Journal of Cell Science 108, 2261-2271 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Differential expression of bcl-2 in intestinal epithelia

Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia

Anita J. Merritt[1,2], Christopher S. Potten[1], Alastair J. M. Watson[2,3], Dennis Y. Loh[4], Kei-ichi Nakayama[4], Keiko Nakayama[4] and John A. Hickman[2,*]

[1]CRC Department of Epithelial Biology, Paterson Institute, Wilmslow Road, Manchester M20 9BX, UK
[2]CRC Molecular and Cellular Pharmacology Group, School of Biological Sciences, Stopford Building (G38), University of Manchester, Manchester M13 9PT, UK
[3]Department of Medicine, University of Manchester, Hope Hospital, Salford M6 8HD, UK
[4]Howard Hughes Medical Institute, Departments of Medicine, Genetics and Molecular Biology, Washington University School of Medicine, St Louis, MO 63110, USA

*Author for correspondence

SUMMARY

The cell-positional incidence of both spontaneous and damage-induced apoptosis of epithelial cells was assessed in longitudinal sections of the crypts of small intestine and colon of BDF1 mice. This was compared, using immunohistochemistry, with the pattern of expression of bcl-2, a suppressor of apoptosis. In the small intestine, apoptosis was maximal around cell position 4 from the base of the crypt; this closely corresponds to the position considered to contain the stem cells. In the colon, however, apoptosis was not confined to the area considered to harbour the stem cells (position 1 and 2). Instead, apoptosis was attenuated and distributed along the length of the crypt. Some cells at the base of murine colonic crypts expressed bcl-2 protein, whereas bcl-2 was absent in the crypts of the small intestine. Most pertinently, bcl-2 was absent from small intestinal crypt cells at positions 4-5 (the stem cell region). The importance of the expression of bcl-2 to the attenuation of apoptosis in stem cells was confirmed by analysis of the levels of both spontaneous and induced apoptosis in homozygously bcl-2 null C57BL/6 mice: in colonic crypts the level of spontaneous apoptosis rose significantly, and selectively at the base of the crypt, in comparison with crypts from wild-type animals. In contrast, there was no rise in spontaneous apoptosis in the small intestinal crypts from the bcl-2 null animals. Analysis of sections of human colon and small intestine also showed that expression of bcl-2 was confined to the base of the colonic crypt. The attenuation of apoptosis by bcl-2 in the region of the stem cells of the colonic crypts may dispose these to neoplastic transformation. Indeed, analysis of human carcinomas revealed expression of bcl-2, which in some samples was reciprocal with the expression of p53.

Key words: apoptosis, bcl-2, bcl-2 null mice, p53, epithelium, colon carcinogenesis

INTRODUCTION

The conservation of tissue architecture requires a precise maintenance of the balance between cell proliferation, differentiation and loss. Cell loss is not normally an accidental process but instead is programmed. A controlled cell loss is also necessary for the deletion of damaged cells, particularly those whose survival may have the potential to promote the development of malignancy. Programmed cell deaths have a highly conserved morphology, described as apoptosis (reviewed by Wyllie et al., 1980). Some of the features of the molecular control of apoptosis have emerged recently. Perhaps one of the most significant amongst these is the expression of the gene *bcl*-2: its 26 kDa protein product bcl-2 delays or inhibits apoptosis but has no effect on proliferation (reviewed by Reed, 1994). bcl-2 plays a critical role in the development of the immune system, as well as attenuating apoptosis stimulated by, for example, withdrawal of exogenous survival factors or the imposition of genomic damage. Inhibition of apoptosis by bcl-2 is considered to occur through its interactions with a pro-apoptotic homologue, Bax (Oltvai et al., 1993). Other homologues of the bcl-2/bax family of proteins have emerged, such as bcl-x (Boise et al., 1993b). In addition, a rheostat-type of mechanism, balancing pro- and anti-apoptotic signals may also be mediated by members of the ICE (interleukin-1β cleavage enzyme) family of proteases (Wang et al., 1994). Although the expression of bcl-2 was at first considered to be restricted to cells of the lymphoid lineage it has, since become clear that it is more widely distributed, for example in epithelia, including those

of the gastrointestinal tract (Hockenbery et al., 1991; Lu et al., 1993; Krajewski et al., 1994).

We wished to investigate the cell positional expression of bcl-2 in the epithelial cells of small intestine and colonic crypts because previous investigations of ours had suggested a hierarchy in the proclivity of cells to undergo spontaneous or damage-induced apoptosis at these two sites of the intestinal tract (reviewed by Potten, 1992). These differentials in both spontaneous and damaged-induced apoptosis were difficult to reconcile with an expression pattern for bcl-2 which had been reported to be uniform throughout the crypts of both the small and large intestine (Hockenbery et al., 1991). In particular, we had observed that the numbers of cells undergoing spontaneous apoptosis was greater in murine small intestinal crypts when compared with the colon, a difference which was amplified after damage (Potten et al., 1992), and that the apoptotic deletion of cells in the small intestine, spontaneously or after carcinogen treatment, was greatest in the region of what has been suggested to be the stem population at approximately cell position 4 (see Fig. 1C), above the Paneth cells, numbering from the base of the crypt. In contrast, in crypts of the colon, there was an overall attenuation of apoptosis with no focus around the stem cell population, which in this site is located at the very base of the crypt (positions 1-2, Fig. 1D) (Li et al., 1992). These observations led to the hypothesis that a difference in the amounts and position of apoptosis in the crypts of the small intestine and colon might contribute to an explanation of the greater incidence of carcinomas in the colon than in the small intestine, where only 1% of intestinal tumours are found (Goligher, 1980): damaged stem cells in the small intestine would be removed by what was termed an 'altruistic' apoptosis whereas stem cells in the colon might survive an initiating event (Potten et al., 1992, 1994).

We report here that the expression of bcl-2 is not uniform in the intestinal epithelia. Instead, immunohistochemistry shows that, in both the BDF1 mouse and man, bcl-2 expression is maximal in the colonic crypt, where it is expressed at the base, in a position coincident with the stem cells. In contrast, expression in the small intestine was found to be greatly attenuated, including cells at the stem cell position, which readily undergo spontaneous and damage-induced apoptosis. Both spontaneous and DNA damage-induced apoptosis were very significantly increased at the base of the colonic crypts of mice rendered homozygously null for the expression of bcl-2 (Nakayama et al., 1994), data that strongly support the idea that expression of the *bcl*-2 gene plays a significant role in determining the greater survival potential of colonic stem cells, and may promote the retention of cells with a damaged genome, which then go on to become malignant.

MATERIALS AND METHODS

Estimation of epithelial cell apoptosis in murine intestinal crypts

BDF_1 mice or C57BL/6 homozygously bcl-2 null mice (Nakayama et al., 1993) were fed a diet of 'RM1 expanded' (Special Diet Services, Witham, Essex, UK) and water ad libitum. Ten- to twelve-week-old male BDF_1 mice or 7- to 10-week-old homozygously bcl-2 null or wild-type mice of either sex, genotyped as described previously (Nakayama et al., 1994) were irradiated using ^{137}Cs γ-rays, at 3.6 Gy/min. From a group of four treated or four control mice, intestines were dissected, fixed in Carnoy's fixative, transverse intestinal sections were prepared from the entire length of the small intestine and colon, as described (Merritt et al., 1994), and between 150 and 200 half crypts were scored for apoptosis, on a cell positional basis, as described in detail previously (Ijiri and Potten, 1983, 1987; Li et al., 1992; Harmon et al., 1992).

Identification of apoptosis by terminal deoxynucleotide transferase labelling

This was performed using a slight modification of the Apoptag method (Oncor, Gaithersberg, MD) in order to verify our previous estimates of the position and incidence of apoptotic cells in the gastrointestinal tract, when haematoxylin/eosin staining was routinely used. In brief, 3 μm paraffin sections of small intestine from an irradiated mouse (3 hours after 8 Gy) were dewaxed and taken through an ethanol series before washing with phosphate-buffered saline (PBS). Sections were permeablised with proteinase K (20 μg/ml in PBS) and then washed in PBS. Endogenous peroxidase activity was blocked by incubating sections in 0.3% hydrogen peroxide solution for 15 minutes, followed by washing in PBS. Two drops of the equilibration buffer were applied to each specimen for 5-10 minutes at room temperature. The terminal deoxynucleotide transferase (TdT) mixture (Apoptag) was prepared as directed, but then diluted 1:5 with distilled water. At this strength, we found a reduction of non-specific background staining, particularly of the nuclei of the villi of the small intestine. Slides were incubated with or without the enzyme mixture for 1 hour at 37°C before the reaction was terminated by washing sections for 10 minutes in the 'stop wash' solution provided. Digoxigenin-labelled dNTPs were treated with antidigoxygenin-peroxidase for 30 minutes at room temperature. Sections were washed in PBS and developed with 0.05% diaminobenzidine and 0.03% hydrogen peroxide in PBS for 5 minutes, followed by a weak haematoxylin counterstain.

Immunohistochemistry

Normal BDF_1 mouse, homozygously bcl-2 null C57BL/6 mouse, or human colon and small intestine were fixed in formal saline. All tissue samples were embedded in paraffin and processed for immunohistochemistry. Ten sections were prepared from each of four of the different strains of mice; nine samples of normal human colon and seven of normal small intestine were obtained. A rabbit polyclonal antibody raised against a synthetic peptide of murine bcl-2 protein (amino acid residues 44 to 55) was a kind gift from Gerard Evan (ICRF, London) and was used alone or together with blocking peptide to ensure specificity. The murine monoclonal antibody to human bcl-2 protein (DAKO-bcl-2, 124) was purchased from DAKO (High Wycombe, UK). Sections were microwaved at 90°C for 25 minutes in citrate buffer, pH 6.0, then all samples were routinely blocked for 30 minutes in 1:10 (v/v) normal goat serum (murine samples) or horse serum (human samples) diluted in phosphate-buffered saline (PBS) prior to addition of antibody. The antibody to murine bcl-2 was diluted 1:1000 (v/v) in PBS plus 0.5% bovine serum albumin and that to the human antibody 1:100, then incubated with the sections for 60 minutes. After a PBS wash, the preparations were then incubated either in biotinylated goat anti-rabbit IgG (Vector, Peterborough, UK) diluted 1:200 in PBS for 60 minutes, or biotinylated horse anti-mouse IgG (Vector, Peterborough, UK) used at 1:200. Samples were then sequentially washed in PBS and in either avidin-D FITC (Vector, Peterborough, UK) diluted 1:200 in PBS for 60 minutes or ABC peroxidase (Vector, Peterborough, UK) for 60 minutes. Peroxidase-stained sections were developed with diaminobenzidine. Control preparations of murine tissues were incubated in the absence of primary antibody or with the murine antibody absorbed with synthetic peptide at a molar ratio of peptide:antibody of 1:1. Immunostaining was evaluated by both conventional and confocal microscopy (Biorad-MRC 600). Lymphocytes in Peyer's patches within each section were used as internal positive controls. Immunostaining of

p53 in the human samples was performed essentially as above but using the murine monoclonal antibody clone 1801, which recognises both the wild-type and mutant forms of p53 (Oncogene Science, Cambridge).

Analysis of human biopsy samples

Fresh biopsy samples were obtained either at colonoscopy or following surgical resection. Samples were prepared as for normal tissue (above) and analysed for expression of bcl-2 and p53 protein. Seven samples of small intestine, 9 of colon and 8 colorectal tumours were obtained, with 5 of the tumours staged according to the Duke's classification.

RESULTS

Distribution of spontaneous and radiation apoptosis

Fig. 1 (C and D) shows the typical morphological appearance of apoptosis in the crypts of murine small intestine and colon after irradiation. Apoptosis at each cell position was scored by the appearance of both apoptotic cells and/or apoptotic bodies inside an epithelial cell, at each cell position, numbering from the base of the crypt. Fig. 1 (A and B) illustrates the difference in the incidence of spontaneous and radiation-induced apoptosis at each cell position in the crypt in these two areas of the gastrointestinal tract of normal mice, recorded as an apoptotic index. The distribution of apoptotic cells, after 1 Gy of irradiation, shows a peak centred on position 4 in the small intestine. This is the position previously ascribed to the stem cells of the small intestinal crypt (Potten, 1992). No pronounced peak of radiation-induced apoptosis was observed in the colon, where the numbers of apoptotic cells, recorded from 200 half crypts, were reduced in comparison to the small intestine. The stem cells of the colonic crypt, assigned to position 1-2 (Potten, 1992), were not associated with high levels of apoptosis.

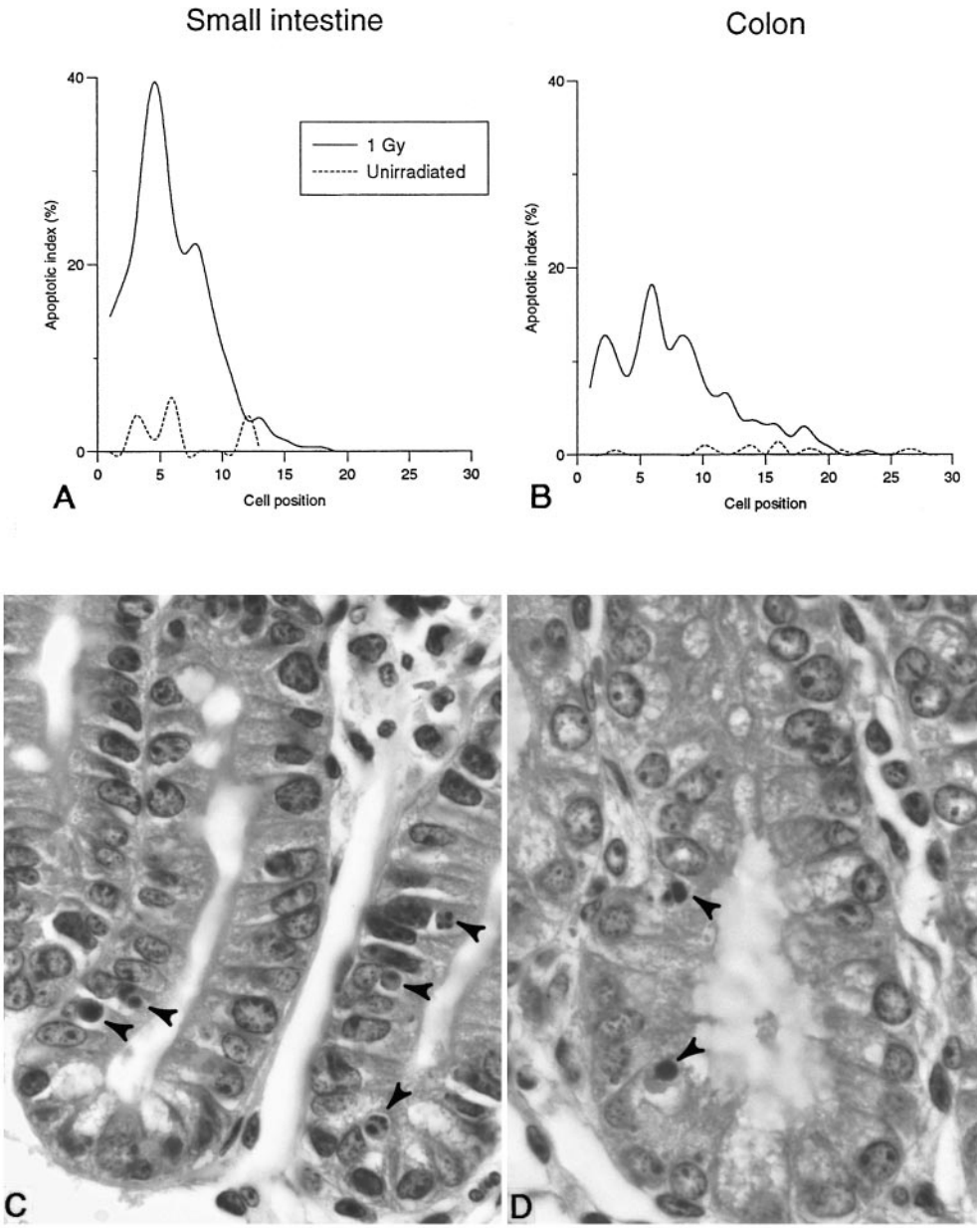

Fig. 1. Spontaneous and radiation-induced apoptosis in murine small intestine (A and C) and colon (B and D). (A and B) The positional distribution of spontaneously apoptotic cells in the crypts taken from control animals or those sacrificed 3 hours after 1 Gy of whole body γ-irradiation. (C and D) Haematoxylin- and eosin-stained transverse sections of the crypts; arrowheads, apoptotic bodies. C and D, ×1000.

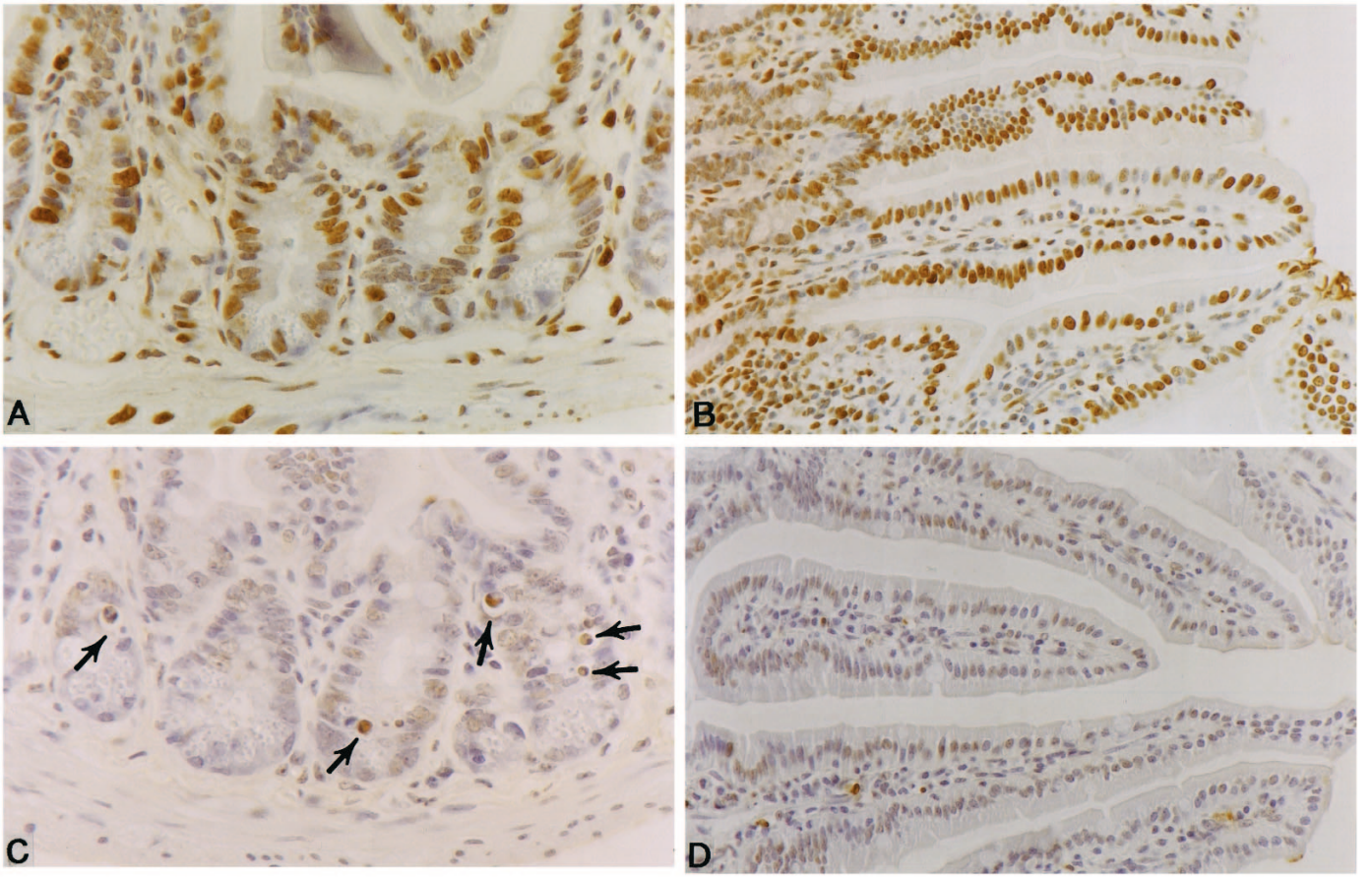

Fig. 2. Detection of apoptosis in the crypts of the small intestine by terminal deoxynucleotide transferase labelling of DNA strand breaks. (A and B) Normal murine small intestine crypts (A) and villi (B) prepared and treated according to a standard protocol (Oncor, see Materials and Methods); (C and D) the results of terminal deoxynucleotide transferase labelling by a modified protocol (see Materials and Methods): arrows, apoptotic cells. B and D, ×100; A and C ×250.

In order to validate the results obtained in Fig. 1 using another method of detection of apoptotic cells, we also used the terminal deoxynucleotidyltransferase reaction to detect cells with DNA having fragments with free 3′-OH ends (Gavrieli et al., 1992). Fig. 2 shows a representative result from terminal deoxynucleotidyltransferase labelling of the irradiated small intestine. Essentially, judicious use of this technique for the detection of apoptotic cells validates the data for both the positional and quantitative distribution of apoptotic cells in the crypts scored from examination of haematoxylin/eosin-stained sections (see Fig. 1C and D). Indeed, in some of the positive cells condensed chromatin may be observed (Fig. 2C). However, in our hands, the standard protocol provided an overestimation of the extent of apoptosis in the crypt and, like Gavrieli et al. (1992), we found that the cells of the villi also underwent labelling, although we have found no morphological evidence of significant apoptosis here (Fig. 2B). This is discussed further below but this technique was not routinely employed in the quantitative estimations of the incidence and cell position of apoptosis.

Distribution of bcl-2 protein in the murine intestinal tract

Immunohistochemical staining of the bcl-2 protein, using normal human and murine tissue and two different antibodies, produced a consistent pattern of staining in both species. Fig. 3 shows the distribution of bcl-2 protein in the base of the crypts of transverse sections taken from the colon and the small intestine of BDF_1 mice. There was little expression of bcl-2 protein in any cells of the murine small intestine (B and F). Peyer's patches from the same section are shown (D), with the lymphocytes showing strong positivity of bcl-2 staining. The expression of bcl-2 at high levels also appeared to be in some mesenchymal cells and from occasional cells, assumed from their position and morphology to be intraepithelial lymphocytes. In contrast to the minimal staining of the murine small intestine, the murine colon expressed bcl-2 protein in a few of the cells at the base of the crypts, where the stem cell compartment is considered to be (Potten, 1992) (Fig. 3A and E). In order to verify the specificity of staining of this antipeptide antibody to murine bcl-2 we also stained the normal mouse thymus: this showed a clear discrimination between areas of the medulla, which are positively stained, and reduced staining of the cortex (data not shown). Close inspection of the peroxidase-stained sections of the intestinal epithelia and the Peyer's patches (Fig. 3) and fluorescently labelled preparations (data not shown) suggested that bcl-2 staining was cytoplasmic, with a particular association with the nuclear membrane. Preincu-

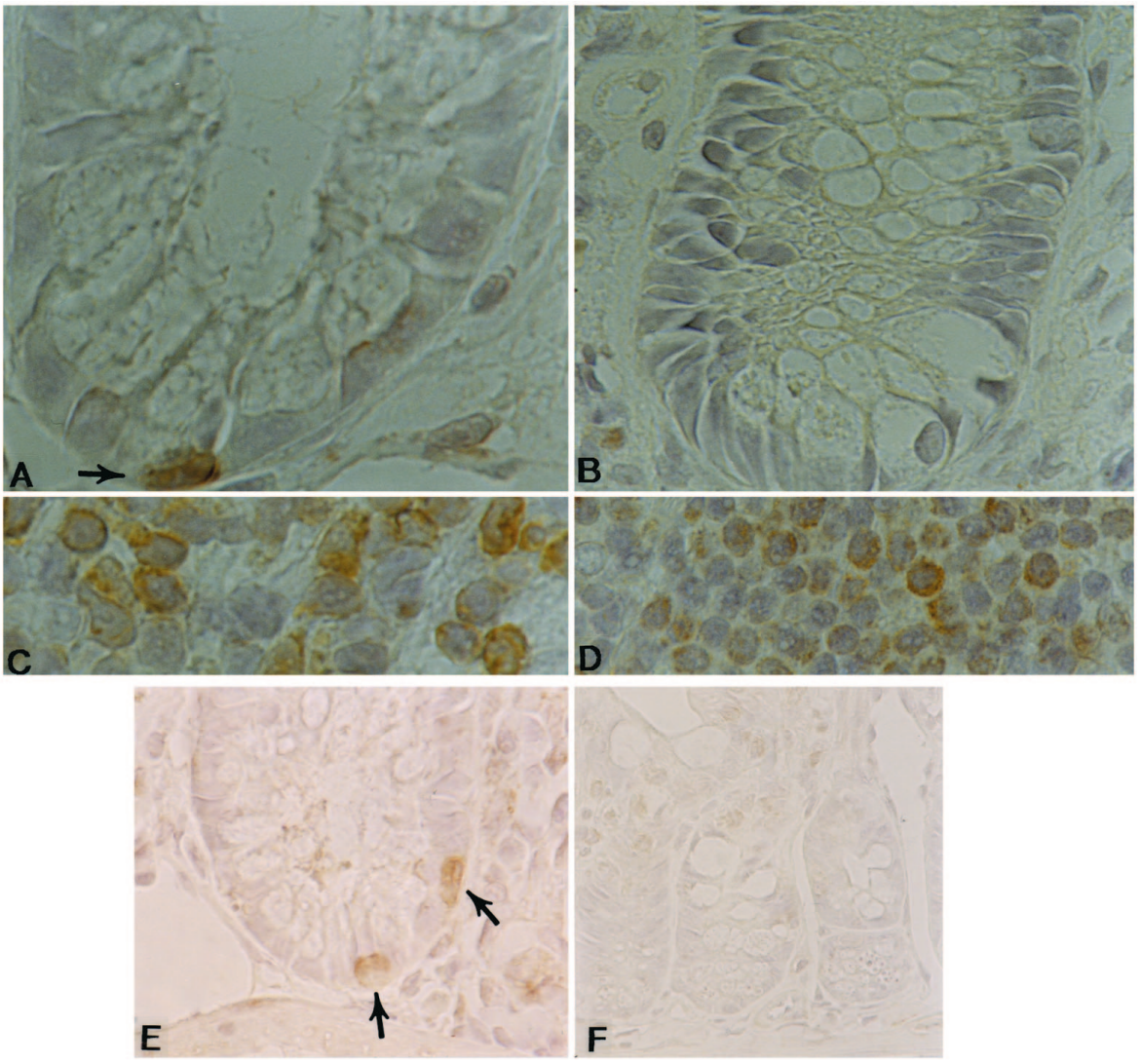

Fig. 3. Distribution of bcl-2 protein in: (A) a murine colonic epithelial crypt, (B) a small intestine crypt. The arrows show examples of cells with peroxidase staining for bcl-2 protein. (C and D) Positive staining of the lymphoid cells from a Peyer's patch from adjacent tissue from the same section. (E and F) Lower power pictures of colon and small intestine from other mice showing the same pattern of staining as seen in A and B. A and C, ×1300; B and D, ×1000; E, ×600; F, ×250.

bation of the antibody for 1 hour with the peptide sequence to which it was raised (equal amount of protein) completely inhibited staining.

Distribution of spontaneous and irradiation-induced apoptosis in bcl-2 null mice

The pattern of distribution of bcl-2 in the small intestine (essentially undetectable) and colon (restricted to the base of the crypts) (Fig. 3) strongly suggested that bcl-2 might play an important role in the differential distribution of apoptosis at these two sites of the gastrointestinal tract (Figs 1 and 2). To formally test this hypothesis the patterns of apoptosis were measured in homozygously bcl-2 null mice, generated by homologous recombination (Nakayama et al., 1994). Fig. 4B shows there was a profound increase in the numbers of spontaneously apoptotic cells at the lowest cell positions of the colon, corresponding to those positions where we had observed bcl-2 staining (Fig. 3), in comparison to the crypts from both the homozygously bcl-2 positive C57BL/6 mice and the wild-type BDF_1 mice (compare with Fig. 1). In contrast, the incidence of spontaneous apoptosis in the small intestinal crypts of the null mice and homozygously positive mice was not altered (Fig. 4A). These differential patterns of the expression of apoptotic cells were preserved after irradiation (Fig. 4C and D), when there was an increased incidence at the base of colonic crypts of the null mice but little change in the small intestinal crypts from null or homozygously positive animals.

Distribution of bcl-2 protein in the human intestinal tract

Analysis of tissues from the normal human gastrointestinal tract (Fig. 5) yielded similar results to those observed in the mouse: there was only very sporadic bcl-2 staining in crypts of the 7 samples of fresh human small intestine investigated, consistent with what we suspect to be the occasional intra-

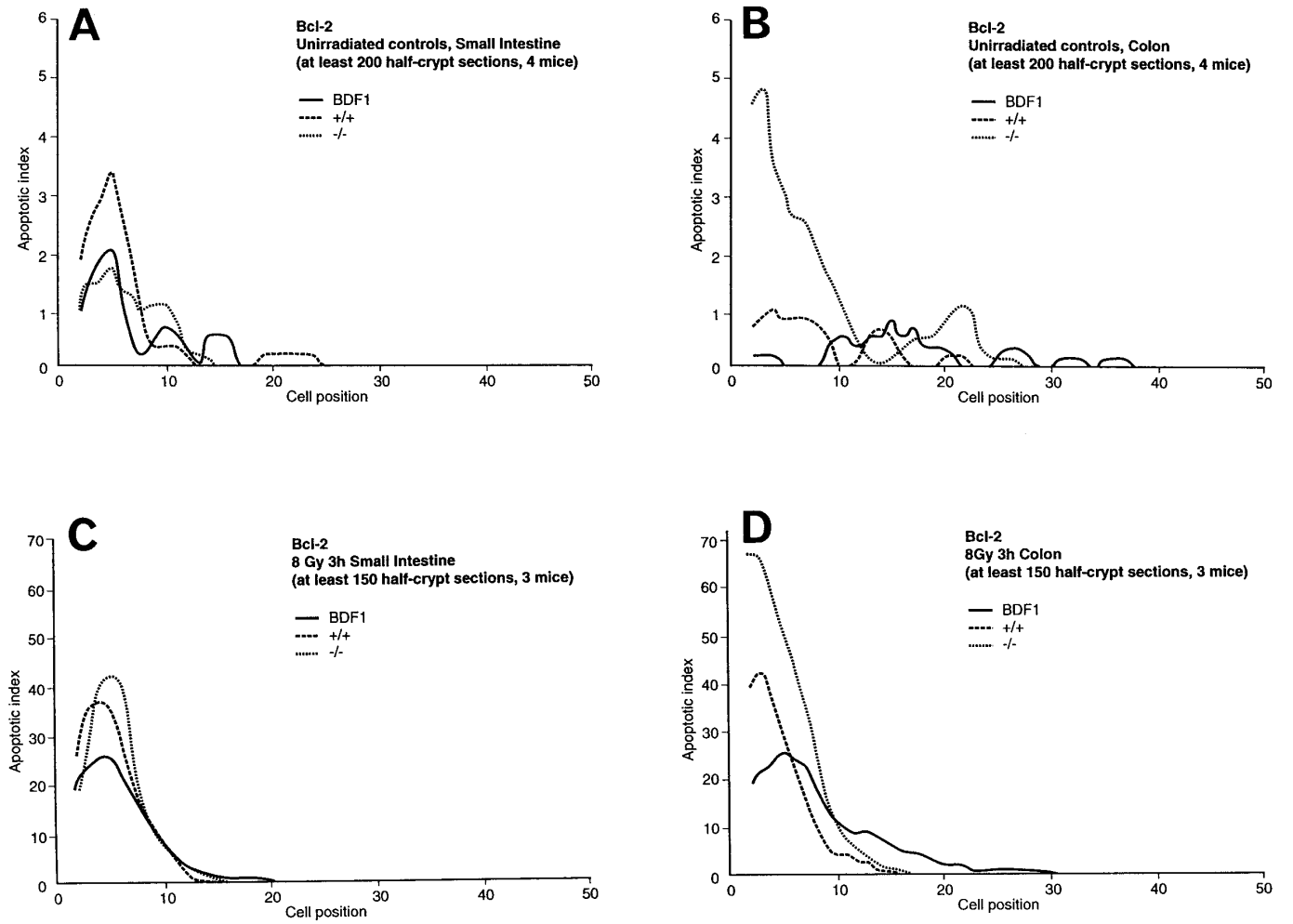

Fig. 4. Spontaneous and radiation-induced apoptosis in homozygously null (–/–) wild-type (+/+) C57BL/6 murine small intestine and colon. The graphs show the positional distribution of spontaneously apoptotic cells in the crypts taken from control animals or those killed 3 hours after 8 Gy of whole body γ-irradiation. The data for BDF_1 mice are shown for comparison. A. Levels of spontaneous apoptosis in the small intestine; B, levels of spontaneous apoptosis in the colon; C and D, levels of radiation-induced (8 Gy) apoptosis in the small intestine and colon, respectively.

epithelial lymphocyte (Fig. 5F and G, where no positive cells are shown). In contrast, normal human colonic crypts showed prominent peroxidase immunostaining for bcl-2 protein in some cells at the very base of the crypt (Fig. 5A,B,D,E), essentially as has been reported recently (Hague et al., 1994). Positive staining of lymphocytes was observed in Peyer's patches of colon (C) and small intestine (H).

Expression of bcl-2 and p53 in human carcinomas

Analysis of bcl-2 protein in seven carcinomas by immunohistochemistry showed that many tumours were positive with a cytoplasmic distribution of bcl-2, the levels of which varied between samples (Fig. 6). A well differentiated colonic adenocarcinoma (Dukes stage B) showed a positive cytoplasmic distribution in many areas of the tumour (Fig. 6A), similar to the pattern reported recently by Hague et al. (1994). When the same sample was stained for the presence of p53 protein, which is encoded by a gene considered to be mutated in later stages of tumour progression (Fearon and Vogelstein, 1990), the protein was shown to be present in the nucleus of some scattered cells, throughout the tumour (Fig. 6B). Peroxidase immunostaining of another colonic adenocarcinoma (Duke's stage C with liver metastases) also showed intense staining of bcl-2 protein in the cytoplasm in some areas with sharp delineation of positive and negative areas (Fig. 6C). In this case the expression of p53 in an adjacent section (D) appeared to be the reciprocal of that of bcl-2, with bcl-2 positive areas being p53 negative and vice versa (Fig. 6C and D). Areas strongly positive for p53 were often negative for bcl-2 (Fig. 6F and E). This reciprocity between the expression of the bcl-2 and p53 genes was found in other tumours (data not shown) and we are continuing to accumulate data so as to attempt to determine the relationship between disease stage and the expression of bcl-2 and p53.

DISCUSSION

The determination of a survival hierarchy in a tissue, which is reflected reciprocally in the ability of its cells to undergo

apoptosis, will be dependent upon factors which modulate the stimuli for the engagement of cell death (such as DNA damage repair) and upon the expression of genes which attenuate the apoptotic process itself. Bcl-2 appears to belong to the latter class of modulators (see Introduction). The crypts of the small intestine and colon provide interesting examples of different positional hierarchies of survival and apoptosis (reviewed by Potten, 1992). In the small intestine, spontaneous levels of apoptosis exceeded those observed in the colon and, remarkably, were maximal in the stem cell region above the base of the crypt, at position 4, particularly after irradiation (Fig. 1A and C). In contrast, in the colonic crypts there was no peak of either spontaneous or radiation-induced apoptosis, particularly at their base, where the stem cells are harboured (Potten, 1992) (Fig. 1B and D). This differential distribution of apoptosis in the region of the stem cells may reflect the greater number of stem cells in the small intestine compared with the colon, but the best estimates at present suggest similar numbers (about 4) in each site (Potten and Loeffler, 1990). We have suggested that an occasional excess of stem cells, perhaps generated by symmetric stem cell division, might be deleted by spontaneous or non-damage-induced apoptosis (Loeffer et al., 1992; Potten, 1992; Potten et al., 1994; Merritt et al., 1994). Recent studies demonstrated that the homozygously null p53 genotype showed no change in the numbers of spontaneous apoptotic cells in the small intestine and colon, compared to the wild-type genotype, suggesting that a DNA damage-driven p53 response, perhaps initiated during replication, is not a stimulus for these cells to be deleted spontaneously (Merritt et al., 1994). Previous claims that apoptotic cell death occurs along the length of the villus of the small intestine, by measurement of DNA strand breaks in cells (Gavrieli et al., 1992), are not concordant with the present study (Fig. 2) or with any of our previous morphological analyses of apoptosis in the crypts (Ijiri and Potten, 1983, 1987; Potten et al., 1992; Harmon et al., 1992), where morphological evidence of apoptosis in the villus was extremely rare. We have previously suggested that the occasional apoptotic cell observed in the villus is probably derived from cells undergoing cell death at lower positions, in the crypt (Potten and Allen, 1977). In our hands, the observations of DNA strand breaks along the villus by the terminal transferase reaction (Gavrieli et al., 1992) were the result of an excessive sensitivity of the preparations, as discussed by Hall et al. (1994), and were not accompanied by a classical apoptotic morphology (Figs 1 and 2).

The analysis of bcl-2 staining in the murine small intestinal and colonic crypts (Fig. 3) presented a pattern which nicely reflected their different relative propensities to undergo spontaneous or damage-induced apoptosis: thus there was little or no staining in the positions suggested to harbour small intestinal stem cells (position 4) which readily undergo apoptosis, but there was expression of bcl-2 at the position of the colonic stem cells (position 1), which do not readily undergo apoptosis (see Fig. 1). This differential pattern of bcl-2 staining was also observed in sections of normal human small intestine and colon (Fig. 5). A recent report of bcl-2 staining in colonic crypts adjacent to adenomatous and carcinomatous tissue also suggested that the base of the colonic crypt was the most heavily stained (Hague et al., 1994). Hockenbery et al. (1991), in a wide-ranging survey of bcl-2 staining in various tissues, suggested that enterocytes undergo a programmed cell death after terminal differentiation. In support of their claim *bcl*-2 expression was restricted to the lower regions of the crypts of both small intestine and colon and absent in the villi. However, as discussed above, we have rarely observed apoptosis in differentiated enterocytes and, like Hague et al. (1994) and Krajewski et al. (1994), we find the expression of bcl-2 was most intense at the base of the colon (Figs 3 and 5) although absent in the small intestine. Thus our analysis of the morphology of apoptosis by conventional staining and terminal deoxynucleotide staining (Figs 1 and 2) does not support an analysis of cell death on the villi, nor does it support the observations of expression of *bcl*-2 in the small intestine (Hockenbery et al., 1991), which we conclude was due to non-specific staining. The importance of the expression of bcl-2 to the survival of epithelial cells at the base of the colonic crypts was confirmed in experiments which used homozygously bcl-2 null animals (Nakayama et al., 1994). There was an increase in the levels of spontaneous apoptosis observed in cells at the base of the colonic crypts in the null animals compared to the homozygously positive animals in which bcl-2 was expressed at this position (Fig. 4B). In contrast, no such increase in spontaneous apoptosis was observed in the small intestinal crypts of the null animals (Fig. 4A), confirming that bcl-2 expression is not important in controlling the survival of these cells (Fig. 3). This pattern was recapitulated after irradiation (Fig. 4C and D), strongly supporting our contention that bcl-2 plays a significant role in attenuating both spontaneous apoptosis (for which the stimulus is unknown) and that induced by potentially carcinogenic DNA damage.

The distribution of apoptosis found in the small intestine (Fig. 1), might suggest that bcl-2 expression might be expected to be restricted to those cells in the small intestinal crypt which do not readily undergo either spontaneous or induced apoptosis. Examples would be the Paneth cells at the very base of the crypt or the rapidly proliferating cells in the mid crypt (Fig. 1). The attenuation of apoptosis in the small intestine epithelial cells at positions away from the stem cells at position 4, which readily undergo apoptosis (see Fig. 1), must be mediated by other factors, since we observed negligible bcl-2 staining at any position in this tissue, nor was the incidence of apoptosis increased in the bcl-2 null animals (Fig. 4). Whether the diminished apoptosis here is the result of a modulation of the stimulus (enhanced DNA repair, for example) or due to factors controlling the apoptotic process itself (such as increased trophic factor signalling or the effects of the extracellular matrix) remains to be determined. Studies are currently underway to estimate the expression of the homologues of *bcl*-2, such as *bax*, *bcl*-x_S and *bcl*-x_L (Boise et al., 1993b; Oltvai et al., 1993), in these tissues as it is likely that they may also regulate epithelial cell survival and apoptosis. Indeed, a recent report from Reed's group (Krajewski et al., 1994) shows significant immunohistochemical staining of bax in the small intestine at positions, like the Paneth cells, in which apoptosis is attenuated, suggesting that modes of promoting survival other than bcl-2 must contribute here.

The overall reduced level of radiation-induced apoptosis observed in the colonic epithelial cells, in comparison with those from the small intestine (Fig. 1), could also result from their differential capabilities to repair damage. However, expression of *bcl*-2 has been shown to provide a survival

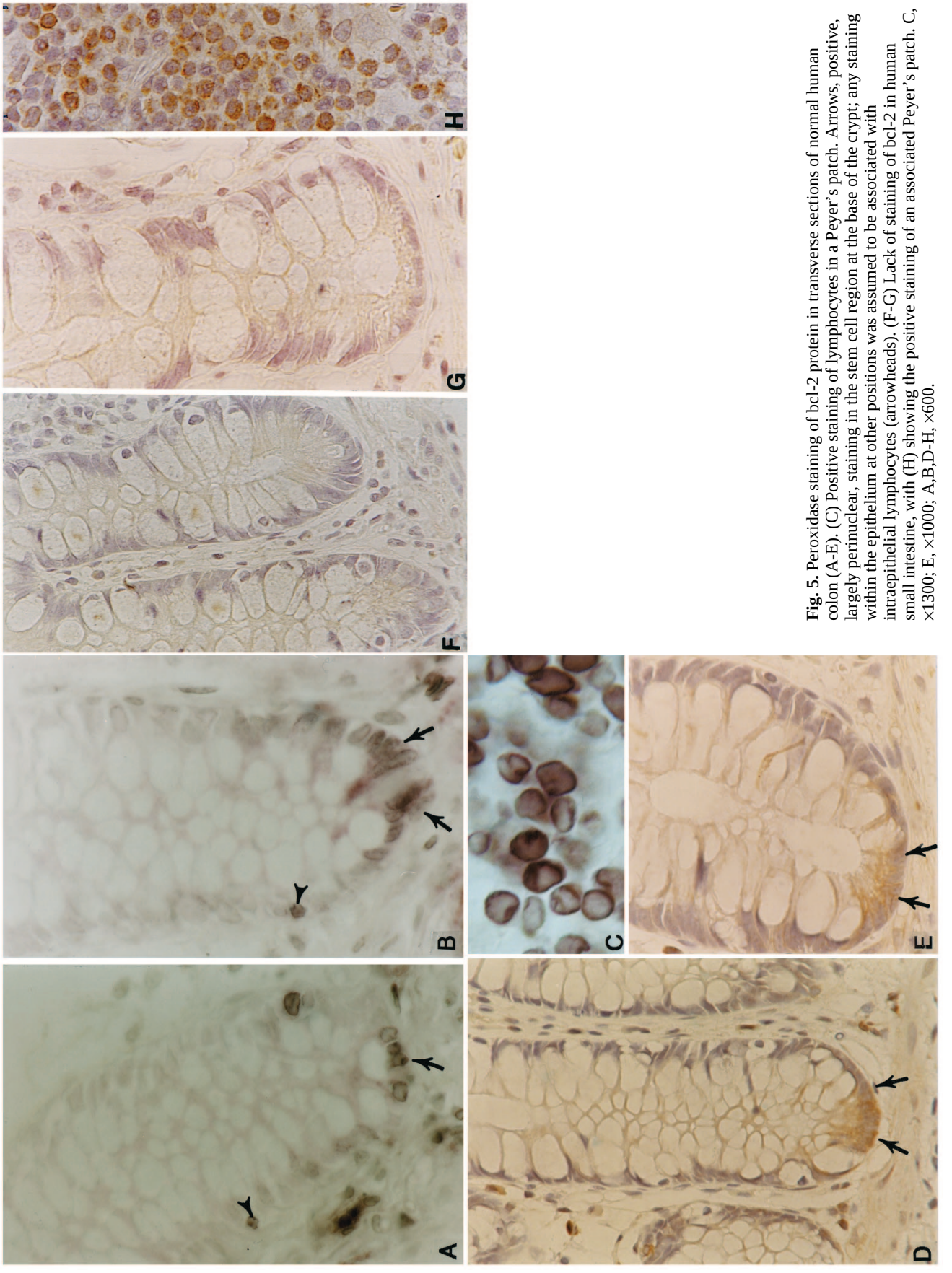

Fig. 5. Peroxidase staining of bcl-2 protein in transverse sections of normal human colon (A-E). (C) Positive staining of lymphocytes in a Peyer's patch. Arrows, positive, largely perinuclear, staining in the stem cell region at the base of the crypt; any staining within the epithelium at other positions was assumed to be associated with intraepithelial lymphocytes (arrowheads). (F-G) Lack of staining of bcl-2 in human small intestine, with (H) showing the positive staining of an associated Peyer's patch. C, ×1300; E, ×1000; A,B,D-H, ×600.

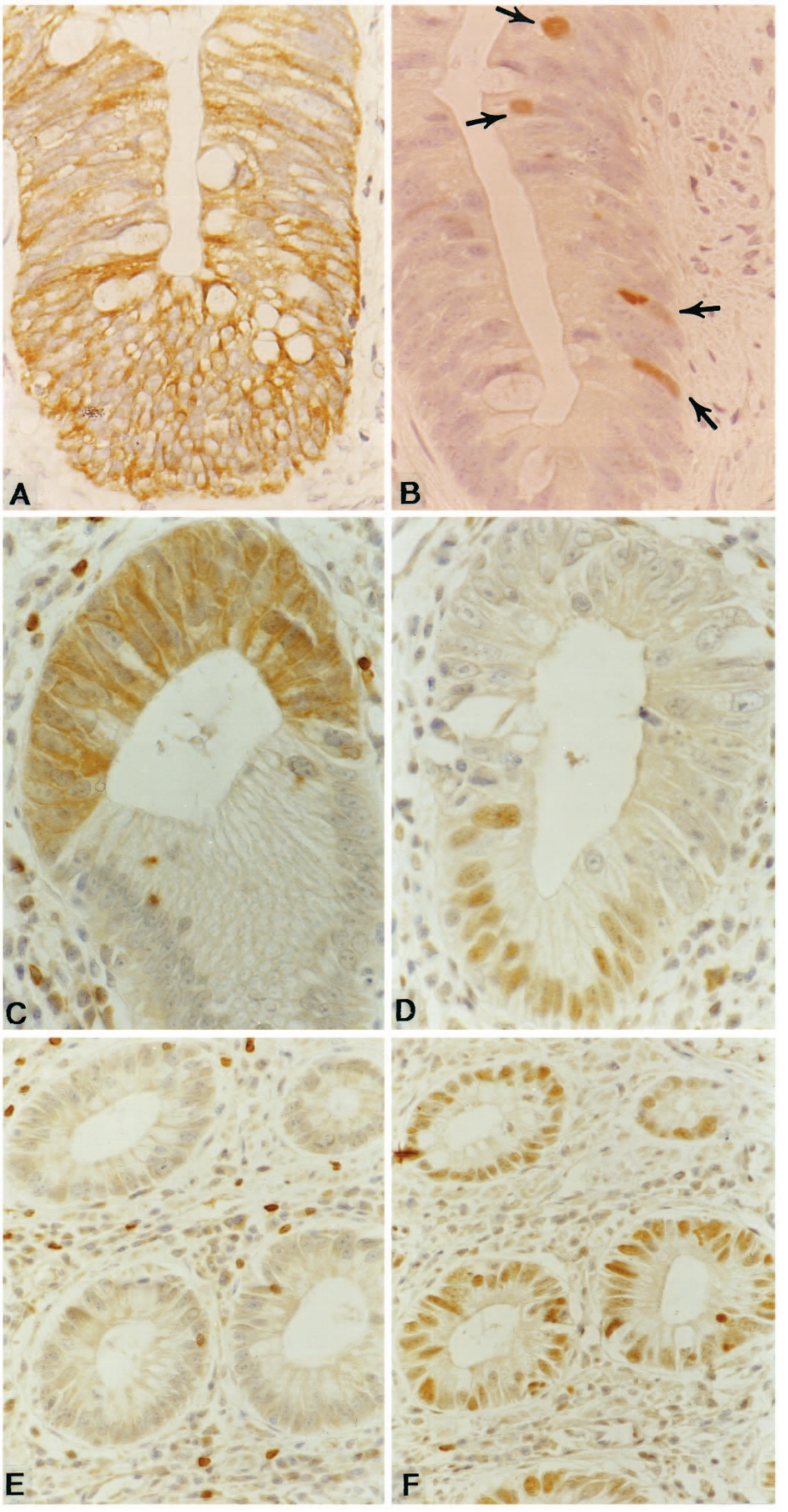

Fig. 6. Peroxidase staining of a 5 µm sections of a formalin-fixed, paraffin-embedded sections of human colon carcinomas for bcl-2 (A,C,E) and p53 protein (B,D,F). (A and B) Moderately differentiated invasive human adenocarcinoma (Dukes stage B), bcl-2 is fairly homogeneously expressed but p53 is sporadically expressed. (C and D) From a Dukes stage C adenocarcinoma, showing the reciprocity of staining for bcl-2 and p53. A different region from the same Dukes stage C tumour (E and F) showed no bcl-2 staining but positive staining for p53. B-D, ×750; A,E,F, ×600.

advantage to cells where equal amounts of cellular damage had been induced either by radiation (Collins et al., 1992) or DNA damaging drugs (Miyashita and Reed, 1992, 1993; Walton et al., 1993; Fisher et al., 1993). The persistence of cells in the colon which carry a damaged genome, because of the expression of *bcl*-2, would be a critical early event of the carcinogenic process. These may then progress to undergo further damage, as proposed by the schemes of Fearon and Vogelstein (1990). For example, somatic mutations of the *MSH2* gene, preventing DNA mis-match repair, have recently been described in hereditary nonpolyposis colorectal cancer (Parsons et al., 1993; Papadopoulos et al., 1994). Whilst mismatched repair is likely be very important in initiating neoplasia, the cell must first have the capability to survive the resultant mutations: bcl-2 expression may, perhaps with other factors, promote that survival. Furthermore, the selective positional expression of bcl-2 in colonic epithelia is accompanied by an attenuated expression of the product of the tumour suppressor gene p53: we recently showed that p53 was potently induced by radiation in the stem cells of the small intestine but not those of the colon (Merritt et al., 1994). p53 expression was cell-positionally coincident with apoptotic cells in the small intestine. Thus, attenuated p53 function in colonic stem cells, which express bcl-2 protein, could be envisaged to provide conditions strongly promoting the survival of mutated or genetically unstable cells. Recent molecular biological evidence suggests that p53 expression may attenuate that of bcl-2 (Miyashita et al., 1994); our data of an inverse relationship between bcl-2 and p53 expression in the small intestine are concordant with this. Another event in the progression of colon cancers appears to be a change in the expression of the oncogene c-*myc* (reviewed by Fearon and Vogelstein, 1990). Constitutive expression of c-*myc* initiates apoptosis in a variety of cell types (Askew et al., 1991; Evan et al., 1992) and the expression of bcl-2 abrogates this (Bisonette et al., 1992; Fanidi et al., 1992). The deregulated expression of c-*myc* in the epithelial cells of the small intestine would be predicted to induce apoptosis whereas it should be tolerated in colon stem cells where there is constitutive bcl-2 expression.

The finding of constitutive expression of bcl-2 in colonic cells which may give rise to tumours, suggested to us that adenomas and colon tumours might continue to express significant levels of bcl-2 protein. Those recently surveyed did (Hague et al., 1994). Our limited survey, reported here, supports these findings (Fig. 6), although in some advanced carcinomas we have observed a loss of bcl-2 staining coincident with the appearance of positive staining for p53, as was found in breast carcinomas (Leek et al., 1994), a result different from that of Hague et al. (1994). Recent studies of bcl-2 expression in a variety of cancers, including small cell lung, breast, prostate and neuroblastoma, have all suggested that bcl-2 expression may play a role in disease progression (Pezella et al., 1993; Leek et al., 1994; McDonell et al., 1992; Boise et al., 1993a). The expression of bcl-2 in colon tumours may also be important in considering the systemic treatment of colon adenocarcinomas with chemotherapy: we showed that transfection and expression of bcl-2 provides significant resistance to the cytotoxicity of thymidylate synthase inhibitors, such as 5-fluorodeoxyuridine, used in the treatment of colon cancer (Fisher et al., 1993). The dominance of survival genes in inhibiting apoptosis has been suggested recently to pose a greater impediment to genotoxic cancer chemotherapy than the loss of p53 (Strasser et al., 1994).

It is tantalizing to speculate as to why *bcl*-2 expression should be different in the stem cells of the small intestine and the colon, and how it is regulated. The numbers of stem cells in the colon may be more limited than in the small intestine, so that expression of bcl-2 may protect a potentially more vulnerable stem cell population. The constitutive expression of bcl-2 in the colon may also serve the function of protecting the colonic epithelia from initiating apoptosis in response to constant exposure to dietary toxins being concentrated in this region, where the rate of passage through the gut is reduced in comparison to the small intestine, or to differences in intestinal flora. Whatever the reason, the expression of a gene which limits toxin-induced apoptosis then presents a Janus-like character: the advantageous survival of cells exposed to toxins but also the survival of those cells that have mutations and deletions of genes considered to be involved in the early stages of carcinogenesis.

We thank the Cancer Research Campaign for funding, Gerard Evan for generous supply of murine anti-bcl-2 antibody, and Oncor for the gift of an Apoptag kit. Bill Moser and Eric Wheeldon kindly advised and assisted with some preliminary experiments. Peter Hall and Caroline Dive provided helpful comments on the data and the manuscript. This paper is dedicated to the memory of the late Dr John Storey, who died from colon cancer.

REFERENCES

Askew, D. S., Ashmun, R. A., Simmonds, B. C. and Cleveland, J. L. (1991). Constitutive c-*myc* in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-1922.

Bissonnette, R. P., Echeverri, F., Mahboubi, A. and Green, D. R. (1992). Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. *Nature* **359**, 552-554.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Castle, V. P., Heidelberger, K. P., Bromberg, J., Ou, X., Dole, M. and Nunez, G. (1993a). Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavourable histology and N-*myc* amplification. *Am. J. Pathol.* **143**, 1543-1550.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. (1993b). *bcl*-x, a *bcl*-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 1-20.

Collins, M. K. L., Marvel, J., Malde, P. and Lopez-Rivas, A. (1992). Interleukin 3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. *J. Exp. Med.* **176**, 1043-1051.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M. and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128.

Fanidi, A., Harrington, E. A. and Evan, G. I. (1992). Cooperative interactions between c-*myc* and *bcl*-2 proto-oncogenes. *Nature* **359**, 554-556.

Fearon, E. R. and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767.

Fisher, T. C., Milner, A. E., Gregory, C. D., Jackman, A. L., Aherne, G. W., Hartley, J. A., Dive, C. and Hickman, J. A. (1993). *bcl*-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. *Cancer Res.* **53**, 3321-3326.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, A. (1992). Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.*, **119**, 493-501.

Goligher, J. C. (1980). *Surgery of the Anus, Rectum and Colon*, 4th edition, pp. 375-378. Balliere Tindall, London.

Hague, A., Moorghen, M., Hicks, D., Chapman, M. and Parascava, C. (1994). Bcl-2 expression in human colorectal adenomas and carcinomas. *Oncogene* **9**, 3367-3370.

Hall, P. A., Coates, P. J., Ansari, B. and Hopwood, D. (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J. Cell Sci.* **107**, 3565-3577.

Harmon, B. V., Takano, Y. S., Winterford, C. M. and Potten, C. S. (1992). Cell death induced by vincristine in the intestinal crypts of mice and in a human Burkitt's lymphoma cell line. *Cell Prolif.* **25**, 523-536.

Hockenbery, D. M., Zutter, M., Hickey, W., Nahm, M. and Korsmeyer, S. J. (1991). Bcl-2 protein is restricted in tissues characterized by apoptotic cell death. *Proc. Nat. Acad. Sci. USA* **88**, 6961-6965.

Ijiri, K. and Potten, C. S. (1983). Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation. *Br. J. Cancer* **47**, 175-185.

Ijiri, K. and Potten, C. S. (1987). Further studies on the response of intestinal crypt cells of different hierarchical status to cytotoxic drugs. *Br. J. Cancer* **55**, 113-123.

Krajewski, S., Krajewski, M., Shabaik, A., Miyashita, T., Wang, H.-G. and Reed, J. C. (1994). Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am. J. Pathol.* **145**, 1323-1336.

Leek, R. D., Kaklamanis, L., Pezella, F., Gatter, K. C. and Harris, A. L. (1994). Bcl-2 in normal human breast and carcinoma, association with ER positive EGFR negative tumours and in situ cancer. *Br. J. Cancer* **69**, 135-139.

Li, Y. Q., Fan, C. Y., O'Connor, P. J., Winton. D. J. and Potten, C. S. (1992). Target cells for the cytotoxic effects of carcinogens in the murine small bowel. *Carcinogenesis* **13**, 361-368.

Loeffer, M., Birke, A., Winton, D. and Potten, C. S. (1992). Somatic mutation, monoclonality and stochastic models of stem cell organisation in the intestinal crypt. *J. Theor. Biol.* **160**, 471-491.

Lu, Q. L., Poulsom, R., Wong, L. and Hanby, A. M. (1993). *Bcl*-2 expression in adult and embryonic non-haematopoietic tissues. *J. Pathol.* **169**, 431-437.

McDonnell, T. J., Troncoso, P., Brisbay, S. M., Logothesis, C., Chung, L. W. K., Hsieh, J-T., Tsu, S.-M. and Campbell M. L. (1992). Expression of the protooncogene *bcl*-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* **52**, 6940-6944.

Merritt, A. J., Potten, C. S., Kemp, C. J., Hickman, J. A., Balmain, A., Lane, D. P. and Hall, P. A. (1994). The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Res.* **54**, 614-617.

Miyashita, M. and Reed, J. C. (1992). Bcl-2 gene transfer increases relative resistance of S49. 1 and WEHI17. 2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.* **52**, 5407-5411.

Miyashita, M. and Reed, J. C. (1993). Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukaemia cell line. *Blood* **81**, 151-157.

Miyashita, M., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Lieberman, D. A., Hoffman, B. and Reed, J. C. (1994). Tumour suppressor gene p53 is a regulator of *bcl*-2 and *bax* gene expression in vitro and in vivo. *Oncogene* **9**, 1799-1805.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H. and Loh, D. Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidneys disease, and lymphocyopenia. *Proc. Nat. Acad. Sci. USA* **91**, 3700-3704.

Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.

Papadopoulos, N., Nicolaides, N. C., Wei, Y.-F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Peterson, G. M., Watson, P., Lynch, H. T., Peltomaki, J.-K., de la Chapelle, A., Kinzler, K. W. and Vogelstein, B. (1994). Mutation of a MutL homolog in hereditary colon cancer. *Science* **263**, 1625-1628.

Parsons, R., Li, G-M., Longley, M. J., Fang, W., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K. W., Vogelstein, B. and Modrich, P. (1993). Hypermutability and mismatch repair deficiency in RER+ tumor cells. *Cell* **75**, 1227-1236.

Pezzella, F., Turley, H., Kuzu, I., Tungekar, M. F., Dunnill, M. S., Pierce, C. B., Harris, A. L., Gatter, K. C. and Mason, D. Y. (1993). bcl-2 protein in non-small-cell lung carcinoma. *N. Engl. J. Med.* **329**, 690-694.

Potten, C. S. and Allen, T. D. (1977). Ultrastructure of cell loss in intestinal mucosa. *J. Ultrastruct. Res.* **60**, 272-277.

Potten, C. S. and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, uncertainties and pitfalls: lessons for and from the crypt. *Development* **110**, 1001-1019.

Potten, C. S. (1992). The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. *Cancer Metast. Rev.* **11**, 179-195.

Potten, C. S., Li, Y. Q., O'Connor, P. J. and Winton, D. J. (1992). A possible explanation for the differential cancer incidence in the intestine, based on the distribution of the cytotoxic effects of carcinogens in the murine large bowel. *Carcinogenesis* **13**, 2305-2312.

Potten, C. S., Merritt, A. J., Hickman, J. A., Hall, P. A. and Faranda, A. (1994). Characterisation of radiation-induced apoptosis in the small intestine and its biological implications. *Int. J. Rad. Biol.* **65**, 71-78.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**, 1-6.

Strasser, A., Harris, A. W., Jacks, T. and Cory, S. (1994). DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms, inhibitable by *bcl*-2. *Cell* **79**, 329-339.

Walton, M. I., Whysong, D., O'Connor, P. M., Hockenbery, D., Korsmeyer, S. J. and Kohn, K. W. (1993). Constitutive expression of human *bcl*-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res.* **53**, 1853-1861.

Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J. (1994). *Ich*-1, an *Ice/ced*-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* **78**, 739-750.

Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int. J. Cytol.* **68**, 251-305.

(Received 10 November 1994 - Accepted, in revised form, 7 March 1995)