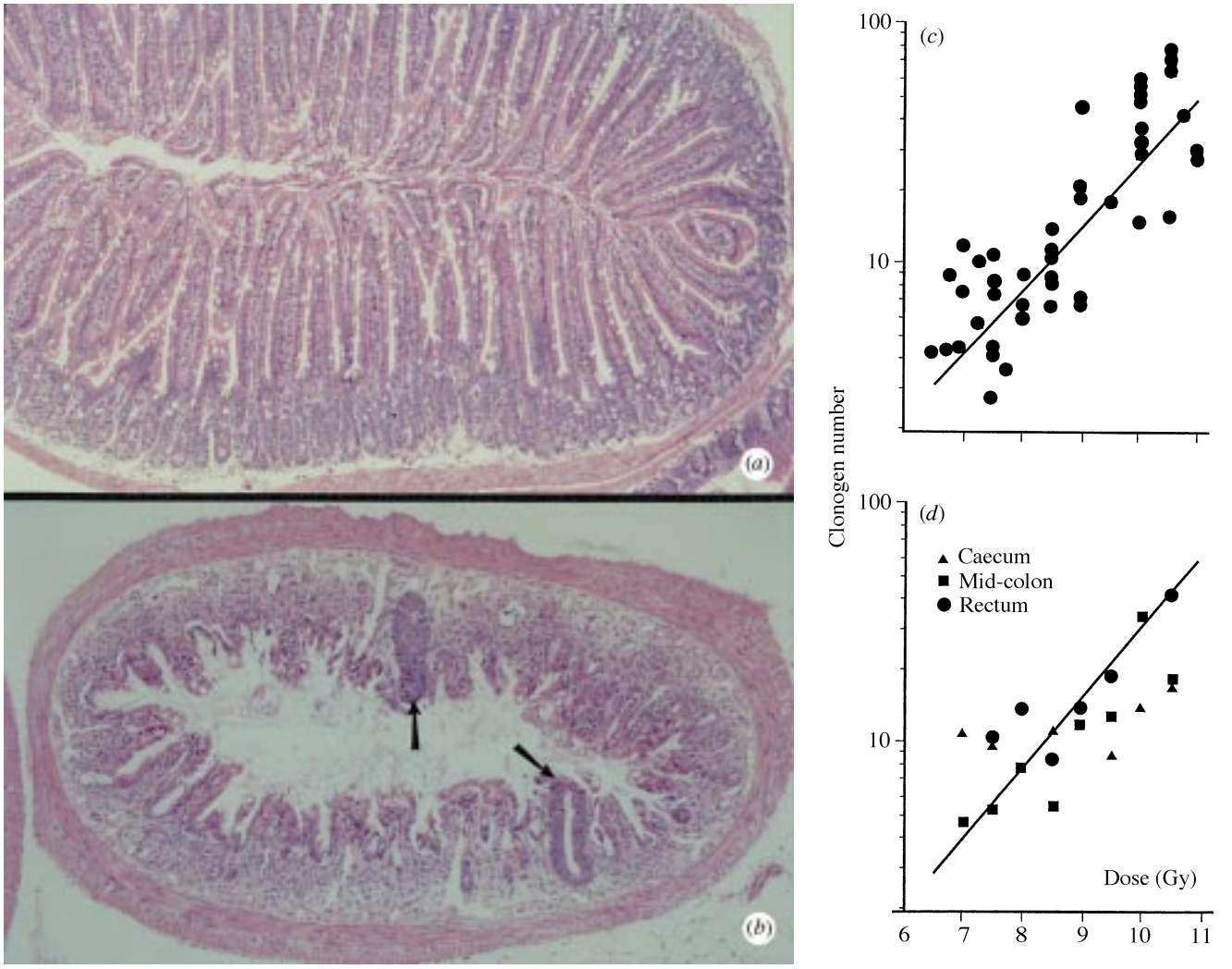

Figure 2. Cross-sections of mouse small intestine: (*a*) controls and (*b*) 4 days after 14.5 Gy showing regenerating colonies (crypts). Analysis of crypt survival data can provide estimates for the number of clonogenic stem cells per crypt, which is similar in (*c*) small and (*d*) large intestine and varies with the level of damage induced (or radiation dose) (in addition, see Cai *et al*. (1997)).

induced in the crypt: the more damage, the more cells are recruited into the clonogenic compartment (figure 2). At moderate doses of radiation there are about six clonogenic cells per crypt and at higher doses the number appears to be about 36 in both the small and large intestine (Hendry *et al*. 1992; Cai *et al*. 1997).

Such studies, together with the results of many years of other experiments, have shown that each crypt in the small intestine of the mouse contains about 250 cells in a flask-shaped structure, with approximately 16 cells in an annulus in the mid-point of the crypt. There are about 150–160 rapidly proliferating cells and about 30 differentiated, functional Paneth cells at the base of the crypt. Most of the proliferating cells are dividing twice a day, whereas cells at the very base of the proliferative compartment, immediately above the Paneth cells, seem to be cycling about once a day. A combination of cell proliferation studies and mathematical modelling suggests that the crypt could be maintained under steady-state conditions by between four and six ultimate stem cells. These produce dividing transit cells that form a proliferative lineage in the mid-crypt region (figure 3).

3. THE STEM CELL HIERARCHY

Our current model for the organization of the crypt stem cells is that the stem cell compartment is itself hierarchical, with three tiers. At the base of the hierarchy there are four to six ultimate-lineage, ancestor stem cells, which are very easy to kill by small doses of radiation. They die by apoptosis and this can be easily detected. If these cells are all killed their immediate daughters or siblings, which are much more radioresistant, possessing a good repair capacity, can take over the function of the ultimate stem cells replacing this first tier of stem cells and therefore maintaining the crypt. If higher doses of radiation are delivered (which would never be encountered in nature) and these six second-tier stem cells are also killed, there is a third tier of up to about 24 cells with even greater resistance (better repair) that again can regenerate the earlier stem cell tiers, the crypt and the epithelium (Potten & Hendry 1995). This makes a total of about 36 stem and clonogenic cells per crypt (see figure 4*d*). The remaining 120 proliferating cells appear to possess no clonogenic stem cell properties.

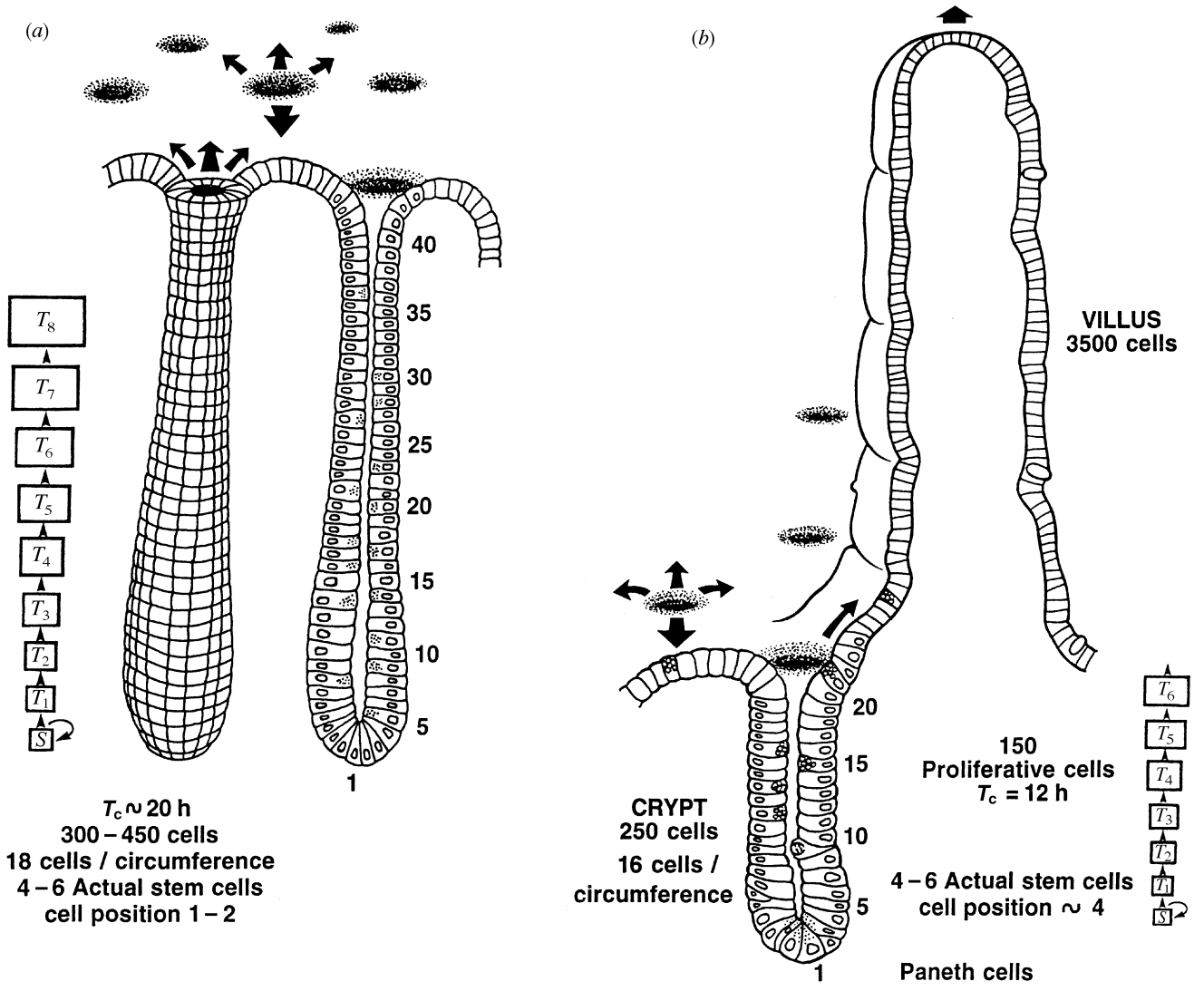

Figure 3. Diagram showing the three-dimensional architecture, sectional profiles and presumed cell lineage organization for (*a*) large and (*b*) small intestine. The position of a cell in the lineage can be related to position along the crypt axis.

One of the attractive features of the intestine as a cell biological model is the fact that the position of a cell within the lineage or hierarchy can be related to its topographical position in the tissue in the way that is illustrated in figure 3. This is particularly valuable as there are no markers or means of identification for the stem cells. However, the characteristics, response to injury and the regulatory processes can be investigated and be associated with the position in the crypt at which the stem cells are located.

This is done by selecting longitudinally sectioned crypts and recording information on a cell positional basis. A wide variety of markers for proliferation, differentiation and death can be analysed in this way (figure 4*a*,*b*).

Using this cell positional approach, various features associated with the stem cell position in the small intestine can be determined (Potten 1977; Potten *et al*. 1997). It has been noted that some cells at this position (i) have a slower cell cycle (approximately 24-h cycle in the mouse), (ii) are specifically involved in regeneration following injury, (iii) exhibit a low level of spontaneous apoptosis in healthy mice and humans, (iv) are very radiosensitive and die via apoptosis, and (v) represent the origin of the cell migration pathways.

There are a number of features (summarized later in figure 4) that are more weakly linked to this zone of the crypt. The first point of note here is the close association between this position and the Paneth cells. However, although this is true for mouse and humans, it is not so for all species. Second, the expression of wild-type p53, c-myc, bax and laminin α2/M and β-galactosidase expression in Rosa 11 mice and susceptibility to TGFβ effects all show some weak association with this region. Rosa 11 mice were generated from promoter trap experiments in embryonic stem cells. The, as yet, unidentified promotor drives the expression of β-galactosidase in a characteristic lower-crypt pattern as detected by X-gal.

4. GASTROINTESTINAL CANCER

One of the surprising and, as yet, incompletely understood observations in the gastrointestinal tract of humans is the high risk and incidence of cancers in the large intestine and the extraordinarily low risk and incidence in the small intestine. In fact in some regions of the small intestine cancer is virtually unknown, whereas there are, in contrast, clear 'hot spots' in the large intestine. This is

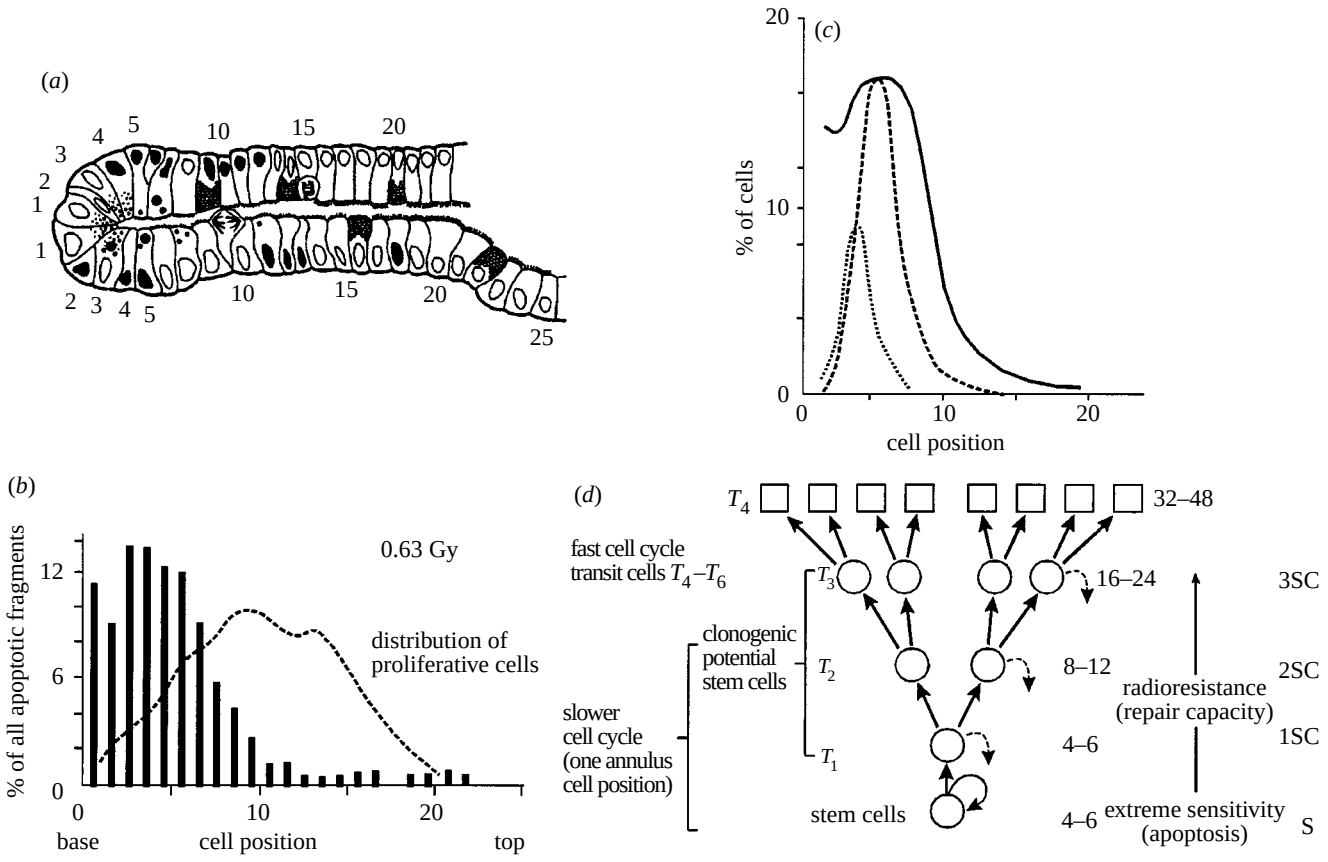

Figure 4. (*a*) Diagram of a longitudinal crypt section showing the cell position numbering system. (*b*) Frequency plots of various parameters can be generated from analysis of such sections. Cell death (apoptosis) 3 h after 0.63 Gy (bars) and frequency plots of a proliferation marker (dotted line) (e.g. labelling with tritiated thymidine, shaded nuclei in (*a*)) are shown. (*c*) The apoptosis frequency plot (solid line) can be compared with the theoretical distribution of actual stem cells (dotted line) and clonogenic stem cells (dashed line) based on mathematical modelling. All of these are centred around cell position four. (*d*) The current model for a three-tiered hierarchical stem cell compartment is also illustrated (modified from various sources). Crypts possess from four to six actual stem cells (S), but many more clonogenic stem cells (SC) that are capable of stem cell function. Cells with clonogenic capacity are denoted by circles. These are thought to have a three-tiered radioresistance of repair capacity (1SC–3SC) with the actual stem cells being very radiosensitive.

surprising bearing in mind the much larger number of potential carcinogen target cells in the small intestine (due to its much greater length) and the fact that the cells here are proliferating much more rapidly.

5. APOPTOSIS

Over a number of years we have been particularly interested in apoptosis and the expression of apoptosis-related genes in relation to the crypt hierarchy in the small and large intestine. We have been interested in why cells die, which cells die, what is the consequence and biological reason for that death, how is the process regulated and how does the tissue react to induced cell death. The results of our studies provided an indication that apoptosis is important in determining the cancer incidence figures and in stem cell homeostasis. I will attempt to summarize the results from a large number of experiments conducted over a period of many years (Potten 1977, 1992; Ijiri & Potten 1987; Merritt *et al.* 1994, 1995; Pritchard *et al.* 1997).

In normal, healthy, small intestinal epithelium in mouse and humans, a low incidence of apoptosis can be observed. We refer to this as 'spontaneous apoptosis'. It is easily recognized and in the mouse, where it can be quantified, it is associated with the stem cell position. The incidence would suggest that between 1% and 10% of the cells at this position are dying at any time. This form of apoptosis is similar to that seen in development in that it is both damage and *p53* independent (it occurs in *p53* knockout mice). In contrast, in the large intestine spontaneous apoptosis is very rare indeed and when it is seen it does not appear to have any particular association with the stem cell position.

Radiation, some chemotherapeutic cytotoxic drugs and some mutagens and carcinogens induce apoptosis within the stem cell position in a period of 3–6 h in the small intestine. This type of early, 'damage-associated apoptosis' is totally *p53* dependent although there is a late wave of apoptosis at about 24 h, which is believed to be associated with the regeneration process, involving the clonogenic stem cells described earlier, and this is *p53* independent (Merritt *et al.* 1997). Extremely small doses (0.01–0.05 Gy) of radiation induce elevated levels of apoptosis in the stem cell position in the small bowel. The number of apoptotic cells increases with increasing dose up to a point when about six cells per crypt have been killed, which is seen after a dose of about 1 Gy. Above this dose, few additional cells die via apoptosis at this early time. In the large

Table 1. *Apoptosis*

	small intestine	large intestine
spontaneous	easily recognized but rare stem cell associated (1–10% stem cells dying) *p53* and damage independent	even rarer not stem cell associated *p53* independent
induced by radiation, some drugs and carcinogens	early apoptosis/totally *p53* dependent stem cell associated peaks at 3–6 h induced by very low radiation doses late apoptosis/*p53* independent	not stem cell associated (when *bcl-2* is expressed)
other drugs	mid and upper crypt cell specific all cells can undergo apoptosis	lower frequency

Table 2. p53 *protein*

(Both small and large intestines.)

wild-type protein expressed early (2–4 h) after radiation (coincident in time and cell position with apoptosis)
not expressed in apoptotic cells but in other cells at stem cell position
$p21^{Waf1/1/cip1}$ expressed at similar time but slightly higher up crypt

p53 nulls
spontaneous apoptosis normal
developmental apoptosis normal
radiation-induced apoptosis completely absent

mutant *p53*
heavily expressed (mutant form) in late stages of colon cancer sequence (carcinomas)

Table 3. bcl-2 *protein*

	small intestine	large intestine
	not expressed	expressed at stem cell position in mice (variable) and human (always) increased expression at crypt base near adenocarcinomas in humans expressed in adenomas, lower expression in carcinomas
bcl-2 nulls	no effect on spontaneous or radiation-induced apoptosis	increased spontaneous apoptosis in nulls increased radiation-induced apoptosis.
bcl-2 gene family	bag, bax not expressed in crypt some bax expressed at crypt base bad expressed after irradiation bad, bax, bcl-$x_{L/S}$ expressed on villus	bag, sporadic cell expression bax, bak, bcl-$x_{L/S}$ expressed on intercrypt table

Table 4. *Role of apoptosis*

	small intestine	large intestine
spontaneous apoptosis	removes occasional excess stem cells part of stem cell homeostasis stable stem cell population and crypt	not as effective in controlling stem cell numbers *bcl-2* can override the homeostatic process stem cell numbers may drift upwards with time (more cells at carcinogenic risk) hyperplastic crypts may be formed
damage-induced apoptosis	very efficient damage-detection system no attempt to repair (misrepair prevented) efficient protection mechanism for DNA (carcinogenic) damage	*bcl-2* prevents apoptosis damage removal (protection mechanism) damage or misrepaired damage may persist; increased cancer risk developing cancers express *bcl-2*: chemoresistant

intestine, apoptosis is also induced by radiation and some drugs. However, as *bcl-2* is being expressed (although expression in the mouse seems to be rather variable) it is assumed that this is responsible for the radiation-induced apoptosis being lower per unit dose than in the small intestine and less strongly associated with the stem cell position. In the small intestine a range of other cytotoxic drugs can induce apoptosis in cells at all other positions in the crypt, which indicates that all the cells are capable of activating the apoptosis programme but only do so after an appropriate type of damage. Indeed there are indications that after appropriate damage (e.g. ischaemia), all epithelial cells including villus cells can initiate apoptosis.

6. *p53*, *p21*$^{Waf1/1/cip1}$ AND APOPTOSIS

In both the small and large intestine, wild-type p53 protein is expressed early after radiation exposure (2–4 h), and in the small bowel its expression, in terms of time and cell position, is coincident with that observed for apoptosis (Merritt *et al.* 1994). However, it is not expressed in many of the apoptotic cells but is expressed in other cells at the stem cell position. These we believe are the clonogenic or regenerative stem cells outlined above. The *p53*-related gene *p21*$^{Waf1/1/cip1}$ is expressed at a similar time at the same position but also at slightly higher positions up the crypt. When the *p53* gene is deleted (*p53* knockout mice) the radiation-induced apoptosis is totally absent, supporting the suggestion that *p53* (and also *p21*$^{Waf1/1/cip1}$) is involved in cell cycle checkpoints and repair in clonogenic stem cells. Mutated *p53* expression is also seen commonly in the later stages (carcinomas) in the colon cancer development sequence, consistent with the view that transformed cells have less efficient cell cycle checkpoint controls.

7. THE *bcl-2* GENE FAMILY

The anti-apoptotic or cell-survival gene *bcl-2* is not expressed in the small intestine. However, it is expressed at the stem cell position in the mid-colon of mice although the expression is rather variable and weak. It appears to be routinely expressed in the stem cell position at the bottom of the colonic crypts in humans (Merritt *et al.* 1995). In human colon adjacent to adenocarcinomas the expression of bcl-2 increases at the base of the crypt and expression is maintained in the early stages of the development of cancer (i.e. in adenomas), although its expression decreases in carcinomas (Watson *et al.* 1996). The expression of bcl-2 and p53 during tumour development therefore appears to be reciprocal.

When the *bcl-2* gene is deleted the spontaneous and radiation-induced apoptotic yields in the small intestine are unaffected, which is not surprising as *bcl-2* was not expressed in this region of the gut. However, in the large intestine in mice, increased spontaneous apoptosis was observed in *bcl-2* knockout animals, particularly in our initial studies when our *bcl-2* colony was newly established (Merritt *et al.* 1995). Similarly, an increase in apoptotic yield in colon was seen after radiation in the *bcl-2* knockout mice. *Bcl-2* is a member of a large family of genes. The expression of some of the other members has also been investigated (Wilson & Potten 1997, 1998; see also

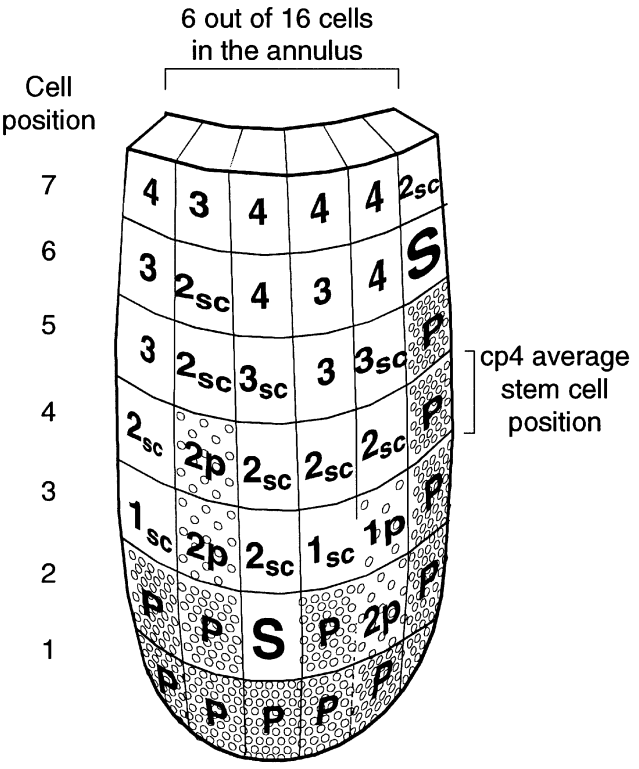

Figure 5. The actual stem cells (S) are situated within an annulus of about 16 cells on top of the highest Paneth cell (P) in each circumferential position. The stem cells generate daughter cells that become Paneth lineage cells (1P, 2P, P) or that enter a longer crypt lineage (transit cells 1–6). The first three steps in the transit lineage are still uncommitted and can act as clonogenic stem cells (SC 1–3); see figure 4. (Taken from Potten *et al.* (1997).)

figure 6). In the small intestine, bag and bax were apparently not expressed in the crypt generally, although there was some bax expression at the crypt base. Bad, bax, and bcl-$x_{L/S}$ are all expressed at some level on the villus. Bad expression increases in the crypt after radiation. In the large intestine, bag seems to be sporadically expressed on occasional cells in the crypt and bax, bak, bcl-$x_{L/S}$ are all expressed on the intercrypt table.

8. GENERAL CONCLUSIONS

These observations on apoptosis in the gut are summarized in tables 1–3 and the conclusions that can be drawn are summarized in table 4. It is suggested that the spontaneous apoptosis in the small intestine is part of the stem cell homeostatic process. It is involved in removing occasional excess stem cells perhaps produced by an occasional inappropriate symmetric division. Mathematical modelling suggests that up to 5% of the stem cell divisions might be symmetric. The consequence of this homeostatic process, which seems to be very precise (tightly regulated), is that we have a stable stem cell population and a stable crypt in the small intestine. The implications are that the stem cells are able in some way to detect a single extra stem cell, i.e. if their numbers go from six to seven. Instructions are then given for one of these cells to commit suicide. One must bear in mind that a single extra stem cell may generate a lineage of

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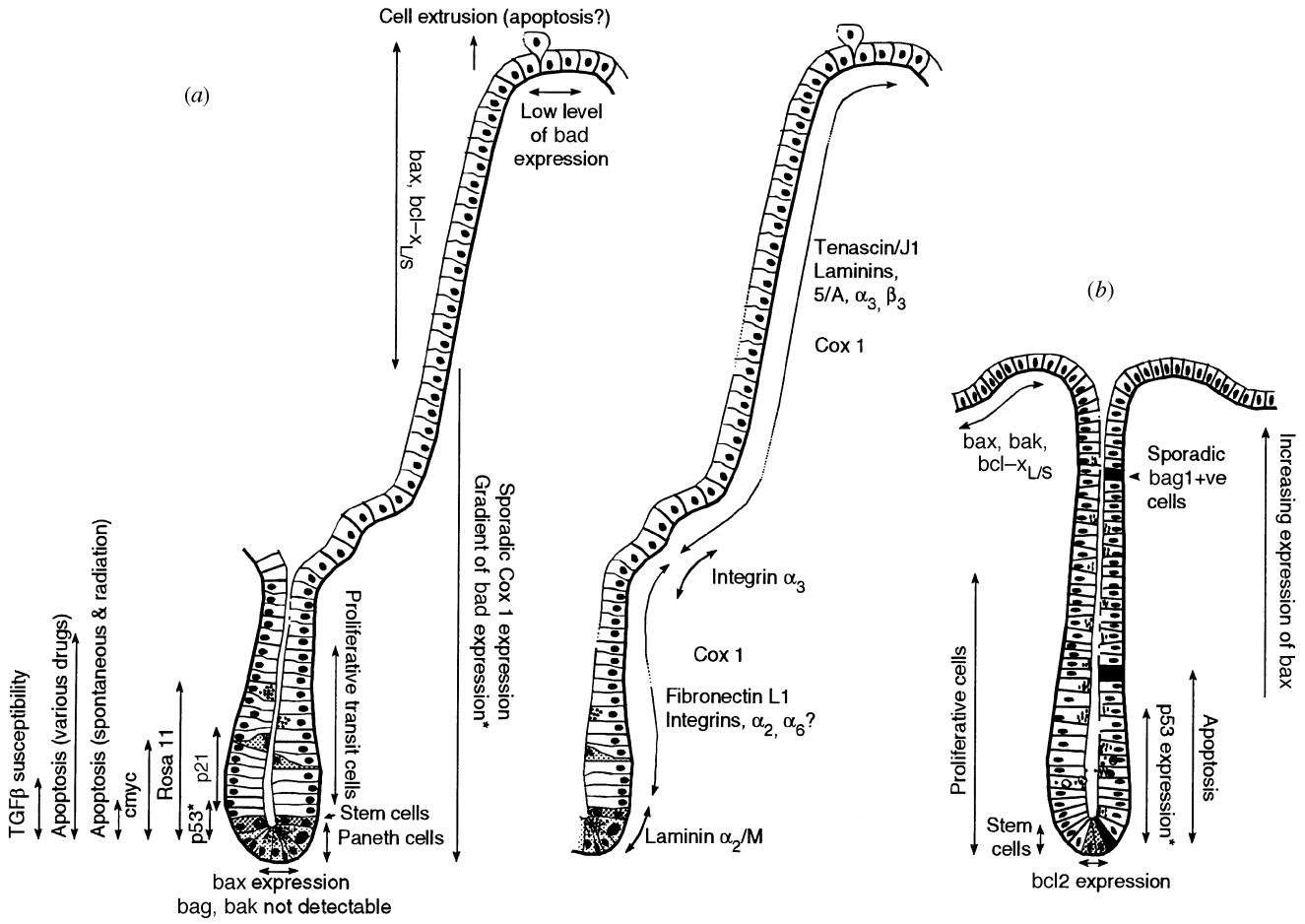

Figure 6. Diagram showing the spatial (cell positional) expression of various apoptosis-related genes, integrins and extracellular matrix adhesion molecules in the small and large intestine. Those marked with an asterisk represent expression seen following radiation injury. (Taken with modifications from Potten (1997) and Potten *et al.* (1997).)

64–128 extra cells, which would severely distort the architecture of the crypt and render it hyperplastic in appearance. The hypothesis is that in the large intestine this homeostatic mechanism is not operating as efficiently when bcl-2 is expressed. Stem cell numbers may as a consequence gradually drift upwards with time producing more cells at carcinogenic risk and occasional hyperplastic crypts.

After exposure to a DNA-damaging agent such as radiation, in the small intestine the stem cells appear to have an extremely efficient damage-detection mechanism as they seem to be capable of detecting radiation–DNA interaction events at the level of about one per nucleus, i.e. one per entire DNA nuclear content. The cells do not attempt to repair the damage but rather activate the suicide apoptosis pathway and this is somehow controlled by the *p53* gene. The damage, and the cell carrying the damage, is removed and this represents an extremely efficient protection mechanism for the small intestine. Other cells at the stem cell position upregulate p53 and p21$^{Waf1/1/cipl}$ and use cell cycle check points to allow repair to occur. These cells can then function as regenerative or clonogenic stem cells. In the large bowel this protective mechanism is not operating as effectively owing to the action of bcl-2. Damage or misrepaired damage could in principle persist, thus increasing the cancer risk. Cancers that arise in the large bowel would arise from cells inherently expressing bcl-2 and therefore might continue to do so, rendering them particularly chemoresistant. Figure 4*c* shows the low dose (0.63 Gy), radiation-induced apoptosis, cell positional distributions for the small intestine, together with the distribution for a proliferation marker (tritiated thymidine-labelling index). Theoretical distributions for the steady-state ultimate stem cells and the stem and clonogenic compartments derived from mathematical modelling studies are also shown. The degree of overlap for the peak values and the spreads on the distributions are evident.

It is suggested that the stem cell compartment in a small intestinal crypt has an extremely efficient method of detecting changes in stem cell numbers. A single extra stem cell (from six to seven) or the deficiency of a single stem cell (from six to five) appears to be easily detectable. In the former case one cell is instructed to commit altruistic suicide (the spontaneous apoptosis), and in the latter case changes in proliferation in cells in the stem cell position are almost instantly detected (Potten 1991). This is remarkable when one bears in mind the complex three-dimensional configuration of the crypt as illustrated in figure 5. The stem cells are believed to sit in the position of the first non-Paneth cell, but as one moves around the crypt the position of the highest Paneth cell varies considerably from as low as cell position 1 to as high as cell position 7. The average of this first non-Paneth cell position

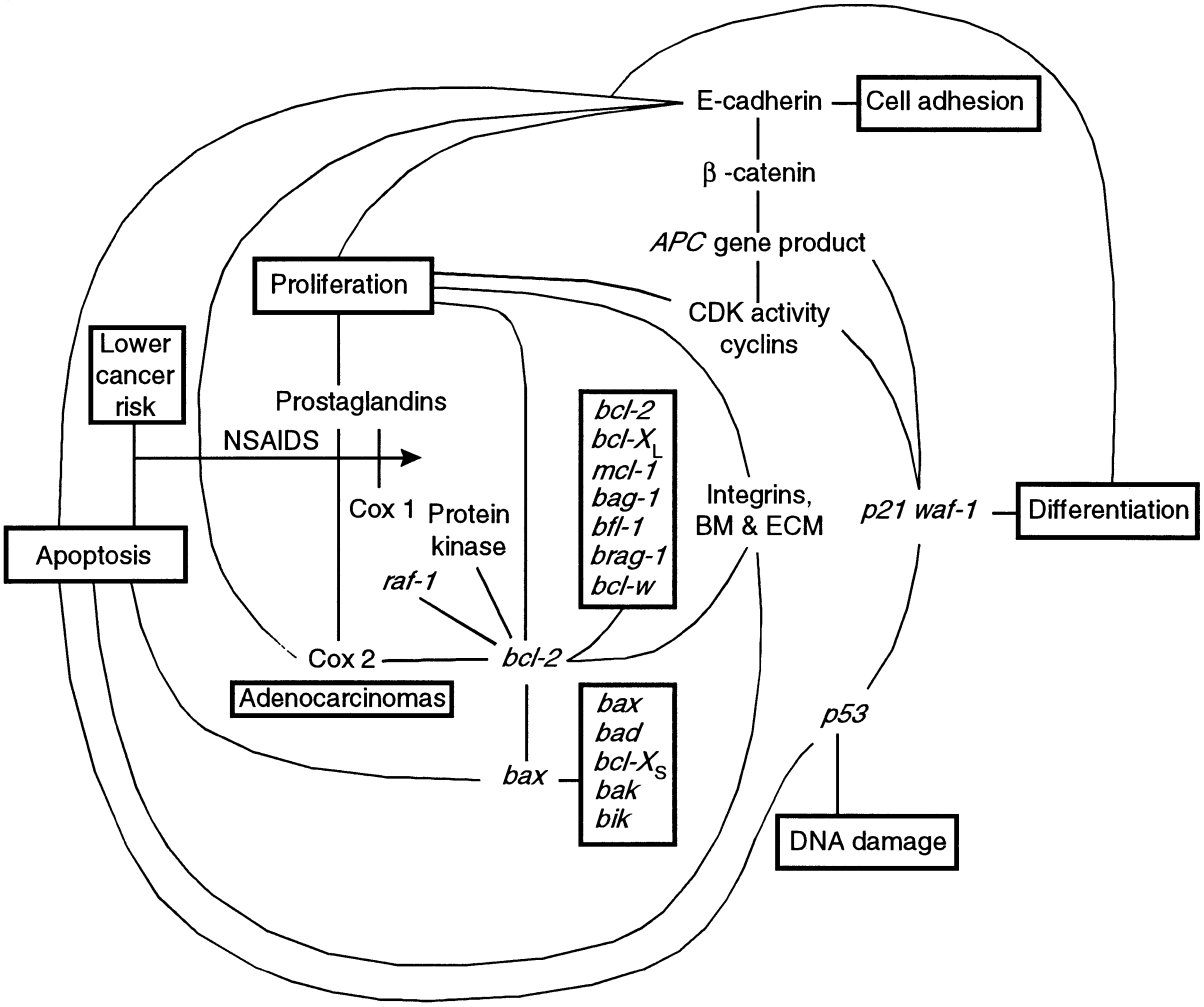

Figure 7. Some probable molecular, genetic and biochemical interactions that determine proliferation, differentiation, apoptosis and cancer risk. The lines indicate interactions that influence the processes shown in peripheral boxes. No distinction is made between positive or negative influences. The understanding of the interactions remains speculative in many cases. (Taken from Potten (1997).)

is at cell position 4. This undulating annulus contains 16 cells of which between four and six are the ultimate stem cells. So stem cells may be displaced from each other not only in the crypt circumference but also in the crypt long axis. How they know their numbers and the mechanisms involved in their homeostasis remains one of the intriguing challenges for the future (Potten *et al.* 1997).

The spatial distribution of proliferation markers and genes, cell death and genes associated with cell death, basement membrane and extracellular matrix proteins, and differentiation and differentiation-related gene products are gradually being determined in relation to cell position and hence cell hierarchy as illustrated in figure 6. The interaction between these various processes and their regulatory or modulatory roles in the overall regulation of cell proliferation, differentiation and apoptosis remains a major challenge, but one that current cell biological and molecular biological approaches seem well suited to address. A few of the interactions between proliferation, differentiation and apoptosis are illustrated in figure 7 and some of these interactions represent areas of current research activity. I show these figures not to overwhelm the reader with the complexity of the network of interacting processes, but to illustrate that it is indeed a complex network that we are trying to understand and that we are now beginning to unravel some of the interactions. In particular, the role of basement membrane proteins and extracellular matrix molecules is an area likely to prove particularly fruitful (see Beaulieu 1992; Hermiston & Gordon 1995; Kedinger *et al.* 1997; Probstmeier *et al.* 1990; Simo *et al.* 1991). Interactions between proliferation and apoptosis, the *APC* gene and the *p53* gene and DNA damage have already been suggested. A second area of current active research concerns the cyclo-oxygenase (Cox) enzymes and the prostaglandin biosynthetic pathway. The studies into inhibitors of these enzymes and the role of non-steroidal anti-inflammatory drugs (NSAIDS) not only offers the potential for further insight into the regulatory processes, but may have important clinical implications in terms of colonic cancer treatment and even cancer preventative measures.

The work summarized here spans a period from about 1974 to the present and would not have been possible without the dedicated help of many assistants and collaborators and the continued support of the Cancer Research Campaign.

Particularly, I am grateful to Caroline Chadwick, Catherine Booth, Jim Wilson, Anita Merritt, Steve Roberts, Mark Pritchard, John Hickman, Alastair Watson and Jolyon Hendry.

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