Virchows Arch (1998) 432:415–426
© Springer-Verlag 1998

ORIGINAL ARTICLE

Gerda Partik · Patrizia Kahl-Rainer · Roland Sedivy
Adolf Ellinger · Wilfried Bursch · Brigitte Marian

Apoptosis in human colorectal tumours: ultrastructure and quantitative studies on tissue localization and association with bak expression

Received: 22 July 1997 / Accepted: 27 October 1997

Abstract Apoptotic cell death in human tumours has been demonstrated by electron and light microscopy. In adenomas, fragmented and apoptotic nuclei and signs of phagocytosis have been observed close to the basement membrane. In carcinomas the characteristic structures were apoptotic bodies with small fragments of chromatin. DNA fragmentation was shown by in situ end-labelling. Quantitative assessment of apoptosis and proliferation revealed a high apoptotic index (AI) in all types of adenoma (tubular: 1.77±0.35%, tubulovillous: 2.38±0.41%; villous: 3.3±0.39%) as well as loss of compartmentalization of proliferating and dying cells. In carcinomas a shift towards proliferation was evident, as shown by lower AIs than in adenomas (0.9±0.68% and 1.1±0.12% for moderately and poorly differentiated tumours), higher Ki67 indices (38.32±2.23% and 57±3.89%, respectively) and higher mitosis (0.9±0.56% and 1.21±0.17%, respectively). However, apoptosis was observed in all tumours and is available as a target for therapeutic intervention. Expression of the apoptosis related proteins bcl-2 and bak also reflected loss of compartmentalization. While bcl-2 did not show a consistent relationship to AI in tumour specimens, bak was positively correlated with apoptosis in 4 of 8 adenomas and 4 of 7 carcinomas, suggesting a role for this protein in the induction of apoptosis in a subset of tumours.

Key words Apoptosis · *bcl-2* · *bak* · Colorectal carcinogenesis

G. Partik · P. Kahl-Rainer · W. Bursch · B. Marian (✉)
Institute of Tumour Biology – Cancer Research,
University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria
e-mail: brigitte.marian@univie.ac.at
Tel.: (43) 1-401 54/234, Fax: (43) 1-406 0790

R. Sedivy
Institute of Clinical Pathology, University of Vienna,
Vienna, Austria

A. Ellinger
Institute of Histology and Embryology, University of Vienna,
Vienna, Austria

Introduction

In the liver and other organs, premalignant and malignant lesions have higher rates of cell turnover than normal tissue, reflected in increased proliferation as well as increased active cell death [4, 13, 31, 32]. They also are more sensitive than normal cells to modulation by growth signals [11, 28, 33]. In the colon, cell turnover is high even in normal mucosa because of the necessity for constant tissue renewal supported by few stem cells at the bottom of the crypt. Cells then undergo three or four divisions while they migrate up the crypt and become terminally differentiated resting cells. Finally they die and are shed into the colonic lumen [21, 22]. These cells are removed from the tissue very fast, so that the exact nature of the cell death process is hard to determine. Characteristic apoptotic structures – condensed nuclei, apoptotic bodies – are rarely observed in normal mucosa, although DNA strand breaks considered to be characteristic of apoptosis can be shown in cells at the very top of the crypts by in situ end-labelling (ISEL) at a low incidence [10]. A conflicting report describes high rates of cell death in normal mucosa, which can be inhibited by mutation of the *APC* gene [3]. However, in this study cell death was determined by detection of DNA fragmentation, a method that does not specifically identify apoptosis but may also indicate other types of cell death [12].

A high incidence of apoptosis has been described in colorectal adenomas [2] and carcinomas [36]. A qualitative and quantitative study comparing morphology and incidence of cell death in normal, premalignant and malignant tissues has not been reported, and information about the mechanisms controlling cell turnover and induction of cell death in tumours is inadequate.

The occurrence of active cell death in the tumours indicates that some measure of response to endogenous growth control is still in effect and might be modulated by therapeutic intervention. A prerequisite for any such strategy is detailed knowledge of the signal transduction mechanisms involved. In this regard, proteins of the bcl family are of interest, because they are involved in the

regulation of active cell death in many cell types. Expression of bcl-2 and bak in the colon and in colorectal tumours has been reported by others [18, 19, 26] and may well be involved in endogenous regulatory mechanisms, so that their expression level and association with apoptosis is of prime interest.

The present study of human colorectal tumours was untertaken with three specific aims. We were anxious to characterize the type of cell death taking place within the tumours unequivocally. Because different types of cell death have been described that differ in their morphological characteristics and because of the lack of specificity of molecular characteristics, we used strict morphological criteria at light and electron microscopic levels for our study, and cell turnover was estimated by light microscopic counting on tissue sections using morphological criteria for apoptosis and mitosis and the proliferation marker Ki67 to determine cell turnover in normal mucosa, adenomas and carcinomas. We also wished to investigate whether bcl-2 and bak, which have been associated with apoptosis in other systems, are temporally and spatially related to apoptosis in human colorectal tumours.

Materials and methods

Tissue specimen of 48 adenomas and 55 carcinomas and adjacent normal tissue from the resection margins were obtained from the Institute of Clinical Pathology, University of Vienna. They were routinely fixed in 4% buffered formalin at 4°C overnight and embedded in paraffin. Haematoxylin-eosin (H&E) stained sections were used for pathological diagnosis. H&E staining and immunohistochemical staining for proliferation markers and cell death-associated proteins was performed in serial sections of the same tissue block (10–12 sections/series, 2–4 µm thick).

For morphological analysis, tissue sections were routinely stained with H&E. Two to five areas per tumour were picked out at random, and 2000 cells per area were counted to determine the number of proliferating cells and cells with morphological alterations. Morphological criteria defined by Kerr et al. [17] were used to identify apoptotic cells with condensed or fragmented nuclei and apoptotic bodies with or without chromatin. Small nuclear fragments grouped close together were counted as one fragmented nucleus. Mitotic figures were counted in a separate category from the same areas. Apoptotic and mitotic indices (AI and MI) were determined by counting 2000 cells per area and are presented as percentages of total nuclei.

For immunohistochemistry sections were deparaffinized, rehydrated, and sections for Ki67 and bak stain were heated in 10 mM citrate buffer, pH 6, in a microwave oven at 750 W for 3 cycles of 5 min. After blocking endogenous peroxidase with 2% H_2O_2 and protein binding with 1% goat serum, sections were incubated overnight at 4°C in a humidified chamber with 3 µg/ml of the monoclonal mouse MIB1 antibody against Ki67 nuclear antigen (Dianova, Hamburg, Germany) for determination of proliferation. bcl-2 and bak were stained using the mouse monoclonal antibodies 124 (Dako, Glostrup, Denmark) directed against bcl-2 at a working dilution of 1:50 and bak Ab-1 (Oncogene Research Products, Cambridge, Mass.) at a concentration of 2.5 µg/ml. Negative controls received nonimmune serum. Staining was achieved by use of biotinylated second antibodies raised in goat, binding to avidin–biotin reagents coupled to peroxidase (Vectastain ABC reagents, Vector Laboratories, Burlingame, Calif.), and incubation with 3,3′-diaminobenzidine (Sigma, St.Louis, Mo.) and H_2O_2. Counterstaining was done with haematoxylin.

The Ki67 index was counted from 2000 cells per area and is given as a percentage of total cells. Levels of bcl-2 and bak were determined by scoring staining intensities. To correct for inter-staining variations, intensities were scored relative to staining of the adjacent normal mucosa. The bcl-2 staining at the bottom of the crypts was defined as ++. For bak staining the intensity at the top of normal crypts, as shown in Fig. 4b, was used similarly (++). Scoring was done by two independent investigators.

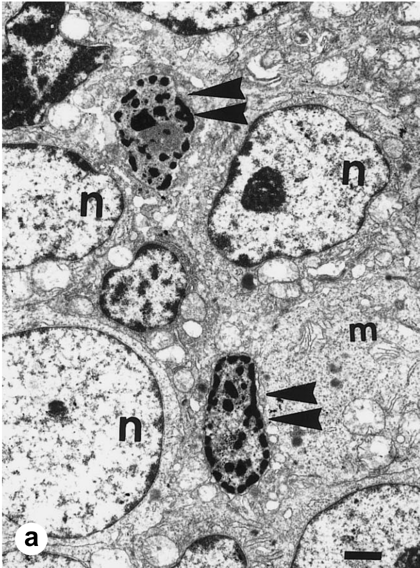
ISEL assays were performed using the Apop Tag In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, Md.) that labels the 3’-OH DNA ends generated by DNA fragmentation. Deparaffinized, rehydrated tissue sections were digested for 20 min at 37°C with 20 µg/ml Proteinase K (Boehringer Mannheim, Germany). Endogenous peroxidase was quenched with 2% H_2O_2 in PBS for 5 min. After 1 h incubation at 37°C with terminal transferase and digoxigenin-labelled UTP, reaction products were detected with an anti-digoxigenin antibody coupled to peroxidase and reaction with 3,3´-diaminobenzidine and H_2O_2. Tissues were counterstained with haematoxylin.

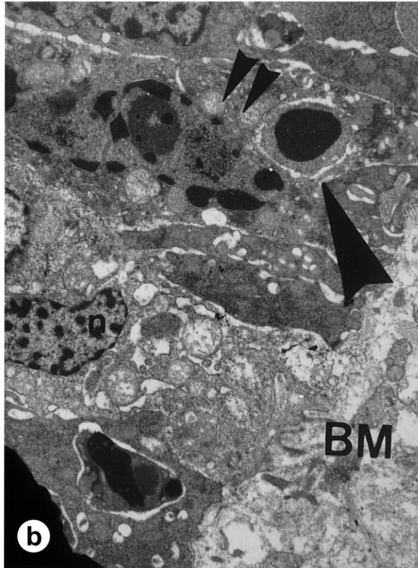
For transmission electron microscopy specimen were fixed with 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH=7.2) for 2 h and postfixed with 1% veronal acetate-buffered osmium tetroxide [30]. After dehydration they were embedded in Epon. Semithin sections were prepared and stained with toluidine blue, and ultrathin sections were stained with 1% ethanolic uranyl acetate for 5 min and alkaline lead citrate for 1 min, and examined in a Philips EM400 transmission electron microscope.

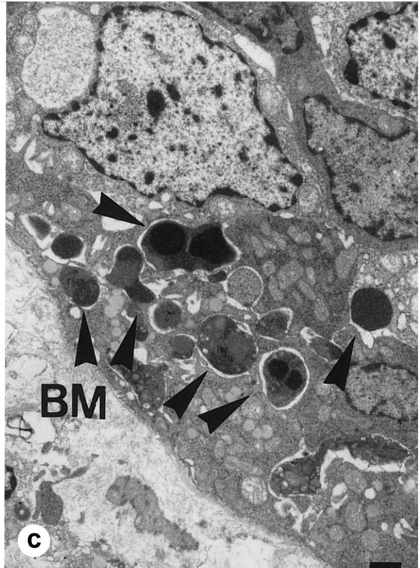
Results

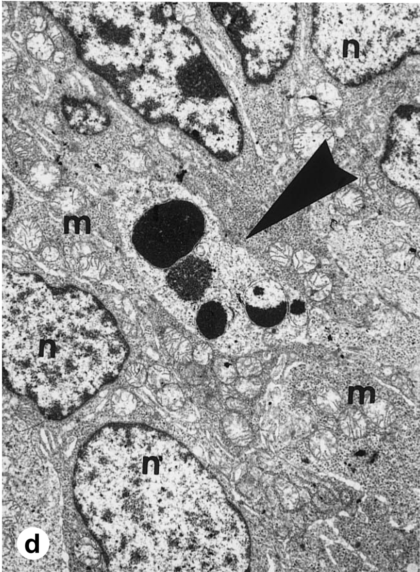
In order to identify apoptotic structures in colorectal tumours tissue specimens obtained from 4 carcinomas and 3 adenomas were fixed in glutaraldehyde and prepared for electron microscopy. The morphology of dying cells was examined by transmission electron microscopy in ultrathin sections at magnifications of 3,700- and 4,800-fold. Figure 1 shows characteristic examples of the structures found in all 7 tumours. We observed cells whose chromatin was condensed to various extents while the cytoplasm and mitochondria were still largely intact (Fig. 1a, b). In addition, there was ample evidence of nuclear and cellular fragmentation (Fig. 1c, d): small membrane-bound cell fragments containing condensed organelles (Fig. 1c) and condensed, fragmented chromatin (Fig. 1d) were located intercellularly close to the basement membrane (Fig. 1c) or intracellularly after phagocytosis by neighbouring epithelial cells (Fig. 1e, f). Some apoptotic bodies contained chromatin in the characteristic crescent shape (Fig. 1d, f). At late stages degradation took place within phagosomes (Fig. 1e, f).

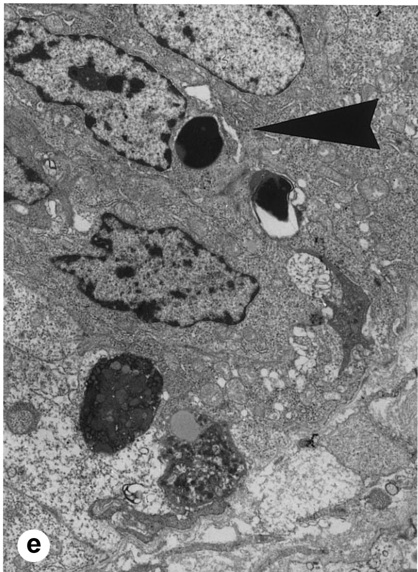
Fig. 1a–i Ultrastructural features of cell death in colonic tumours. ▶ Tissue specimens were prepared for electron microscopy as described in “Materials and methods”, and ultrathin sections examined in a Philips EM400 transmission electron microscope. **a–f** Nuclear condensation at various stages can be identified (**a**, **b** *double arrows*), as can nuclear fragmentation (**d** *arrow*). Cellular fragments can be observed intercellularly (**b**, **c**), in phagosomes (**f**) and intracellularly (**e**) and are indicated by *large arrows*. **a, c** ×4,800. **b**, **d**, **e**, **f** ×3,700 **g–i** Semithin sections stained with toluidine blue. Condensed or fragmented nuclei can be identified in adenomas (**g**, **h**), as can phagosomes (**h**). In the carcinoma specimen (**i**) apoptotic bodies containing small fragments of chromatin can be observed both in the epithelium and in the glandular lumen. In addition, there is necrotic material in the lumen. ×330

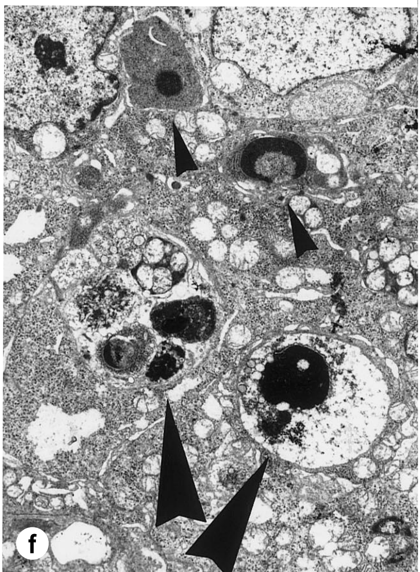

n
n
m
n
a

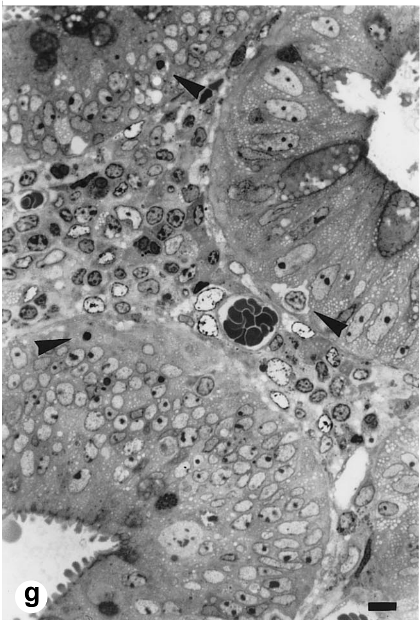

n
BM
b

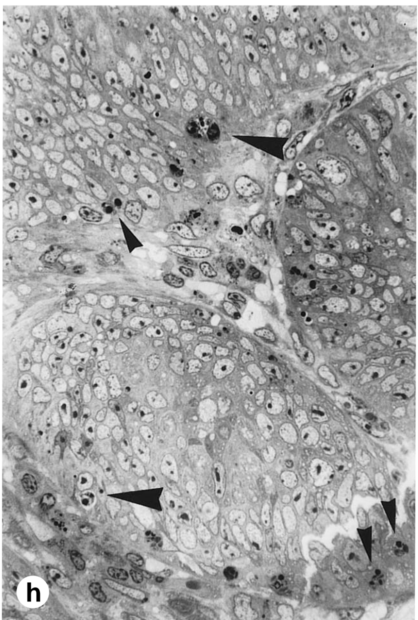

BM
c

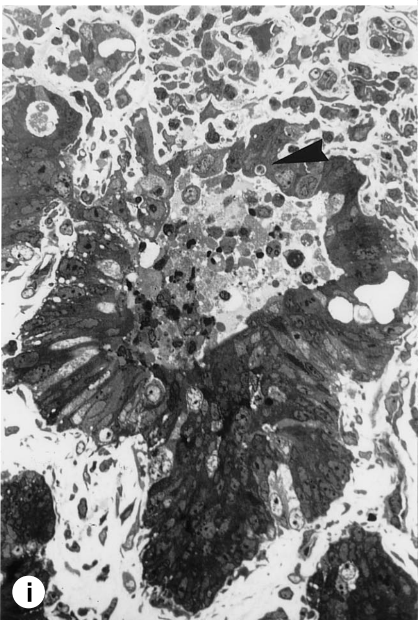

n
m
n
m
n
d


e


f


g


h


i

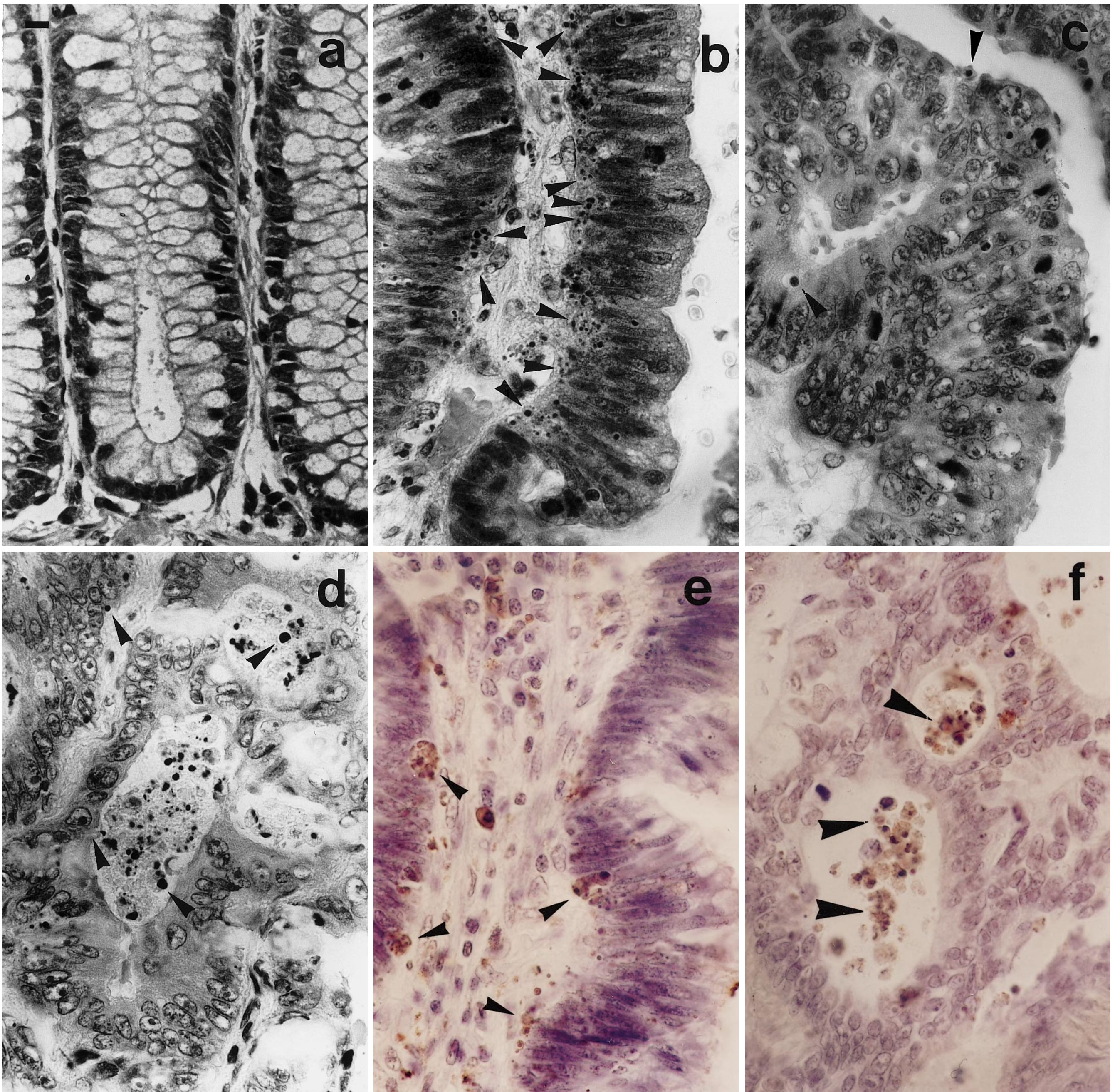


Fig. 2a–f Cell death in human colonic tumours and normal mucosa. Tissue specimens were obtained from **a** normal mucosa of the resection margin, **b** an adenoma and **c, d** two different adenocarcinomas and stained with haematoxylin-eosin. **e, f** ISEL assay was performed on serial sections shown in **b** and **d** as described in "Materials and methods". *Arrows* indicate fragmented nuclei in the adenoma (**b**, **e**), pyknotic nuclei and apoptotic bodies in the carcinomas (**c**, **d**, **f**). ×330

In summary, the features described above characterize the process of cell death in colorectal tumours as apoptosis. Scanning of semithin sections from the same specimen stained with toluidine blue at light microscopic magnifications showed the distribution of these structures within the tissue (Fig. 1g–i). Condensed and fragmented nuclei, apoptotic bodies containing small tightly packed chromatin remnants (Fig. 1g, h, *arrows*) and, on occasion, even larger phagosomes (Fig. 1h, *large arrow*) were mostly observed close to the basement membrane in adenomas and well-differentiated carcinomas. In moderately to poorly differentiated carcinomas fewer apoptotic structures were observed and their locations were more irregular. However, many dying necrotic cells were present in the glandular lumina (Fig. 1i).

To address the quantitative changes suggested by these first observations a more extensive study was undertaken using formalin-fixed specimen and H&E staining. In such sections apoptotic structures similar to those observed in ultrathin and semithin sections were easily recognizable (Fig. 2b–d)). In normal mucosa, however,

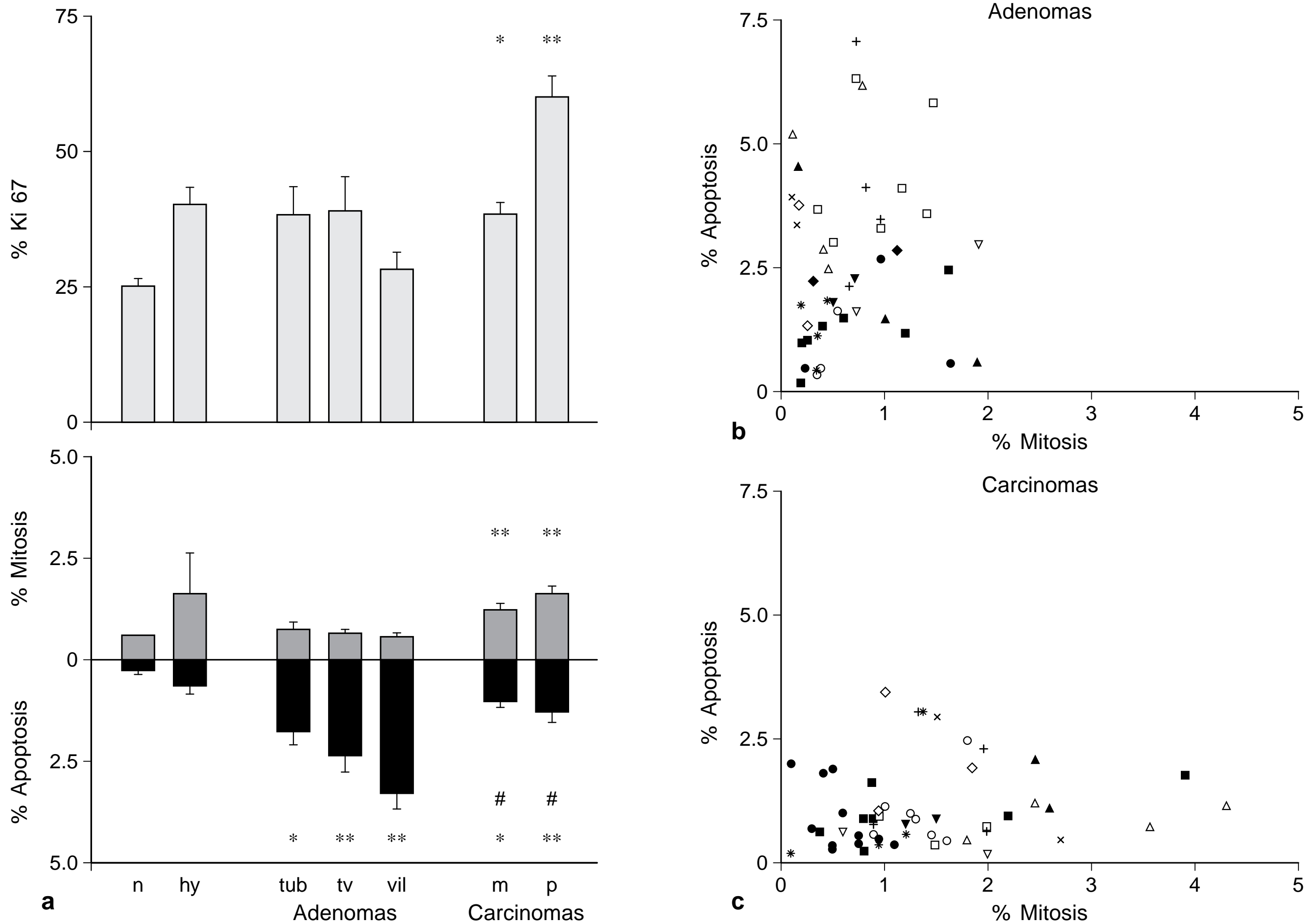


Fig. 3a–c Quantitative assessment of proliferation and apoptosis in colonic tissue specimen. Serial sections were examined for mitotic figures and for characteristic apoptotic structures and stained for proliferating cells using an antibody against the proliferation antigen Ki67 as described in "Materials and methods". **a** Indices of proliferation, mitosis and apoptosis were determined by counting 2000 cells per area and 2–5 areas per tumour. Mean ± SEM. * Different from normal at P<0.05; ** different from normal level at P<0.01; # different from adenoma at P<0.05, according to non-parametric ANOVAR (*n* normal mucosa, *hyp* hyperplastic polyps, *ad* adenoma, *ca* carcinoma, *tub* tubular, *tv* tubulovillous, *vil* villous, *mod* moderately differentiated, *poor* poorly differentiated). **b**, **c** AI was plotted versus MI for individual areas of **b** 13 typical adenomas and **c** 18 typical carcinomas. *Points* with *identical symbols* come from the same specimen

they were observed only rarely and did not appear at specific locations within the crypt (Fig. 2a).

Morphological characteristics and tissue distribution differed significantly between adenomas and carcinomas: in adenomas a high degree of nuclear fragmentation was evident and apoptotic bodies were localized close to the basement membrane as the EM observations had suggested (Figs. 1b, c, e, 2b, e). Groups of three or more fragments were surrounded by a common membrane or halo that suggested that they derived from a single cell (Fig. 2b) and were scored as one apoptotic nucleus for quantification. In contrast, apoptotic bodies with tightly condensed remains of chromatin much smaller than normal nuclei were the predominant feature in carcinomas (cf. Fig. 1). These were dispersed irregularly throughout the tissue (Fig. 2c). In addition, in poorly differentiated carcinomas apoptotic and increasingly necrotic structures were not only observed intraepithelially, but also within glandular lumina (Fig. 2d). Such areas were excluded from quantitative evaluation because an assessment was not possible from the apoptotic/necrotic agglomerations. They might, however, be secondary consequences of massive apoptosis and contribute to total cell loss.

Incubation of tissue sections with terminal transferase in an ISEL assay detected DNA strand breaks in many of these cells, but also in the necrotic debris shed into glandular lumina (Fig. 2e, f).

Serial sections from a formalin-fixed, paraffin-embedded tissue specimen were used to determine cell proliferation by Ki67-antibody staining and counting of mitotic figures and apoptotic bodies by evaluation of nuclear morphology. All together a total of 48 adenomas (12 tubular, 16 tubulovillous and 20 villous) and 55 carcinomas (4 displaying a high, 29 an intermediate and 22 a poor degree of differentiation) were analysed separately and compared with normal mucosa from the resection margin. Areas for counting incidence of apoptosis were selected at low magnification, so that two to five areas (according to the size of the tumour) reached diagonally across the section. Clearly necrotic sections – especially

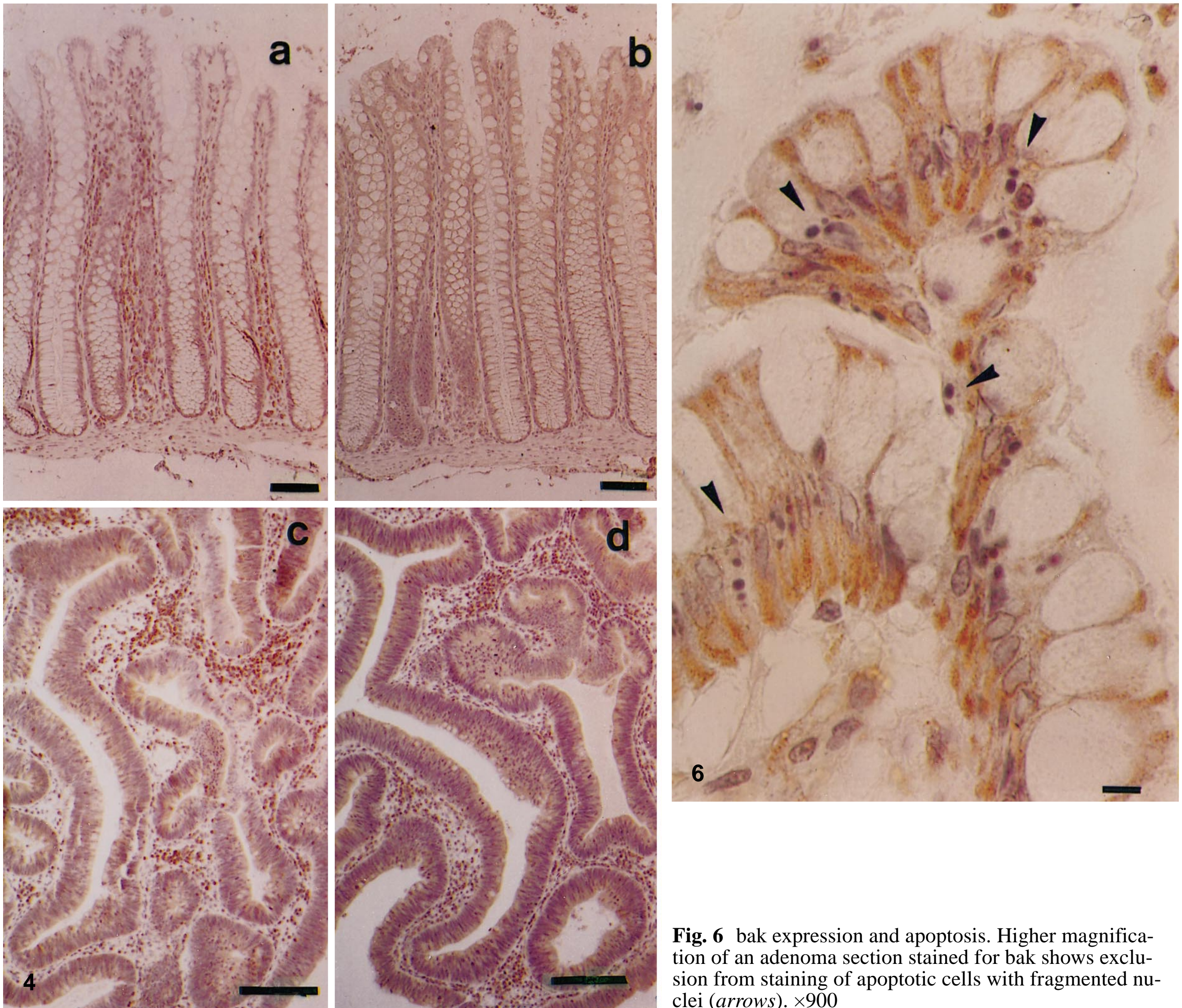


Fig. 6 bak expression and apoptosis. Higher magnification of an adenoma section stained for bak shows exclusion from staining of apoptotic cells with fragmented nuclei (*arrows*). ×900

Fig. 4a–d bcl-2 and bak expression in normal mucosa and tumour tissue. Serial sections from a tumour specimen and corresponding normal mucosa were stained with antibodies against **a, c** bcl-2 and **b, d** bak, as described in "Materials and methods". **a, b** Normal mucosa, showing reverse gradients for bcl-2 and bak staining, respectively. ×63. **c, d** In the carcinoma coexpression in the same area is observed. ×125

in the carcinomas – and apoptotic/necrotic agglomorations in glandular lumina, as shown in Fig. 2d, were excluded from quantification. For quantitative assessment, nuclei with condensed or fragmented chromatin and apoptotic bodies with small chromatin remnants, as shown in Figs. 1 and 2, were counted as apoptosis. An ISEL-positive reaction was not taken into account as it was also seen in necrotic areas. Proliferation was determined by immunohistochemical staining for the Ki67-proliferation antigen and quantified from the same areas as AI. The data are summarized in Fig. 3a: in normal mucosa variations between different specimen were negligible: about 25% Ki67-labelled cells and 0.6% mitotic cells were observed, and these were localized in the lower half of the colonic crypts. AI was as low as 0.28%, and thus too low to identify a preferred localization. Hyperplastic polyps that are not regarded as premalignant lesions showed increased proliferation (40.3±4.5% Ki67-positive cells and 1.64±1.1% MI) and a moderately increased AI of 0.7±0.21% (range 0.2–1.45%).

In adenomas AIs reached a maximum of 7.1% and increased with progression: from 1.77±0.35% (range 0.2–4.6) in tubular adenomas to 2.38±0.41% (range 0.45–7.1) in tubulovillous adenomas and 3.30±0.39% (range 0.35–6.3) in villous adenomas. Regarding proliferation we observed large variations between different areas. Averages were 38.45±5.48%, 39.0±6.91% and 27.69±3.5% Ki67-positive cells in tubular, tubulovillous and villous adenomas, respectively. This does not represent a significant increase over the proliferation indices in normal mucosa. With an overall range of 12–65%, individual areas even showed decreased proliferation com-

Table 1 Bcl2 and Bak-expression in relation to growth parameters in adenomas (*MI* mitotic index, *AI* apoptotic index, *Tub* tubular, *Tv* tubulovillous, Vil villous, *Mod* moderate)

Patient	Stage	Dysplasia	MI	AI	bcl2[a]	bak[a]
1	Vil	Mod	0.7	6.35	++	+++
	Vil	Mod	1.45	5.90	++	++
	Vil	Mod	1.15	4.15	++	+++
	Vil	Mod	0.35	3.70	++	++
	Vil	Weak	0.95	3.35	++	+
	Vil	Weak	0.5	3.05	+	+
2	Tv	Mod	0.1	2.85	−+	++
	Tv	Mod	0.65	2.60	+	+
3	Vil	Weak	0.7	2.30	−+	++
	Vil	Mod	0.5	1.80	−+	+
4	Tv	Mod	0.7	7.10	++	+
	Tv	Mod	0.8	4.15	−	−
	Tv	Mod	0.95	3.50	+	+
	Tv	Mod	0.65	2.15	++	+
5	Vil	Mod	0.1	3.95	+	+
	Vil	Mod	0.15	3.40	+	+
6	Vil	Mod	0.2	1.70	−	+
	Vil	Mod	0.7	1.40	−+	+
7	Tub	Severe	0.15	4.60	++	+
	Tub	Mod	1.9	0.66	+	−
8	Vil	Weak	0.55	1.65	−	−
	Vil	Weak	0.38	0.50	−	−

[a] bcl2- and bak-staining intensity was scored relative to normal mucosa as described in "Materials and Methods"

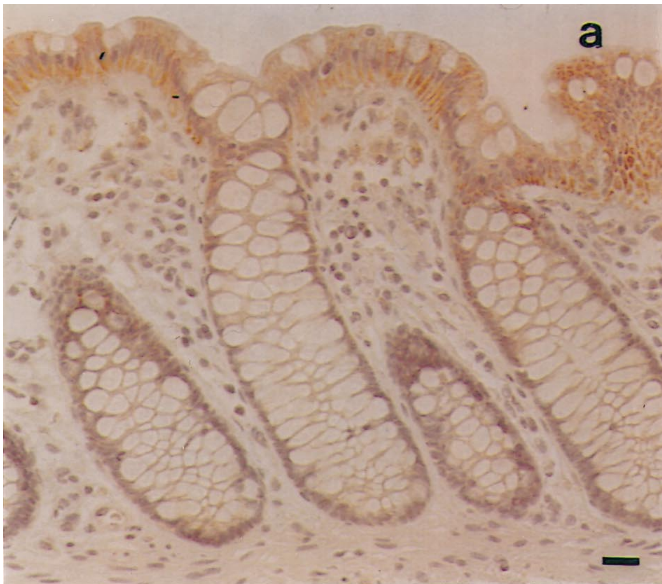
Table 2 Bcl2 and Bak-expression in relation to growth parameters in carcinomas (*nd* not done)

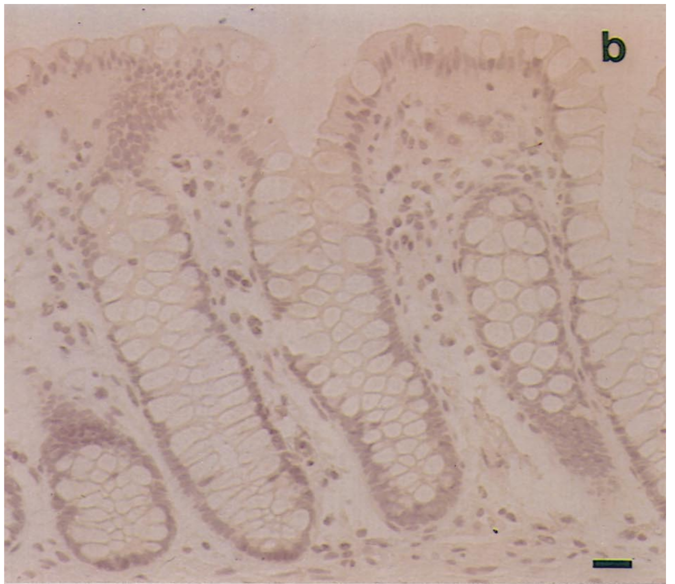
Patient	Dukes'stage	Differentiation	MI	AI	bcl2	bak
4	C1	Well	1.35	3.10	++	+++
	C1	Well	1.2	0.60	+++	+
	C1	Well	0.95	0.40	+++	++
	C1	Well	0.1	0.20	+++	+
7	B	Poor	2.45	2.15	+	−
	B	Poor	2.6	1.15	+	−
9	A	Poor	3.9	1.80	−	++
	A	Poor	2.2	1.00	−	++
10	C1	Poor	1.5	3.00	−	+
	C1	Poor	2.7	0.50	−+	−
11	B	Mod	1	1.15	−	−+
	B	Mod	1.25	1.00	−	−
	B	Mod	1.3	0.90	−	−
	B	Mod	1.45	0.60	−	−
12	B	Mod	1.4	1.65	+++	+
	B	Mod	0.7	0.80	++	−
13	C	Mod	1.3	3.10	+	−
	C	Mod	1.95	2.35	nd	−

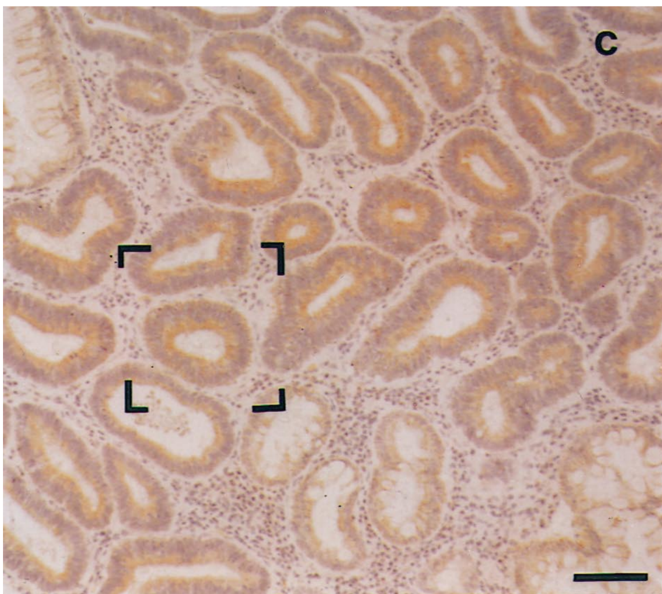
pared with normal mucosa, while others were actively proliferating. The MI followed a similar course, with averages of 0.77±0.2%, 0.65±0.1% and 0.57±0.1% in the progressing classes of adenoma and an overall range of 0.1–1.9% clearly including levels in the normal mucosa.

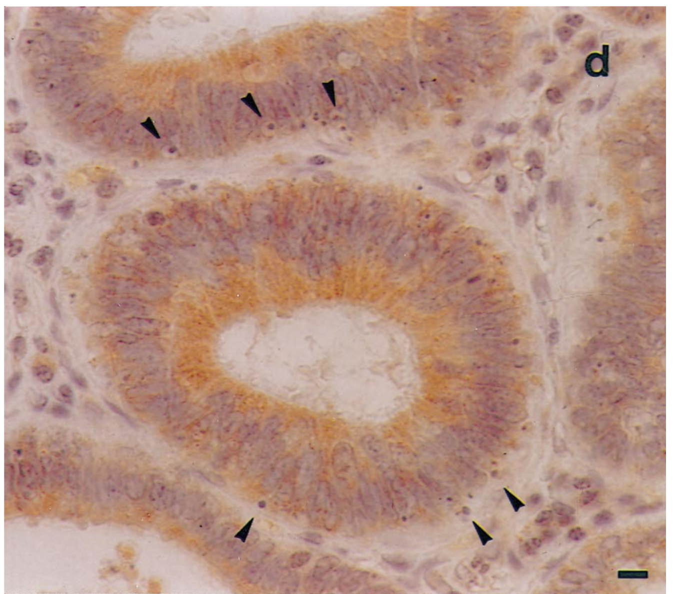
The wide range of both AI and proliferation indices indicated that proliferation and apoptosis are still compartmentalized. However, when AI was plotted against MI for individual areas no consistent relationship between them was observed (Fig. 3b). In some tumours we even observed a positive linear relationship between proliferation and apoptosis instead of the inverse relationship characteristic for normal mucosa. (eg: Figure 3b).

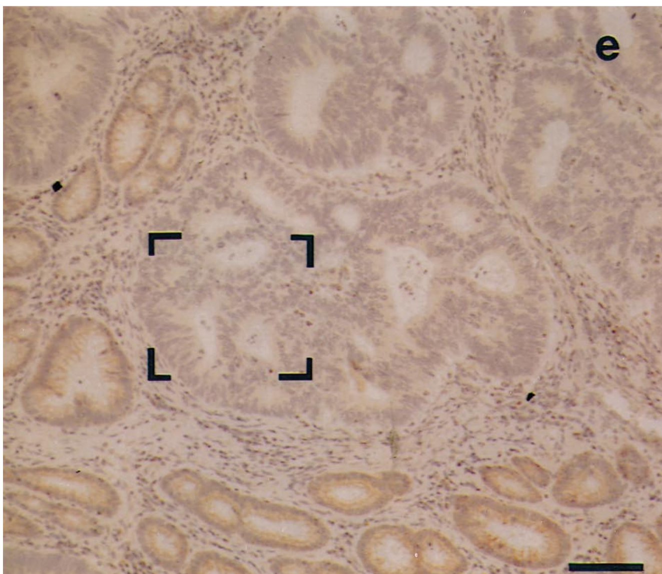
In carcinomas AI was 1.08±0.68% (range 0.25–3.1) for moderately differentiated carcinomas and 1.1±0.12% (range 0.2–5.85) for poorly differentiated tumours (Fig. 3a). There were not enough specimens with a high degree of differentiation to evaluate this category. Proliferation indices were increased above normal: moderately differentiated tumours contained 38.32±2.23% Ki67-pos-

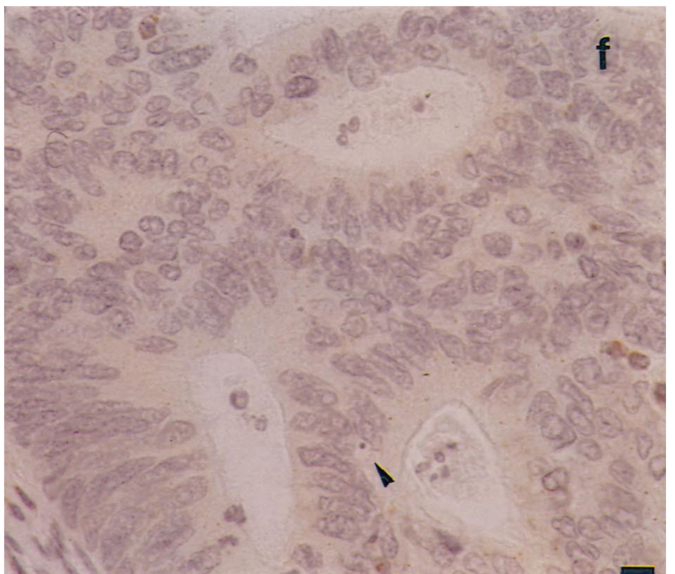

a


b


c


d


e


f

itive cells (range 20–51) and 0.9±0.56% mitosis (range 0.1–1.35) and in poorly differentiated tumours the indices were 57±3.89% and 1.21±0.17%, respectively, with ranges of 28–82% and 0.1–4.3% for Ki67 and mitosis. In summary, this represents a shift towards proliferation in carcinomas as compared with adenomas. However, AI was still higher than in normal epithelium, and additional cell loss was observed in areas of necrosis. In spite of the positive relationship between proliferation and apoptosis when different classes of tumours were compared, there was no consistent relationship at the level of individual areas (Fig. 3c).

Normal mucosa and characteristic areas of high and low AI selected from 8 carcinomas and 9 adenomas and tumour margin areas were stained for bcl-2 and bak proteins as described above. In normal mucosa, bcl-2 was expressed strongly in lymphocytes in the connective tissue and in a few epithelial cells at the lowest part of the proliferation compartment, while bak was present in the resting differentiated cells at the top of the crypts and decreased along a lumen-to-crypt gradient (Fig. 4a, b). In tumour specimens this mutual exclusiveness was lost and coexpression of both proteins was observed in 22/36 areas in 15 tumours (both adenomas and carcinomas; Fig. 4c, d, Tables 1, 2).

Bcl-2 staining was present in a much larger cell population in tumours than in normal mucosa, but staining intensity differed widely both between different specimens and between different areas within the same tumour. Assessment of staining intensities relative to the cells present at the bottom of normal crypts showed that bcl-2 levels were decreased in 5 of 9 adenomas and in 5 of 8 carcinomas and increased in the remaining 4 tumours (1 adenoma and 3 carcinomas). When areas were compared with regard to AI the bcl-2 expression pattern was not related to either proliferation (mitosis) or apoptosis in any way (Tables 1, 2).

Immunoreactivity to the bak protein in adenoma specimens also displayed a wide variety of staining patterns, from high intensity to undetectable, but within bak-positive areas staining was usually present in all cells (Fig. 5c, d). Higher magnification (Fig. 5d, *arrows*) shows a high incidence of fragmented nuclei in such areas. Clearly apoptotic cells with condensed or fragmented nuclei, however, were bak negative (Fig. 6, *arrows*). Most of the carcinoma specimens, on the other hand, had low or undetectable levels of bak (Fig. 5e, f).

Staining intensities of bak protein were semiquantified by scoring relative to the intensity at the top of normal crypts as described above. The data are presented in Tables 1 and 2 for adenomas and carcinomas, respectively. Different areas within one specimen were listed individually and arranged by decreasing AI. In contrast to bcl-2 staining, bak reactivity correlated with AI in 4 of 8 adenomas and in 4 of 7 carcinomas. In most of the remaining specimens bak staining was below normal levels and none of the specimens displayed a reverse correlation between bak and AI.

◀ **Fig. 5e–f** bak expression in normal, premalignant and malignant tissue. Tissue specimens of normal mucosa, adenoma and carcinoma from the same patient were stained for bak. For details see "Materials and methods". **a** Normal mucosa displays a top-to-bottom gradient of bak expression. **b** Negative control. **c, d** Staining is still high in many areas within adenomas, but **e, f** decreased in carcinomas. The *marked areas* in **c** and **d** are shown at larger magnification in **e** and **f,** respectively; *arrows* indicate apoptotic structures that are bak negative throughout. **a–d** ×150, **d, f** ×500

Discussion

We have studied cell death in premalignant and malignant human colonic tumours by histological and ultrastructural methods. At the electron microscopic level early stages of cell death became manifest as chromatin condensation at the nuclear margin, while the nuclear and cellular membranes were still intact. Cell fragments were localized both intercellularly and intracellularly after phagocytosis by neighbouring epithelial cells. The morphological features observed were consistent with apoptosis, a form of active cell death [1, 5, 17, 37]. At the light microscopical level, cells with condensed chromatin or fragmented nuclei and also apoptotic bodies with or without chromatin could be observed. In addition, DNA fragmentation was shown in situ by the terminal transferase reaction.

In adenomas the prevalent morphology was characterized by a high degree of nuclear fragmentation. Apoptotic cells were located close to the basement membrane, and there was evidence of phagocytosis by neighbouring epithelial cells. In carcinomas the morphology changed, displaying mainly apoptotic bodies with only single small fragments of chromatin left. These were not located basally, but more often extruded into glandular lumina, where they underwent secondary necrosis. This suggests that phagocytosis is no longer the preferred way of removal of dead cells or is insufficient for this purpose. As a consequence, there were large areas of glandular lumina filled with both apoptotic and necrotic cell material. Like the fragmented nuclei in adenoma specimens, this material, including the necrotic debris, is ISEL positive.

In normal mucosa apoptotic structures were rarely observed. This agrees with observations reported by others demonstrating only a few ISEL-positive cells at the top of colorectal crypts [10] or little evidence of apoptosis throughout the crypts even after induction by DNA-damaging agents [23]. The low level of apoptosis to remove damaged cells from the tissue is a characteristic of the colorectal mucosa that discriminates it from small intestinal epithelium and has been discussed in terms of the high risk of carcinogenesis in the large versus the small bowel [23]. Independently of any damage, the high rate of cell production in the intestine requires an equally high rate of cell death to maintain homeostasis of cell numbers. New cells are produced at the bottom of the crypts and then migrate up towards the lumen, while differentiating and acquiring a resting state. Finally they die

and are shed into the colonic lumen. Recently it has been reported that the terminally differentiated cells rapidly induce DNA fragmentation, and the conclusion was drawn that they undergo apoptosis [3]. However, they do so only after isolation of the cells in single cell suspensions. Plating of epithelial cell aggregates on collagen gels inhibits this induction of cell death [35]. From these data it has to be assumed that terminally differentiated cells at the top of the crypts are already destined to undergo cell death, but are prevented from actually dying by tissue survival factors.

The compartmentalization of proliferation and cell death described above may account for the low incidence of apoptosis we observed in normal mucosa: even though *rates* of apoptosis have to be high extrusion of dying cells into the lumen may be fast enough to keep *incidence* low. Furthermore, this spatial arrangement makes any determination of AI in histological specimen highly dependent on the conditions of tissue collection, fixation and preparation. Apoptotic cells and even more DNA strand breaks could be induced by tissue autolysis, that is a general problem in the colon [27]. ISEL cannot be regarded as a correct measure of apoptosis in this context as it is also positive in necrosis or autolysis [12].

The tissue specimen in our study were put into formalin for fixation immediately after surgery in the operating room so that no accumulation of apoptotic cells after removal from the organism was possible. AI and proliferation markers (MI and Ki67) were determined from paraffin sections. This may account for the differences between our data and Bedi [3] as well as Arai and Kino [2].

In normal mucosa ISEL-positive and apoptotic cells were extremely rare throughout the crypt. Even though MI was 0.6% and the Ki67 index about 25%, AI did not exceed 0.28%. A top-to-bottom gradient for apoptosis could not be observed, probably because of the very low incidence preventing local differences from reaching significance. Consequently, we have used the average incidence throughout the crypt as the reference value for both apoptosis and proliferation.

In contrast, AI was increased in adenomas, where it reached as much as 7% in some areas, representing a more than 10-fold increase above normal levels. Dying cells were distributed throughout the tumour tissue and no longer exclusively at the luminal surface. A recent study by Moss et al. [25] revealed a reversal of the migration pattern in adenomas, so that proliferating cells are located at the surface of the tumour and dying cells accumulate within the tissue and have to be removed by phagocytosis. The process would make apoptotic cells more visible and increase AI. We have obsered this pattern in some, but not all, of our specimens. In addition, we observed a loss of compartmentalization. Proliferating cells were still concentrated to form areas with high proliferation indices and others with mainly resting Ki67-negative cells in this study and in our previous work [16]. The inverse relationship with cell death was lost, however.

The same loss of interdependence between proliferation and apoptosis was observed in carcinomas. In addition, AI was significantly lower than in adenomas, while MI and Ki67 was increased, indicating a shift towards proliferation in the malignant tumours. However, apoptosis was still observed and AI was higher than in normal mucosa. In addition, there was cell loss by necrosis, which may by a secondary consequence of excessive apoptosis or due to insufficient vascularization and hypoxia. Apoptosis in carcinomas correlated both with proliferation markers (Ki67, MI) and with loss of differentiation, suggesting that cell turnover can provide a selective advantage for tumour progression.

Expression of the cell-death related proteins bcl-2 and bak showed both deregulation at the cellular level and disturbance of tissue architecture. The proteins were mutually exclusive in normal mucosa, bcl-2 staining a subset of Ki67-positive cells in the lowest part of the proliferation compartment and bak being expressed in the upper third of the crypt in the resting, terminally differentiated cells. This agrees with the presumptive functions of the proteins: bcl-2 protects stem cells from undergoing cell death after DNA damage and preserves tissue integrity [24]; bak is involved in the early phases of apoptosis and expressed in cells destined to die [18, 19]. In both adenomas and some carcinomas coexpression of both proteins at the tissue level has been observed in this study. While those three areas expressing bcl-2 only were highly proliferating areas, their MI was not the highest observed, so that bcl-2 alone cannot be regarded as the marker for a tumour proliferation compartment. Similarly, even high expression of bcl-2 did not cause lower AI, suggesting that not all the immunoreactive bcl-2 was active. Bak, however, was positively related to apoptosis in a subset of the tumour areas analysed.

High AI was also observed by Arai and Kino [2] in tubular adenomas. We observed such high levels of apoptosis in all types of adenomas. This provided a mechanism to keep the size of adenomatous polyps stationary for prolonged periods of time in spite of continuing proliferation and explain the slow growth of adenomatous polyps in vivo [29]. A similar phenomenon has been described by Sarraf and Bowen [32] in a study of tumour kinetics using a murine sarcoma model: they described apoptosis in these tumours in terms of an attempt at autoregulation of tumour size that ultimately fails. Increases in both proliferation and apoptosis have previously been observed in liver tumours and premalignant lesions both in experimental animals and in humans [4, 6, 13, 28, 33, 34, 38]. The mechanistic basis for this process is not yet known and might well be tissue specific. In experimental liver carcinogenesis it has been shown that apoptosis in premalignant or malignant cells can be the target for preventive intervention [13].

In the colon, in vitro data suggest that deregulation of proliferation during carcinogenesis provides a growth advantage for premalignant cells [8, 9, 14, 15], but no data are yet available to assess whether this is balanced by similar stimulation of apoptosis. The biological consequences in the colon are self evident, however: as colorectal carcinogenesis is driven by the accumulation of

genetic defects in tumour cells [7] to which cycling cells are more sensitive, increased cell turnover can serve as a motor of tumour progression while apoptosis keeps tumour size in check. Our observation that AI increases with loss of differentiation in carcinomas is confirmed by the study of Tatebe et al. [36], who showed that AI is higher in metastasis than in primary tumours of the colon. Similarly in cervical cancers Levine et al. [20] observed that poor radiation sensitivity and poor prognosis were associated with a high AI.

With regard to the mechanisms controlling cell death, bcl-2 levels and tissue localization do not suggest a regulatory role for the protein in colorectal tumours. Bcl-2 expression was decreased in tumours compared with the levels in the normal stem cell compartment, in agreement with a previous study by Krajewski et al. [19]. In addition, it was not related to AI in any consistent way. Bak levels, however, while decreased in tumours and especially in carcinomas [26], correlated with AI in a subset of tumours – both adenomas and carcinomas.

Our data confirm that apoptosis takes place in premalignant and persists in malignant tumours and is available as a target for therapeutic intervention. However, the morphology and tissue localization of apoptotic structures and their relationship to proliferation reflect increasing disturbances of tissue structure. Our survey demonstrates a loss of compartmentalization concerning both growth behaviour and the expression of bcl-2 and bak. This might well reflect alterations in growth-regulatory mechanisms, and these will have to be explored in in vitro studies.

Acknowledgements We want to thank Prof. R.Schulte-Hermann for critical discussion of the manuscript. This work was supported by a grant from the Herzfelder'sche Familienstiftung, Vienna, Austria.

References

1. Alison MR, Sarraf CE (1995) Apoptosis: regulation and relevance to toxicology. Hum Exp Toxicol 14:234–247
2. Arai T, Kino I (1995) Role of apoptosis in modulation of the growth of human colorectal tubular and villous adenomas. J Pathol (Lond) 176:37–44
3. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR, Jones RJ (1995) Inhibition of apoptosis during development of colorectal cancer. Cancer Res 55:1811–1816
4. Bursch W, Lauer B, Timmermann-Trosiener I, Barthel G, Schuppler J, Schulte-Hermann R (1984) Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumour promoters. Carcinogenesis 5: 453–458
5. Bursch W, Fesus L, Schulte-Hermann R (1992) Apoptosis ("programmed" cell death) and its relevance in liver injury and carcinogenesis. In: Dekant W, Neumann HG (eds) Tissue specific toxicology: biochemical mechanisms. Academic Press, London, pp 95–115
6. Columbano A, Ledda Columbano GM, Rao PM, Rajalakshmi S, Sarma DS (1984) Occurrence of cell death (apoptosis) in preneoplastic and neoplastic liver cells. A sequential study. Am J Pathol 116:441–446
7. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumourigenesis. Cell 61:759–767
8. Friedman E, Urmacher C, Winawer S (1984) A model for human colon carcinoma evolution based on the differential response of cultured preneoplastic, premalignant, and malignant cells to 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res 44: 1568–1578
9. Friedman E, Isaksson P, Rafter J, Marian B, Winawer S, Newmark H (1989) Fecal diglycerides as selective endogenous mitogens for premalignant and malignant human colonic epithelial cells. Cancer Res 49:544–548
10. Gavrieli Y, Sherman Y, Ben Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
11. Grasl-Kraupp B, Bursch W, Ruttkay-Nedecky B, Wagner A., Lauer B, Schulte-Hermann R (1994) Food restriction eliminates preneoplastic cells through apoptosis and antagonises carcinogenesis in rat liver. Proc Natl Acad Sci USA 91: 9995–9999
12. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (tunel assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21:1465–1468
13. Grasl-Kraupp B, Ruttkay-Nedecky B, Müllauer L, Taper H, Huber W, Bursch W, Schulte-Hermann R (1997) Inherent increase of apoptosis in liver tumors: implications for carcinogenesis and tumor progression. Hepatology 25:906–912
14. Kahl-Rainer P, Marian B (1994) Retinoids inhibit protein kinase C-dependent transduction of 1,2-diglyceride signals in human colonic tumour cells. Nutr Cancer 21:157–168
15. Kahl-Rainer P, Karner-Hanusch J, Weiss W, Marian B (1994) Five of six protein kinase C isoenzymes present in normal mucosa show reduced protein levels during tumour development in the human colon. Carcinogenesis 15:779–782
16. Kahl-Rainer P, Sediviy R, Marian B (1996) Protein kinase C tissue localization in human colonic tumours suggests a role for adenoma growth control. Gastroenterology 110:1753–1759
17. Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:39–257
18. Krajewska M, Moss SF, Krajewski S, Song K, Holt PR, Reed JC (1996) Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. Cancer Res 56: 2422–2427
19. Krajewski S, Krajewska M, Reed JC (1996) Immunohistochemical analysis of in vivo patterns of bak expression, a proapoptotic member of the bcl-2 protein family. Cancer Res 56: 2849–2855
20. Levine EL, Renehan A, Gossiel R, Davidson SE, Roberts SA, Chadwick C, Wilks DP, Potten CS, Hendry JH, Hunter RD, et al (1995) Apoptosis, intrinsic radiosensitivity and prediction of radiotherapy response in cervical carcinoma. Radiother Oncol. 37:1–9
21. Lipkin M, Higgins P (1988) Biological markers of cell proliferation and differentiation in human gastrointestinal diseases. Adv Cancer Res 50:1–24
22. Lipkin M, Bell B, Sherlock P (1963) Cell proliferation kinetics in the gastrointestinal tract of man: cell renewal in colon and rectum. J Clin Invest 42:767–776
23. Merritt AJ, Potten CS, Kemp CJ, Hickman JA, Balmain A, Lane DP, Hall PA (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
24. Merritt AJ, Potten CS, Watson AJ, Loh DY, Nakayama K, Nakayama K, Hickman JA (1995) Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. J Cell Sci 108:2261–2271
25. Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR (1996) Inward growth of colonic adenomatous polyps. Gastroenterology 111:1425–1432
26. Moss SF, Agarwal B, Arber N, Guan RJ, Krajewska M, Krajewski S, Reed JC, Holt PR (1996) Increased intestinal Bak expression results in apoptosis. Biochem Biophys Res Commun 223:199–203

27. Moyer MP, Dixon PS, Culpepper AL, Aust JP (1990) In vitro propagation and characterization of normal, preneoplastic and neoplastic colonic epithelial cells. In: Moyer MP, Poste GH (eds) Colon cancer cells. Academic Press, New York, pp 85–136
28. Müllauer L, Grasl-Kraupp B, Bursch W, Schulte-Hermann R (1996) Transforming growth factor β1-induced cell death in preneoplastic foci of rat liver and sensitization by the antiestrogen tamoxifen. Hepatology 23:840–847
29. Muto T, Bussey HJ, Morson BC (1975) The evolution of cancer of the colon and rectum. Cancer 36:2251–2270
30. Palade GE (1952) A study of fixation for electron microscopy. J Exp Med 95:285–297
31. Sarraf CE, Bowen ID (1986) Kinetic studies on a murine sarcoma and an analysis of apoptosis. Br J Cancer 54:989–998
32. Sarraf CE, Bowen ID (1988) Proportions of mitotic and apoptotic cells in a range of untreated experimental tumours. Cell Tissue Kinet 21:45–49
33. Schulte-Hermann R, Ohde G, Schuppler J, Timmermann Trosiener I (1981) Enhanced proliferation of putative preneoplastic cells in rat liver following treatment with the tumour promoters phenobarbital, hexachlorocyclohexane, steroid compounds, and nafenopin. Cancer Res 41:2556–2562
34. Schulte-Hermann R, Bursch W, Grasl-Kraupp B (1995) Active cell death (apoptosis) in liver biology and disease. In: Boyer JL, Ockner RK (eds) Progress in liver diseases. Saunders, Orlando, pp 1–36
35. Sträter J, Wedding U, Barth TFE, Koretz K, Elsing C, Möller P (1996) Rapid onset of apoptosis in vitro follows disruption of β1- integrin/matrix interactions in human human colonic crypt cells. Gastroenterology 110:1776–1784
36. Tatebe S, Ishida M, Kasagi N, Tsujitani S, Kaibara N, Ito H (1996) Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling. Int J Cancer 65:173–177
37. Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306
38. Zerban H, Radig S, Kopp-Schneider A, Bannasch P (1994) Cell proliferation and cell death (apoptosis) in hepatic preneoplasia and neoplasia are closely related to phenotypic cellular diversity and instability. Carcinogenesis 15:2467–2473