The relevance of apoptosis for cellular homeostasis and tumorigenesis in the intestine

Andrew G Renehan FRCS FDS, Simon P Bach FRCS, Christopher S Potten PhD DSc

AG Renehan, SP Bach, CS Potten. The relevance of apoptosis for cellular homeostasis and tumorigenesis in the intestine. Can J Gastroenterol 2001;15(3):166-176. Intestinal epithelium is a rapidly renewing tissue in which cell homeostasis is regulated by a balance among proliferation, growth arrest, differentiation and apoptosis (programmed cell death). Until recently, studies on oncogenesis have focused on the regulation of cell proliferation. The recognition that apoptosis must be understood to comprehend how appropriate cell numbers are maintained and how alterations in any part of the equation can contribute to malignancy has led to an explosion of research in this field. The first half of this review gives an overview of morphology and mechanisms of apoptosis, emphasizing key areas of genetic control such as the *bcl-2* family and *p53*. The second half of the review focuses on the role of apoptosis in normal cellular homeostasis and tumorigenesis in the gastrointestinal epithelium. The importance of understanding the molecular biology of apoptotic pathways in cancer therapy and future directions are also addressed.

Key Words: *Apoptosis; bcl-2 family; Gastrointestinal cancer; Intestinal crypt; p53; Stem cell*

Rôle de l'apoptose dans l'homéostasie cellulaire et la tumorogenèse dans l'intestin

RÉSUMÉ : L'épithélium intestinal est un tissu qui se renouvelle rapidement et dans lequel l'homéostasie cellulaire est régulée par un équilibre entre la prolifération, l'arrêt de croissance, la différenciation et l'apoptose (mort cellulaire programmée). Jusqu'à tout récemment, les études sur l'oncogenèse ont été centrées sur la régulation de la prolifération cellulaire. La reconnaissance que l'apoptose doit être mieux comprise si l'on veut déterminer comment les cellules sont maintenues en nombre approprié et comment le changement de toute partie de l'équation peut contribuer à la malignité a mené à une explosion de recherches dans ce domaine. La première moitié de la présente étude donne une vue d'ensemble de la morphologie et des mécanismes de l'apoptose, en mettant l'accent sur les aspects clés de la lutte génétique, par exemple la famille bcl-2 et p53. La deuxième partie porte sur le rôle de l'apoptose dans l'homéostasie cellulaire normale et la tumorogenèse dans l'épithélium gastro-intestinal. Il est également question de la nécessité de comprendre la biologie moléculaire des voies de l'apoptose dans le traitement des cancers ainsi que des orientations futures.

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CRC Department of Epithelial Biology, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester, United Kingdom
Correspondence and reprints: Professor CS Potten, Epistem Ltd, Incubator Building, Grafton Street, Manchester M13 9XX, United Kingdom.
E-mail cpotten@picr.man.ac.uk
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Apoptosis is an active, energy-dependent process of cell death that occurs during development in response to certain physiological stimuli and also, in steady-state synthesis, in response to cell injury and stress (1). (The term 'apoptosis' is derived from the Greek words describing the dropping off and falling off of pellets from flowers or leaves from trees [2].) This type of cell death involves the deletion of discrete cells within a tissue and differs from necrotic cell death in that the cells that are eliminated by apoptosis are processed without the initiation of an inflammatory response.

The role of apoptosis in tumorigenesis is now well established (reviewed in 3-8). Furthermore, there is increasing recognition that many of the effects of chemo- and radiotherapeutic agents are mediated by apoptosis (reviewed in 2,9-11). The first half of this review gives an overview of the morphology and mechanisms of apoptosis, emphasizing key areas of genetic control, such as the *bcl-2* family and *p53*. The second half of the review focuses on the role of apoptosis in normal homeostasis and tumorigenesis in the gastrointestinal epithelium. The importance of understanding the molecular biology of apoptotic pathways in cancer therapy and future directions are also addressed. Discussion is restricted to the simple epithelium of the hollow organs of the gastrointestinal tract rather than the liver and pancreas.

MORPHOLOGY AND DETECTION OF APOPTOSIS

Morphological features of apoptosis: The seminal work of Kerr et al (2) in 1972, building on earlier observations in vertebrates (12) and insects (13), should be read by those interested in assaying apoptosis because of the excellent photomicrographs that document the morphological features of the process.

The morphological features of apoptosis can be separated, for clarity, into three sequential phases (14). Initially, a cell loses contact with its neighbours and detaches from its substratum; the chromatin becomes condensed into crescent-like caps at the nuclear periphery. There is nucleolar disintegration, compaction of organelles with endoplasmic reticulum dilation, clumping of ribosomal particles, cytoskeletal filament aggregation and cytoplasmic volume reduction. There is also loss of specialized surface structures such as microvilli and junctional structures. In the second phase, blebs of plasma membrane develop, which can split away from the cells. This is a very dramatic process and can give the cells a 'boiling' appearance when viewed on time-lapse video microscopy. Both the nucleus and the cytoplasm split into fragments of various sizes, with the remaining cell becoming a round, smooth membrane-bound body referred to as an 'apoptotic body'. In the third phase, there is progressive degeneration of the residual nuclear material and cytoplasmic structures. At this stage, the plasma membrane becomes permeable to dyes such as tryptan blue. Apoptosis is rapid and is often completed in vivo in 4 to 8 h, but it may be much quicker in embryogenic cell systems and in some cell cultures.

TABLE 1
Techniques for the detection of apoptosis

Traditional microscopy (11)
Light microscopy
Electron microscopy
Acridine orange fluorescence
Biochemical approaches (121)
Agarose gel electrophoresis
Pulsed field electrophoresis
Flow cytometry (reviewed in 122)
Unfixed cells (123)
Fixed cells (eg, propidium iodide [124])
In situ detection of broken DNA strands
5'-triphosphate nick end-labelling (TUNEL) (15)
In situ end-labelling (ISEL) (16)
Recently established techniques
Annexin V immunohistochemical expression (125)
Clusterin immunohistochemical expression (126)
In situ hybridization using digoxigenin-labelled poly (A) oligonucleotide probes (127)

References appear in parentheses

Identification and quantification of apoptosis: The number of techniques available for identifying apoptosis are summarized in Table 1. Because apoptosis is defined by a series of distinct changes in cellular morphology, light and electron microscopy provide the best evidence for detecting and quantifying apoptosis in intestinal epithelium. Many contemporary studies on intestinal apoptosis supplement traditional microscopy with in situ techniques for the detection of broken DNA strands (15,16). In the strictest sense, 'programmed cell death' may be applied to circumstances where death is initiated by a genetic program that leads to autonomous cell destruction. It is now recognized that a cell may undergo 'programmed cell destruction' without fulfilling some, or all, of the morphological criteria of apoptosis (17). When considering apoptosis in the intestinal epithelium, this is not just a matter of semantic debate. For example, epithelial cells are shed from the tips of the intestinal villi. Because the dimensions of the villi tips are remarkably constant, it seems reasonable to assume that the shed cells are undergoing a form of programmed cell death. However, cells complying with the strict morphological definition of apoptosis are rarely seen at the tip of the intestinal villus, raising the question of whether other mechanisms account for cell shedding.

When quantifying apoptosis by morphological features, other caveats to the technique should be borne in mind (18,19). The rapid nature of apoptosis means that in any static analysis, a very small number of apoptotic cells observed at a given instant might, in fact, reflect a very considerable contribution to cell turnover. There are recognized variations in the speed of apoptosis among cell types and in relation to different insults. For example, the half-life of apoptotic fragments following treatment with hydroxyurea is approximately 3.5 h, while following radia-

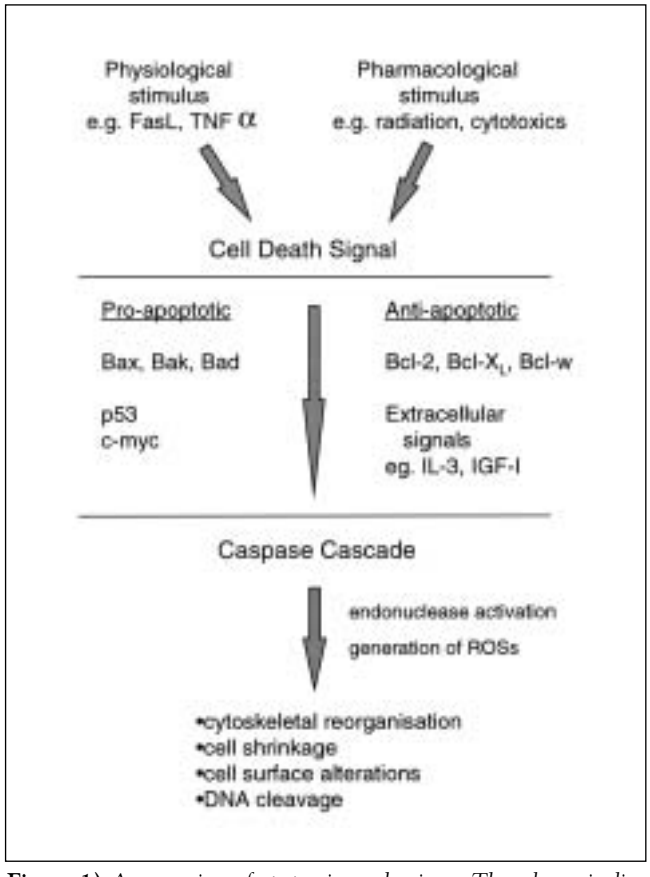

Figure 1) *An overview of apoptotic mechanisms. The schematic diagram illustrates apoptosis initiation, signal transduction, genetic regulation and effector mechanisms. FasL Fas ligand; IGF Insulin-like growth factor; IL Interleukin; ROS Reactive oxygen species; TNF α Tumour necrosis factor alpha*

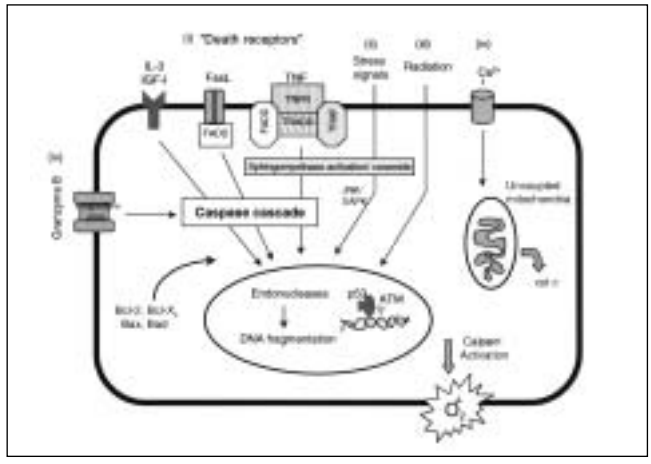

Figure 2) *Schematic diagram illustrating the various pathways of apoptosis resulting in endonuclease cleavage of DNA and regeneration of reactive oxygen species (explained in text). Multiple apoptotic signals converge on caspase activation, many of which are regulated by Bcl-2 family proteins. ATM Ataxia-telangectasia malignancies gene; cyt c Cytochrome c; FADD Fas-associated death domain protein; FasL Fas Ligand; IGF-I Insulin-like growth factor-1; IL-3 Interleukin-3; JNK/SAPK c-Jun kinase/stress-activated protein kinase; TNF Tumour necrosis factor; TNFR Tumour necrosis factor receptor; TRADD TNFR-associated death domain protein; TRAF TNFR-associated factor*

tion it is 15 h (data from our laboratory). A caveat to these remarks is that such half-life measurements relate to the removal of apoptotic bodies by phagocytic digestion and cell migration combined with the true duration of the cell death process. Hall and Coates (20) have described a counting technique termed the 'wandering mean method' in an attempt to overcome some of these limitations.

MECHANISMS OF APOPTOSIS

In its simplest model, the stages of apoptosis can be considered as initiation and signal transduction, genetic regulation (discussed in detail below) and effector mechanisms (Figure 1). There are numerous stimuli of apoptosis acting through a variety of pathways, some of which are as yet poorly defined. Broadly speaking, there are five pathway classes:

- receptor-mediated stimuli, such as glucocorticoids, interleukin (IL)-3 withdrawal and activation of tumour necrosis factor receptor or 'death receptor' family (Fas/CD95/APO-1 and tumour necrosis factor-R1) (reviewed in 21);
- stress signals through the sphingomyelin pathway, including ceramide and c-Jun kinase (reviewed in 22);
- those that induce DNA damage such as x-rays and other forms of ionizing and nonionizing radiation, including *p53* pathways;
- poorly understood pathways such as uncoupling of mitochondria via calcium channels and direct activation of caspase via granzyme B; and
- agents that cause direct physical damage such as heat, cold and ultraviolet light (Figure 2).

The search to understand the downstream effector pathways of apoptosis led to the identification of a whole family of cellular proteases termed 'caspases' (cysteine proteases that cleave after aspartate residues) (reviewed in 23,24). There are at least 13 different types that may be activated in response to different apoptotic signals. Additionally, the distribution of caspase isotypes varies between cells and tissues, and it is likely that different activation systems operate at different sites. The 'classical pathway' includes Bcl-2 family proteins, Apaf-1 (apoptosis proteases-activating factor) and caspase 9 (25). An alternative pathway is ligand-induced aggregation of 'death receptors', leading to activation of caspase 8 via the adapter protein Fas-associated death domain protein (FADD)/Mort1 (reviewed in 26). The precise mechanisms leading to cell death after activation of caspases are generally unclear, although cleavage of DNA by endonuclease is a cardinal feature (27).

GENETIC REGULATION OF APOPTOSIS

***Caenorhabditis elegans* and apoptosis:** Observations made in the nematode *Caenorhabditis elegans* have laid the foundation for understanding the genetic organization of the

TABLE 2
Regulatory proteins of apoptosis

Anti-apoptotic (pro-survival)	Pro-apoptotic
Bcl-2 family	
Bcl-2, Bcl-x_L, Mcl-1,	Bak subfamily:
Bcl-w (128),	Bax, Bak, Bok, Bcl-x_S
	BH-3 only homologues:
	Bid, Bad, Bik, Blk, Hrk, Bim
Others	
RB	p53 family: p53, p73 (59)
Survivin (129)	c-jun (27)
Bcl-10 (130)	c-myc (131)
Bag-1 (132)	

References appear in parentheses

control of mammalian cell death (reviewed in 28,29). During this organism's development, precisely 131 of 1090 somatic cells undergo programmed cell death (30). Eleven genes control this process, of which three, called *ced-9*, *ced-3* and *ced-4* (the *C elegans* 'apoptosome'), have been studied in detail. Normal functioning of *ced-9*, which has striking similarity to the mammalian *bcl-2* gene, is required to prevent cell death (31). Moreover, human *bcl-2* can prevent cell death in *C elegans* and can substitute for *ced-9* in *ced-9*-deficient nematodes. The *ced-3* gene encodes a 2.8 kb mRNA, which has significant homology with the human caspase (32,33). Part of the sequence of Apaf-1 shows striking similarity to that of *ced-4*, while Apaf-2 is homologous to cytochrome c (34).

Mammalian Bcl-2 family genes: The *bcl-2* oncogene was initially discovered as a result of its involvement in the chromosomal translocation t(14;18)(q32;q21) in human B-cell lymphoma (35). Subsequently, it was demonstrated that *bcl-2* prevented the induction of apoptosis in B cells following withdrawal of IL-3 (36). This anti-apoptotic property of *bcl-2* has been demonstrated in many cell systems (reviewed in 37,38). The Bcl-2 protein has a molecular mass of 24 kDa and has been shown to be located in the nuclear membrane, mitochondrial membranes and endoplasmic reticulum (39). Its mechanism of action is not understood, although particular attention has been paid to the possibility that its anti-apoptotic effect is via an antioxidant action (40).

It has become clear that Bcl-2 is only one of a family of related proteins that are involved in apoptosis control (Table 2). These all have a degree of homology with Bcl-2, especially within the conserved domains BH1 and BH2. A third domain, BH3, appears to confer pro-apoptotic activity (41). Bcl-x is one family member that can be alternatively spliced to form either Bcl-x_L (which inhibits cell death) or Bcl-x_S (which promotes apoptosis) (42). Bcl-x protein expression has been demonstrated in a number of tissues, but the distribution is different from that of Bcl-2 (43). Bax (bcl-2-associated x protein) encodes a 21 kDa protein that can either homodimerize or heterodimerize with Bcl-2 (44), and it is proposed that the Bcl-2:Bax ratio determines whether a cell will undergo apoptosis or survival – Bcl-2 in excess protects cells from apoptosis, and Bax in excess (homodimers predominate) induces cell apoptosis. This is known as the Bcl-2/Bax 'rheostat'. Bad and Bak are additional Bcl-2 family pro-apoptotic members that can either homodimerize or heterodimerize with Bcl-2 (45,46). It is likely that these proteins interact with one another and other proteins to different degrees in different cell types to control apoptosis.

The *p53* tumour suppressor gene: The p53 cellular protein was first described by Lane and Crawford (47), and Linzer and Levine (48) as a complex with the simian virus 40 large T antigen in virally transformed rodent cells. Mutant *p53* acts as an oncogene, but wild *p53* is a tumour suppressor gene because transfection into cells suppresses transformation caused by other oncogenes. The gene is located on chromosome 17p in humans, and mutations of *p53* have been found in a wide range of human cancers, including most gastrointestinal malignancies, although the site of mutations depends on tumour type (49-51).

The biochemical function of *p53* is to bind to specific DNA sequences, including the promoters of the *mdm-2* (murine double minute), *Gadd-45* (growth arrest and DNA damage) and *Waf-1/Cip-1* (Cdk-interacting protein) genes. The last of these is probably the most important; the gene product of *Waf-1/Cip-1* is a 21 kDa protein ($p21^{WAF1/CIP1}$) that can bind to cyclin A- and E-dependent kinase II and cyclin D-dependent kinase, preventing progression past the G1 phase of the cell cycle, and may also be involved in controlling G2 progression in some cell types (reviewed in 52).

In 1992, Lane (53) proposed that p53 was the "guardian of the genome". If DNA is damaged, *p53* accumulates and switches off replication to arrest cells in G1 and to allow extra time for repair. If repair fails, *p53* may trigger cell suicide by apoptosis. The importance of this is illustrated by experiments demonstrating that *p53* wild-type mouse embryos, but not *p53* null mice embryos, will readily abort following radiation-induced teratogenesis (54,55). How *p53* induces apoptosis is not clear, and there may be more than one mechanism – p53 alters the balance between Bax and Bcl-2, favouring cell death (56); alternatively, p53 increases the expression of at least 14 genes, termed 'p53-induced genes', of which three are potent generators of reactive oxygen species (57). Whether a cell undergoes *p53*-mediated growth arrest or *p53*-mediated apoptosis is probably related to functional levels of p53 (52) and $p21^{WAF-1/CIP1}$ (58).

There is emerging evidence that, analogous to *bcl-2*, a p53 family of genes is involved in cell cycle control and apoptosis. Two additional members, p51 and p73, have recently been identified (reviewed in 59). Both proteins, at least when overproduced, can mimic the ability of *p53* to induce apoptosis but, in contrast, appear infrequently mutated in human cancers.

Other apoptosis regulatory genes: Recently, an increasing number of transcription activators have been identified in the initiation and manifestation of apoptosis (reviewed in 60). Examples of cell growth inhibitory transcription fac-

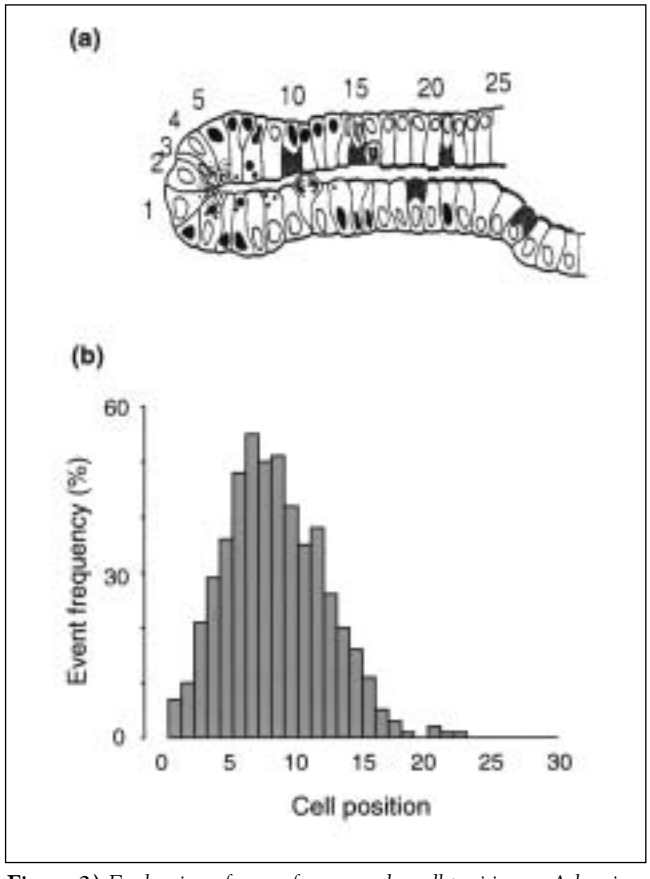

Figure 3) *Evaluation of event frequency by cell position*. **a** *A longitudinal section of a colonic intestinal crypt, illustrating how the position of events up the crypt axis can be determined (position 1 being at the crypt base)*. **b** *When a number of crypt cross-sections are counted, an event frequency at each cell position can be plotted*

tors are insulin receptor substrate-1, signal transduction and activation of transcription (STAT) proteins, and examples of cell growth stimulatory transcription factors are c-myc, AP-1 and nur77.

APOPTOSIS IN THE NORMAL INTESTINE

Intestinal epithelium: The intestinal epithelium is a rapidly renewing tissue in which tissue homeostasis depends on both cell proliferation and cell death (reviewed in 61). Most studies of this renewal process have focused on the control of cell proliferation. However, it is becoming increasingly apparent that the control of cell death is equally if not more important in the regulation of cell numbers and ultimately susceptibility to neoplastic transformation.

Potten and co-workers (reviewed in 62-65) have developed and validated a method for quantifying epithelial cells in relation to their position along the long axis of the intestinal crypt, with position 1 at the base of the crypt (Figure 3). Using this methodology, it is clear that the intestinal crypt has a well defined and polarized topographical organization in which the hierarchy, lineage or cellular 'age' can be assessed by the position of that cell in the tissue. Careful analysis of cell positional behaviour and hierarchies in the crypts of the murine colon and small intestine suggests that the cell renewal in colonic and small intestinal crypts is broadly similar, with a few important differences. In the murine small intestine, there are approximately six stem cells or lineage ancestor cells per crypt located at cell positions 4 to 5, just above the Paneth cells. In the murine colon, the number of stem cells is probably similar, but they are thought to be located at the base of the colonic crypts (cell positions 1 to 2) (66).

Spontaneous and induced apoptosis: When studying apoptosis and using the murine model, intestinal tissue is fixed in Carnoy's fixative, carefully sectioned so that the crypt/villus units can be viewed in longitudinal section and stained with hematoxylin and eosin. Apoptotic bodies and fragments can be readily identified and reliably distinguished from mitotic and normal cells. To quantify apoptosis, each cell position along the long axis of the crypt, counting the base of the crypt as cell position 1, is scored as to whether it contains a normal cell, an apoptotic body or fragment, or a cell undergoing mitosis. These data relating cell status to cell position can be simply analyzed by computer (67). An apoptotic index defined as the total number of cells with one or more apoptotic fragments at that cell position can be calculated. Statistically valid results can be obtained by counting 200 to 300 well orientated half crypt sections from four to six mice (68). Over a decade of performing studies at our laboratory, we have shown that the pattern of spontaneous apoptosis in the small intestine is different from that in the large intestine. In the former, spontaneous apoptotic cells are readily observed but are restricted to the stem cell region (positions 4 to 5), whereas in colonic crypts, spontaneous apoptosis is very infrequent. Some authors report that spontaneous apoptosis occurs predominantly in the outer third compartment of the colonic intestinal crypt (69,70), and others indicate that it occurs within the lower crypt compartments (71); we have observed it occurring in a less topologically restricted way (Figure 4). Critically, however, few apoptotic cells are observed at the base of the colonic crypts, where the stem cells are located. This naturally occurring or spontaneous apoptosis, which is p53-independent, has been interpreted as part of the stem cell homeostatic mechanism. When the process is regressed by bcl-2, the colonic stem cell numbers, and hence carcinogen target cells, may gradually drift upwards with time (63). Additionally, in comparison with the small intestine, the damage-induced apoptosis response in the large intestine is blunted and distributed throughout the crypt. These observations of the differential amounts and position of apoptosis in the crypts led to the hypothesis that damaged small intestinal stem cells were deleted by an 'altruistic' apoptotic process, thereby protecting this site from genetic and carcinogenic damage, whereas in the colon, damaged cells survive with the consequence of increased susceptibility to neoplastic transformation (62).

Investigations of apoptosis at other sites in the gastrointestinal tract are less plentiful. Hall et al (71) found morphological evidence of apoptosis, both at the base of gastric

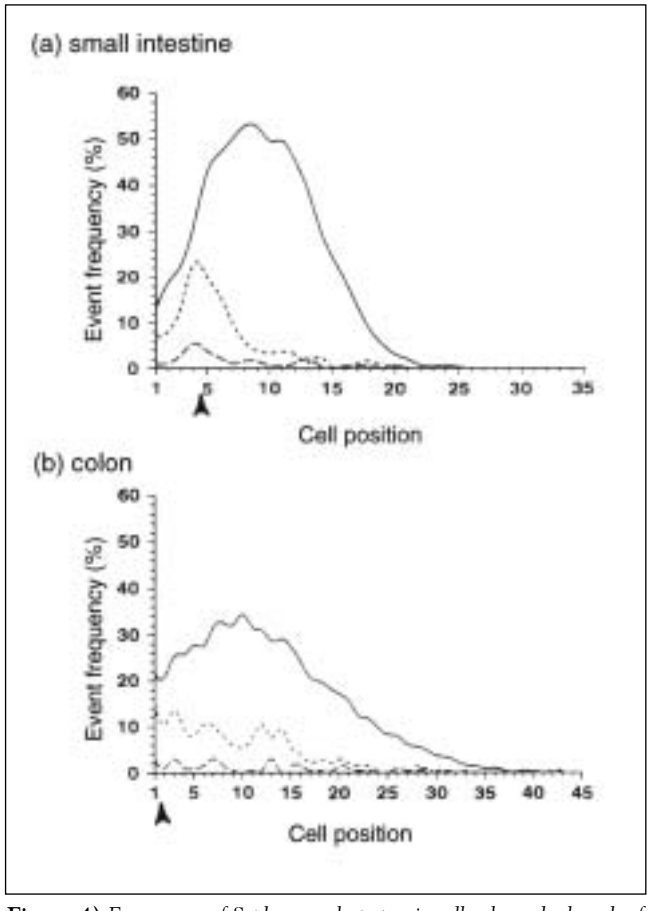

Figure 4) *Frequency of S-phase and apoptotic cells along the length of an intestinal crypt.* **a** *Small intestine.* **b** *Colon. The frequency of cells labelled with tritiated thymidine (S-phase) at each cell position (unirradiated adult mouse) is shown as a continuous line; the frequency of spontaneously occurring apoptosis (unirradiated adult mouse) is shown as a dashed line; and frequency of radiation-induced apoptosis (42 h after 1 Gy gamma-irradiation) is shown as a dotted line. In the small intestine, peak frequency of apoptosis is at cell positions 4 to 6. In contrast, apoptosis is less topographically restricted in the colon. The proposed stem cell positions for small intestine and colon are indicated by the arrowheads*

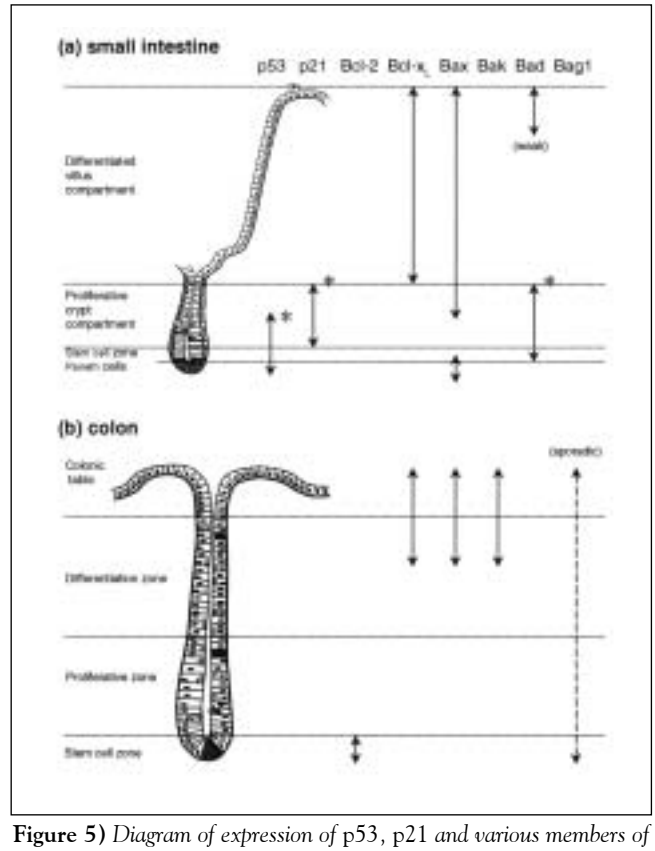

Figure 5) *Diagram of expression of* p53, p21 *and various members of the Bcl-2 family in the small intestinal* (**a**) *and colonic epithelia* (**b**). **Radiation induced*

glands and at the luminal surface, while other studies have reported evidence of apoptosis along the entire length of the gastric mucosal pit (72). *Helicobacter pylori*, the principle cause of type B gastritis and peptic ulcer disease, and classified as a type I carcinogen for gastric cancer (73), regulates gastric cell growth by direct induction of apoptosis. In terms of tumorigenesis, this is paradoxical (see below), but one explanation may be that long term increases in apoptotic rates act as a stimulus to hyperproliferation and the subsequent promotion of neoplasia (74,75).

Bcl-2 family, p53 and apoptosis in the intestinal crypt: Bcl-2 is minimally expressed in the small intestine of both mouse and human but more strongly expressed at the base of colonic crypts in both species, indicating that this may be involved in over-riding the apoptotic (both spontaneous and induced) homeostatic mechanisms in these cells. This hypothesis is supported by the finding that, in *bcl-2* knockout mice, the incidence of spontaneous and induced apoptosis is dramatically increased in the stem cell region of the colon but unchanged in the stem cell region of the small intestine (76).

Immunohistochemical and immunoblot studies from Krajewski et al (77-79), Kitada et al (80), and Wilson and Potten (81) have characterized the intestinal expression of various Bcl-2 family members (Figure 5). Moss et al (69), observing apoptosis mainly at the luminal surface in normal human colonic epithelium, reported a strong positional correlation between Bak expression and apoptosis. Overall, the differential positional expression of pro- and anti-apoptotic factors fits with the hypothesis that their ratios in a given cell ('rheostats') are important in controlling the sensitivity of that cell to apoptosis.

The expression of wild-type p53 in the normal intestinal epithelium is low; therefore, the role of p53 in spontaneous apoptosis was examined in studies comparing normal and p53-knockout mice. Interestingly, the levels of spontaneous apoptosis were similar in both types of mice, indicating that spontaneous apoptosis is p53-independent (68). In contrast, when the changes that occur in p53 expression following exposure to ionizing radiation in the small and large bowel were examined, interesting patterns emerged. In the small intestine, there was a strong p53 immunoreactivity that overlaid the apoptosis cell position incidence frequency plot precisely, whereas radiation damage in the large intestine resulted in increased levels of p53 and apoptosis

occurring at a lower level and distributed along the whole crypt. However, apoptotic bodies were surprisingly negative for p53 protein expression, suggesting that the presence of p53 at cell position 4 (stem cells) is associated with regulation of cell cycle check point genes and/or initiation of repair mechanisms (82). The p53 knockout studies suggested that damage-induced apoptosis is p53-dependent in the small intestine and illustrates the limited ability of colonic stem cells to undergo apoptosis (83).

APOPTOSIS AND INTESTINAL TUMORIGENESIS

Animal models and cancer development: Over the past decade, the experimental use of transgenic models has helped elucidate some of the mechanisms underlying the pivotal role of apoptosis in oncogenesis (reviewed in 84). Transgenic mice bearing a *bcl-2*-immunoglobulin minigene designed to mimic the human t(14:18) translocation highly express *bcl-2* in B cells. As with the human B-cell lymphoma counterpart, lymphoid tissue from these mice progresses spontaneously to a benign hyperplasia and, after many years, to high grade malignant large B-cell lymphoma. The extended latency indicates that *bcl-2* expression alone is insufficient for tumorigenesis and an additional transforming event, such as a rearranged *myc* gene, is required (85).

Additional studies using viral oncoproteins as tools in transgenic mice models have clearly shown a role for apoptosis suppression in tumour progression. Symonds et al (86) used a choroid plexus epithelium (CPE) model – a normally nondividing brain epithelium cell. Expression of a wild T antigen in CPE results in aggressive nonclonal tumour growth, whereas truncated T antigen (T_{121}), which inactivates the pRB (retinoblastoma) protein but not p53, induces very slow growing tumours (87). This indicates that p53 inactivation in these cells contributes to tumour progression rather than initiation. Indeed, tumour growth is retarded by wild-type p53 (88).

Apoptosis in intestinal tumorigenesis: What is the evidence that abnormalities in apoptosis are involved in the development of intestinal tumours? Using morphology to quantify apoptosis, a number of studies have found an increased apoptosis index from normal colonic epithelium through adenomas to the carcinoma stage (89,90). Additional evidence comes from the observation that a number of dietary factors (eg, fibre) and chemopreventive agents (eg, nonsteroidal anti-inflammatory drugs ([NSAIDs]) prevent colonic tumour formation by promoting apoptosis. For example, it has been proposed that the chemopreventive effect of NSAIDs results from increased intestinal apoptosis brought about by decreased expression of Bcl-2 as a result of cyclo-oxygenase (COX)-2 inhibition (91).

A further role of apoptosis in intestinal tumorigenesis may lie at the epithelial-lymphoid cell interface. Fas ligand (FasL) induces apoptosis in sensitive immunocytes (Fas receptor [FasR]/APO-1/CD95 receptor positive) and regulates several immune responses, including contributing to immune privilege. FasL was originally thought to be expressed only in lymphoid cells but has now been shown to be expressed in epithelial cells from many organs. This has been termed the 'Fas counter-attack' (92). FasL expression has been demonstrated in a number of human colon cancer cell lines in vitro (93) and neoplastic colon epithelial cells in vivo (94), suggesting that the Fas counter-attack is a prevalent mechanism of immune evasion in colonic cancers.

Bcl-2 family and p53 expression in intestinal tumours: Many groups have performed (mostly by immunohistochemical means) studies of p53 and Bcl-2 expression in colorectal neoplasia. Accepting the methodological limitations of these approaches (95), a number of general conclusions emerge that suggest that dysregulation of the expression of these apoptosis-controlling genes occurs during colorectal tumorigenesis:

- p53 protein expression occurs more frequently in colorectal carcinomas than in adenomas (96,97) and is associated with a poorer prognosis (98). In sporadic colorectal carcinogenesis, *p53* mutations are likely to be a late event – a hypothesis supported by the observation that p53-null mice do not develop spontaneous colonic tumours (99) and mice with multiple intestinal neoplasia (MIN) mice (which carry a mutation in the adenomatous polyposis coli gene) do not show a change in the spectrum of intestinal tumours when rendered homozygously null for p53 (100).

- Within the conventional adenoma-carcinoma model, adenomas have generally been found to express more Bcl-2 than carcinomas (98,101-104). Watson et al (104) showed that there was reciprocity of expression of Bcl-2 and p53 in some neoplasms that were dually stained for both proteins. This study also demonstrated higher levels of Bcl-2 expression in normal colonic crypts adjacent to carcinomas than in normal crypts more than 5 cm from the tumour, suggesting that changes in Bcl-2 expression occur at an early stage of colorectal tumorigenesis.

- A number of studies have shown that Bcl-2 expression is positively associated with a favourable clinical prognosis, although some data are conflicting (98,105). The finding that colorectal carcinomas that have increased Bcl-2 expression (and consequently, one would suppose less apoptosis) had an improved prognosis is counterintuitive. Although this finding agrees with findings in breast cancer (106) and non-small cell lung cancer (107), it contrasts with findings in other tumours such as lymphoma (108), in which Bcl-2 confers a poorer prognosis. The explanation for this paradox is not fully established, but the finding that colonic adenomas, in general, express more Bcl-2 than adenocarcinomas suggests that carcinomas expressing Bcl-2 are developmentally 'earlier', stage for stage, and hence have a better prognosis.

- One study has shown markedly elevated levels of Bcl-x_L in colonic adenocarcinomas compared with normal intestinal mucosa (109). The study also showed normal expression of the pro-apoptotic protein Bak in adenomas but reduced expression in carcinomas, suggesting that reductions in Bak expression occur early in colorectal tumour progression. Bax expression (pro-apoptotic) was not significantly altered in either adenomas or carcinomas. It has recently been shown that the anti-apoptotic protein Bcl-w is frequently expressed in colorectal adenomas, and particularly in adenocarcinomas, but absent in other carcinomas such as breast, stomach and cervix (110).

THERAPEUTIC MANIPULATION OF APOPTOSIS IN COLORECTAL CANCER

Chemotherapy and radiotherapy: Recent work has demonstrated that virtually all cytotoxic drugs and radiotherapy induce apoptosis in tumour cells (reviewed in 108,111). This discovery highlights future avenues for therapeutic intervention because these diverse cytotoxic treatments have been shown to stimulate a common cell death program. For example, early studies by Ijiri and Potten (67) demonstrated that 18 commonly used cytotoxic drugs and radiation all induced apoptosis in the proliferative compartment of the small intestinal crypts. In vitro and in vivo studies have shown that the early apoptotic events (within 12 to 24 h) observed after administration of cytotoxic drugs or radiation are mediated by DNA damage-induced activation of p53 and, consequently, is completely absent in p53 knockout mice (68,112). However, p53-independent apoptosis may also occur at a later time following cytotoxic insult (82). Consistent with this observation is the fact that, in clinical practice, it has been frequently observed that tumours with mutant p53 are resistant to chemotherapy or radiotherapy. This can now be understood because p53-dependent apoptosis cannot be activated by DNA damage in these tumours (113,114).

COX: There is a wealth of evidence that NSAIDs, which have a principle action of inhibiting COX isoenzymes, COX-1 and COX-2, prevent colorectal cancer (90). The protective effect of NSAIDs may be due to their ability to induce apoptosis, possibly via the inhibition of COX-2 (115). In turn, overexpression of COX-2 increases Bcl-2 expression. Increased apoptosis has been observed in in vivo studies of NSAID-induced colitis (116) and in patients with familial adenomatous polyposis treated with sulindac (117), as well as in in vitro colorectal cell lines following addition of either sulindac or acetylsalicylic acid (118).

Antioxidants: Chemotherapy for disseminated colorectal cancer relies on 5-fluorouracil (5-FU), but its efficacy remains disappointing. Current therapeutic strategies rely on combining 5-FU with other agents that enhance or complement its action (comodulators), such as leucovorin (tetrahydrofolate) and levamisole. Recently, it has been observed that the antioxidants pyrrolidinedithiocarbamate (PDTC) and the water soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylachroman-carboxylic acid enhance 5-FU-induced apoptosis in cultured colorectal cancer cells regardless of their p53 status (119,120). PDTC-induced apoptosis may be mediated via the CCAAT enhancer-binding protein-beta and involve the activation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$. Other new agents that induce apoptosis in transformed cells and that have potential as new anticancer drugs include betulinic acid, paclitaxel (Taxol, Bristol Myers Squibb, Canada) and retinoids (111).

CONCLUSIONS

There are four major messages from this review. First, studies with the small nematode *C elegans* have identified a number of apoptosis-regulating genes – evidence that programmed cell death is an active process under genetic control. Many of these genes have mammalian homologues that seem to regulate mammalian apoptosis. These studies have also led to the identification of the signal transduction pathways of apoptosis and the identification of the caspases. Much remains to be learned in this field. Second, the intestinal crypt is a highly topologically organized system, in which changes can be quantified. These studies have shown that there are fundamental differences in position and function of stem cells, apoptotic processes and regulatory genes in the small intestine versus the large intestine. These differences may explain differences in cancer incidences between these sites. Third, it has become clear that carcinogenesis (and the colorectum is one good example) is characterized by dysregulation of apoptotic programs. Finally, there is an increasing focus on potential manipulation of apoptotic processes, with the hope of being able to interfere with apoptosis regulation and develop new therapeutic concepts.

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