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Lack of *Pseudomelanosis coli* in colonic adenomas suggests different pathways of apoptotic bodies in normal and neoplastic colonic mucosa

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Abstract *Pseudomelanosis coli* is characterized by pigment deposition in the lamina propria and caused by increased epithelial apoptosis. *Pseudomelanosis coli* is absent in colonic neoplasia. The aim of our studies was to investigate this phenomenon in more detail. Apoptotic fragments of epithelial cells and their distribution, cell proliferation (Ki-67, MIB 1 immunostaining), macrophages (CD68 immunostaining), Bcl-2 expression and apoptosis [terminal-deoxynucleotidyl-transferase mediated dUTP fluorescein nick end labeling (TUNEL) assay] were studied in adenomas arising in normal and melanotic colonic mucosa, in normal colonic mucosa and colonic mucosa with pseudomelanosis alone. In adenomas, we found 7.0 apoptotic bodies per 100 epithelial cells in the epithelial layer and only 0.2 apoptotic bodies per high power field (HPF) in the lamina propria. In contrast, in melanotic mucosa 1.7 apoptotic bodies per 100 epithelial cells in the epithelial layer and 2.5 per HPF in the lamina propria were found. Our results show that apoptotic fragments remain in the neoplastic (adenomatous) epithelium and do not reach (at least in higher amounts) the lamina propria. They can, therefore, not contribute to the development of pseudomelanosis in these lesions. However, macrophages are diminished in adenomas. Proliferation (Ki-67) and also Bcl-2 expression are highly increased in adenomas. The pathway of mucosal macrophages is also discussed.

Key words *Pseudomelanosis coli* · Large bowel · Colonic adenoma · Apoptosis

Introduction

Pseudomelanosis (melanosis) *coli* is characterized by deposition of granular brown pigment in macrophages in the lamina propria of colonic mucosa. It results from increased apoptosis of colonic epithelial cells followed by phagocytosis and degradation of residual material by macrophages [8] (Fig. 1). Apoptosis of colonic epithelial cells may be stimulated by laxatives of the anthrachinon type, constipation [3, 21] or inflammatory bowel disease [16]. Several studies have shown that the pigment is neither melanin nor a pure anthrachinon derivative [3, 6, 7, 22]. Because of the staining of pseudomelanosis pigment by Masson-Fontana (positive for melanin), Ziehl-Neelsen (positive for lipofuscin) and periodic acid–Schiff base (PAS) stains (positive for mucins) [11], the nature and origin of the pigment were uncertain for a long time. Recent studies, however, revealed that the pigment consists of lipofuscin and partly digested cell organelles originating from apoptotic enterocytes. Consequently, it resembles residual bodies derived from material sequestered and degraded in lysosomes [6, 7]. In contrast, the brown color of the mucosa in other parts of the digestive tract may have different etiologies. For example in the esophagus it is caused by melanin-producing melanocytes, and in the duodenum it is caused by storage of ferrous sulfide resulting from hemorrhage in the upper gastrointestinal tract [8].

Pseudomelanosis coli spares hyperplastic polyps, adenomas and carcinomas of the colon. This is evident endoscopically in resected specimens as well as in microscopic slides [14]. In contrast to the surrounding melanotic colonic mucosa, these lesions appear pale and are therefore easily detected endoscopically. It is remarkable that pseudomelanosis pigment is absent even in very small adenomatous lesions consisting only of about 10–20 dysplastic crypts. The occasional brown pigment granules seen in pedunculated adenomas resemble hemosiderin and can be detected by Pearl's stain.

The aim of this study was to investigate the phenomenon of the lack of *Pseudomelanosis coli* in colonic adenomas in order to further contribute to the understanding of its pathogenesis. The following factors could be responsible: (1) neoplastic and hyperplastic colonic lesions have suppressed apoptosis and increased proliferation of epithelial cells relative to normal colonic mucosa, (2) in

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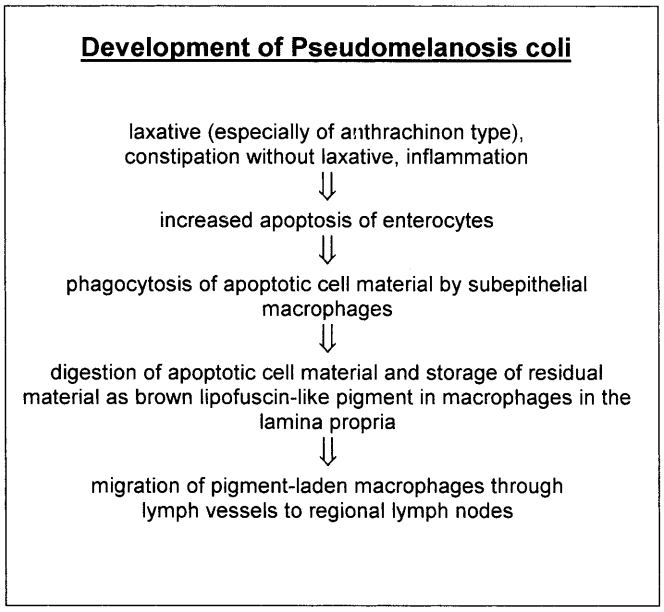

Fig. 1 Hypothetical pathogenesis of *Pseudomelanosis coli*

contrast to the situation in normal colonic mucosa, apoptotic bodies (ABs) cannot reach the lamina propria in these lesions, or (3) lack or low numbers of macrophages ingesting the material derived from apoptotic cells in the lamina propria of these lesions.

We therefore studied (1) the distribution of ABs in hematoxylin and eosin-stained sections, (2) the presence and distribution of apoptosis using a terminal-deoxynucleotidyl-transferase mediated dUTP fluorescein nick end labeling (TUNEL) assay, (3) the expression of apoptosis inhibitor protein Bcl-2, (4) cell proliferation as revealed by Ki-67 (MIB 1) immunostaining, and (5) the presence and distribution of macrophages as revealed by CD68 immunohistochemistry.

Materials and methods

Materials

Seven tubular colonic adenomas (three from rectosigmoid, four from cecum/colon ascendens) with low or moderate degrees of dysplasia associated with *Pseudomelanosis coli* in the surrounding non-neoplastic mucosa were retrieved from the files of the Department of Pathology, University of Graz, School of Medicine. In addition, five tubular adenomas (three from rectosigmoid and two from cecum/colon ascendens) in colonic mucosa without pseudomelanosis, five biopsies with *Pseudomelanosis coli* alone (one from rectosigmoid, two from colon transversum and two from cecum/colon ascendens) and five normal colonic mucosa specimens (two resection margins from rectosigmoid, one from colon transversum and two from cecum/colon ascendens) were studied.

Conventional histology

The specimens were fixed in 8% buffered (pH 7.4) formaldehyde solution, embedded in paraffin, and sections, 5 µm thick, were stained with hematoxylin and eosin (H-E). Histological typing and grading followed standard criteria set by the World Health Organization (WHO).

Immunohistochemistry

Serial sections were deparaffinized, rehydrated and rinsed in distilled water. Antigen retrieval was achieved by microwave treatment at 750 W for 10 min. The Dako ChemMate Detection Kit peroxidase/DAB, (Dako, Glostrup, Denmark) in connection with the Dako TechMate 500/1000 based on an indirect streptavidin-biotin method was used. Monoclonal anti-human Bcl-2 oncoprotein mouse antibodies (Dako), monoclonal mouse antibodies against Ki-67 antigen (MIB 1 clone, Dianova, Hamburg, Germany) and monoclonal mouse anti-human-macrophage antibodies (CD68, KP1 clone, Dako) were used (incubation for 30 min). For CD68 immunostaining, an additional enzymatic digestion with proteinase K (Dako) was performed prior to incubation with the monoclonal antibodies. After washing in Dako ChemMate Buffer Kit, incubation with the secondary link biotinylated antibody was carried out for 30 min. Endogenous peroxidase was blocked in a blocking solution (Dako) for 7.5 min. After washing, sections were incubated with streptavidin-peroxidase (Dako) for 30 min. Finally, the enzymes were visualized using DAB (diaminobenzidine) for 15 min. Counterstaining was performed with hematoxylin.

TUNEL assay

For demonstrating DNA fragmentation, the TUNEL method was applied. Five µm sections were deparaffinized, rehydrated and digested with DNAse-free proteinase K (20 µg/ml; Boehringer-Mannheim, Mannheim, Germany) for 30 min. After washing in distilled water, endogenous peroxidase was quenched with 3% H_2O_2 in methanol. A TUNEL POD Kit (Boehringer-Mannheim) was used according to the manufacturers instructions.

Apoptotic bodies

In addition to cell shrinkage, apoptosis is characterized by dramatic nuclear changes, i.e., chromatin condensation and nuclear fragmentation [4]. To assess the typical late phase of apoptosis, ABs were counted in H-E-stained sections. ABs were defined as clusters of small round to oval, basophilic pyknotic material. Care was taken not to include single fragments or polymorphonuclear leukocytes. These ABs not only contained chromatin clumps but also immunohistochemically detectable keratins and could therefore be related to epithelial cells (unpublished observation). The relatively simple method of counting ABs does, however, not reveal all cells in different stages of apoptosis.

Scoring of cells and statistical analysis

For cell counting in each section, crypts were separated into superficial and basal portions. Ki-67-, Bcl-2- and TUNEL-positive cells were counted in relation to 800 (400 superficial and 400 basal, respectively) neoplastic and 800 non-neoplastic epithelial cells in different fields in each section. The numbers listed in the table and figure are mean values per 100 epithelial cells. CD68-immunostained macrophages were counted at 400× magnification, i.e., 40× objective with a 10×/26.5 ocular (=HPF, high power field) in ten different HPFs in the lamina propria of neoplastic and non-neoplastic areas in each section, respectively. The results are given as numbers of positive cells per HPF. ABs were counted in the same way as described above in the epithelium and in the lamina propria.

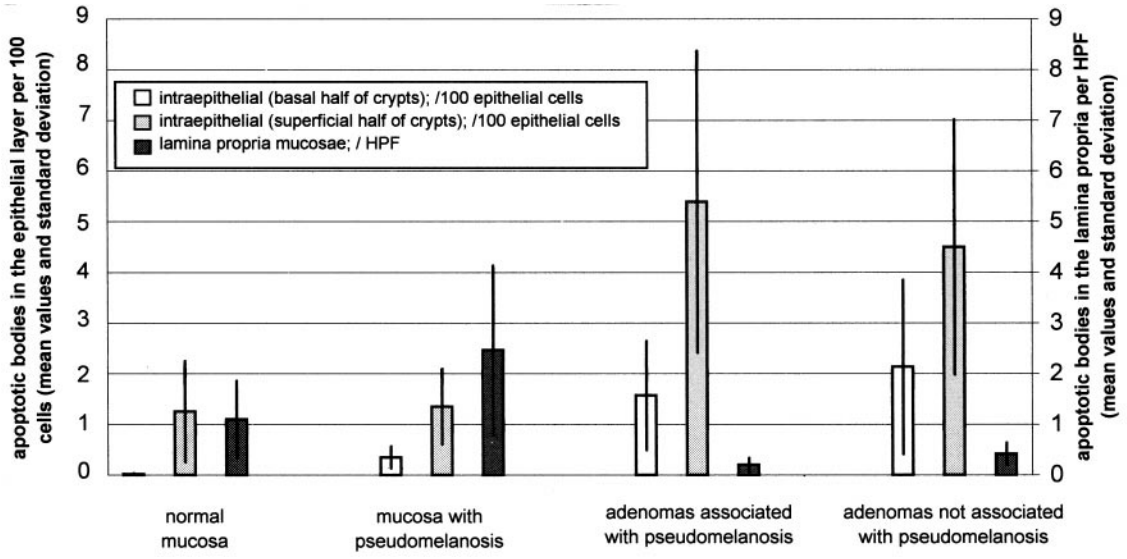

Fig. 2 Distribution of apoptotic bodies in normal and melanotic colonic mucosa and in adenomas (mean values and standard deviation; *n*=7 in adenomas associated with *Pseudomelanosis coli*, *n*=5 in the other groups)

Statistical analysis was carried out using the analysis of variance (ANOVA) test in MS Excel97.

Results

Apoptotic bodies

In normal colonic mucosa, a mean value of 1.3 ABs per 100 epithelial cells and of 1.1 ABs per HPF in the lamina propria mucosae were determined. In melanotic mucosa, apoptosis was slightly increased (1.7 ABs per 100 epithelial cells and 2.5 ABs per HPF in the lamina propria). A completely different distribution of ABs was found in adenomas in that the number of intraepithelial ABs was highly increased (7.0 ABs per 100 epithelial cells, $P<0.03$), whereas only very few ABs were detected in the lamina propria (0.2 ABs per HPF, $P<0.005$) (Fig. 2).

Bcl-2

Immunohistochemical staining for Bcl-2 showed a weak cytoplasmic reaction in colonic columnar epithelial cells. In colonic mucosa with pseudomelanosis, we found a mean value of 7.0 positive cells per 100 epithelial cells in the basal half and no positive cells in the superficial half of the crypts. In adenomas associated with *Pseudomelanosis coli*, mean values of 70 positive cells per 100 epithelial cells in the basal half and of 16.7 per 100 epithelial cells in the superficial half of crypts were determined. In adenomas associated with normal mucosa, we found a mean value of 61.4 positive cells per 100 epithelial cells in the basal half and 20.8 per 100 epithelial cells in the superficial half of the crypts.

TUNEL assay

TUNEL staining revealed a variably strong brown staining of nuclei of epithelial cells. In melanotic colonic mucosa, epithelial cells with positive nuclei were mostly present in the superficial half of the crypts. There was a

Table 1 Summary of results: apoptotic bodies, Ki-67, Bcl-2 and CD68 staining. (numbers are mean±SD). *HPF* high power field

		Normal mucosa	Mucosa with pseudomelanosis	Adenomas associated with pseudomelanosis	Adenomas not associated with pseudomelanosis
Number of cases		5	5	7	5
Apoptotic bodies, basal	/100	0.02±0.02	0.35±0.22*	1.57±1.07*	2.15±1.71
Apoptotic bodies, superficial	/100	1.25±1	1.35±0.74**	5.39±2.98**	4.5±2.51
Apoptotic bodies, lamina propria	/HPF	1.1±0.75	2.46±1.67**	0.2±0.14**	0.42±0.21
Ki-67, basal	/100	27.95±17.47	16.45±10.7***	18.75±6.15***	17.89±8.45
Ki-67, superficial	/100	0.75±0.53	2.25±2.94**	58.36±18.13**	45.21±18.8
Bcl-2, basal	/100	10.25±2.18	7.05±5.49**	70±10.03**	61.43±24.23
Bcl-2, superficial	/100	0±0	0±0**	16.75±10.34**	20.78±10.45
CD68, lamina propria	/HPF	37.04±7.7	34.42±8.28*	13.8±10.95*	18.9±9.62
Pigmented macrophages	/HPF	0±0	9.66±4.53**	0.26±0.59**	0±0

*$P<0.03$, **$P<0.005$, ***not significant. Adenomas associated with pseudomelanosis compared with non-neoplastic mucosa with pseudomelanosis

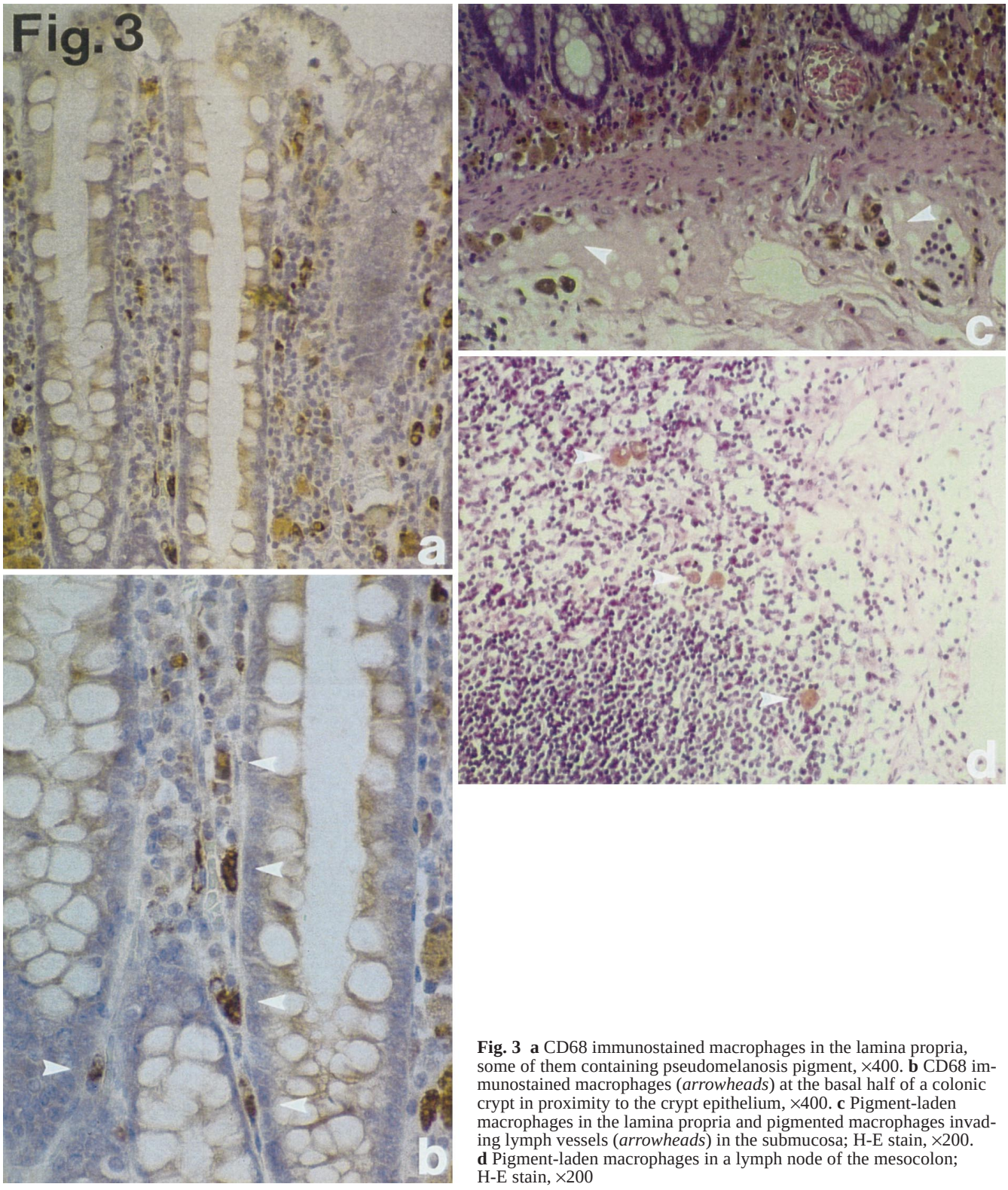

Fig. 3 **a** CD68 immunostained macrophages in the lamina propria, some of them containing pseudomelanosis pigment, ×400. **b** CD68 immunostained macrophages (*arrowheads*) at the basal half of a colonic crypt in proximity to the crypt epithelium, ×400. **c** Pigment-laden macrophages in the lamina propria and pigmented macrophages invading lymph vessels (*arrowheads*) in the submucosa; H-E stain, ×200. **d** Pigment-laden macrophages in a lymph node of the mesocolon; H-E stain, ×200

continuous increase in staining intensity from the base to the luminal surface of the crypt. In adenomas, positive cells occupied only a small area at the surface of the crypt, whereas basal parts of the crypts remained unstained. Neoplastic epithelium, therefore, clearly contained fewer positive cells than normal epithelium. However, in both normal and neoplastic epithelium, many false-positive cells, especially at the edge of the sections, were found. False-negative cells were also obvious despite typical morphological signs of apoptosis [9].

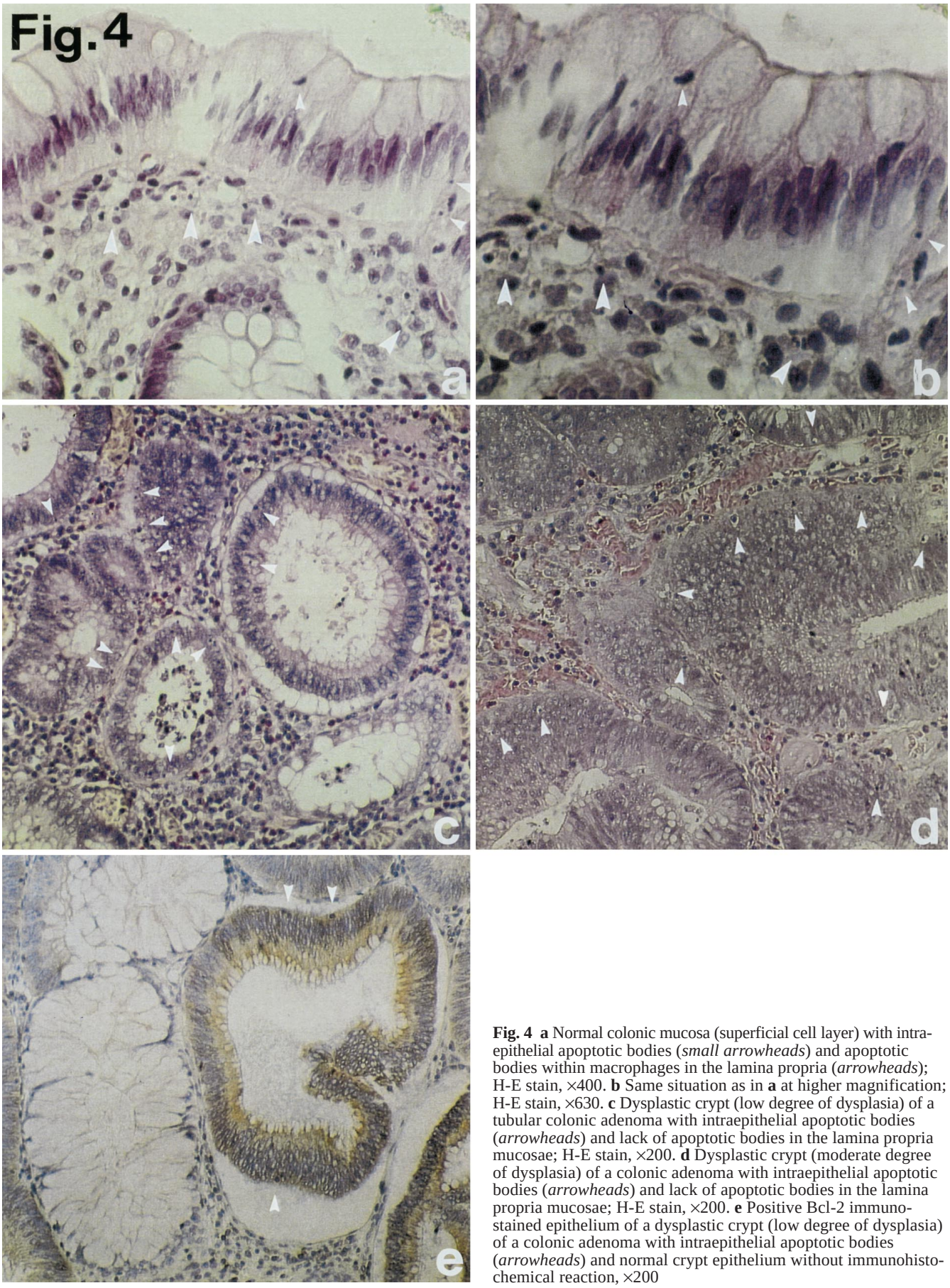

Fig. 4 **a** Normal colonic mucosa (superficial cell layer) with intraepithelial apoptotic bodies (*small arrowheads*) and apoptotic bodies within macrophages in the lamina propria (*arrowheads*); H-E stain, ×400. **b** Same situation as in **a** at higher magnification; H-E stain, ×630. **c** Dysplastic crypt (low degree of dysplasia) of a tubular colonic adenoma with intraepithelial apoptotic bodies (*arrowheads*) and lack of apoptotic bodies in the lamina propria mucosae; H-E stain, ×200. **d** Dysplastic crypt (moderate degree of dysplasia) of a colonic adenoma with intraepithelial apoptotic bodies (*arrowheads*) and lack of apoptotic bodies in the lamina propria mucosae; H-E stain, ×200. **e** Positive Bcl-2 immunostained epithelium of a dysplastic crypt (low degree of dysplasia) of a colonic adenoma with intraepithelial apoptotic bodies (*arrowheads*) and normal crypt epithelium without immunohistochemical reaction, ×200

Ki-67

Immunostaining with Ki-67 (MIB 1) antibody revealed strong nuclear staining. In normal colonic mucosa, we determined a mean value of 27.9 positive cells per 100 epithelial cells in the basal half and 0.7 positive cells per 100 cells in the superficial half of the crypts. In colonic mucosa with pseudomelanosis, a mean value of 16.4 positive cells per 100 epithelial cells was found in the basal half and 2.2 positive cells per 100 epithelial cells in the superficial half of the crypts. At the top of the crypts, no Ki-67 positive cells were detected in either normal mucosa or mucosa with pseudomelanosis. In adenomas associated with *Pseudomelanosis coli*, we found 18.7 positive cells per 100 epithelial cells in the basal half and 58.4 positive cells per 100 epithelial cells in the superficial half of crypts. In adenomas associated with normal mucosa, a mean value of 17.9 positive cells per 100 epithelial cells was found in the basal half and 45.2 positive cells per 100 epithelial cells in the superficial half of the crypts.

CD68

Immunohistochemical staining with CD68 antibodies revealed a strong cytoplasmic reaction of histiocytic cells. In colonic mucosa with *Pseudomelanosis coli*, a mean value of 34.4 CD68-positive macrophages per HPF were found in the lamina propria. In H-E-stained sections, 9.7 macrophages per HPF containing brown pigment were present in the lamina propria, i.e., about one-third of all macrophages contained pigment. In adenomas arising in melanotic mucosa, a mean value of 13.8 macrophages per HPF was found in the lamina propria, 2% of which contained pigment. The pigmented macrophages were, however, only found in marginal zones at the border between the adenoma and the melanotic non-neoplastic mucosa. The results and *P* values are summarized in Table 1.

Discussion

We studied apoptosis in non-neoplastic colonic mucosa with *Pseudomelanosis coli*, in adenomas associated with *Pseudomelanosis coli* and, for comparison, in adenomas in non-melanotic mucosa and normal colonic mucosa by counting ABs in H-E-stained sections, using TUNEL assay and immunohistochemical staining of the oncoprotein Bcl-2, which is considered to be a negative regulator in apoptosis [10]. In agreement with other studies on colonic tumors [1, 5], our results show that the expression of Bcl-2 oncoprotein, an inhibitor of apoptosis, is increased, especially at the base of the crypts in adenomas with and without association with pseudomelanosis (Fig. 4e). Moreover, in agreement with other authors [15, 19], a reduction but not total loss of apoptosis was revealed by TUNEL staining in adenomas. However, the number of ABs was increased in the neoplastic epithelial layer but decreased in the lamina propria of adenomas where apoptotic material could be ingested and degraded by macrophages (Fig. 3a–d). In addition, we observed that ABs were mainly situated in the basal portion of the epithelial layer in close proximity to the basement membrane and apparently did not gain access into the lamina propria (Fig. 3c, d). Our results, therefore, suggest that, in contrast to non-neoplastic colonic mucosa, the absence of *Pseudomelanosis coli* in adenomas is due to impaired transition of ABs derived from the epithelium into the lamina propria. Under these conditions, the substrate for the development of pseudomelanosis, i.e., cell particles, is lacking. In non-neoplastic tissue, ABs are rapidly taken up by either macrophages or adjacent epithelial cells [18]. Therefore, ABs are scarce in non-neoplastic tissue. In liver tissue, for example, ABs are eliminated within 2–4 h after chromatin condensation, resembling the morphological onset of programmed cell death [2].

Impaired transit of ABs from the epithelial layer into the lamina propria in adenomas could be due to decreased permeability of the basement membrane. Walker et al. [21] suggested that in normal colonic mucosa ABs are taken up by intraepithelial macrophages and carried through fenestrae of the basement membrane into the lamina propria. The fenestration of the basement membrane of normal colonic mucosa has been electron microscopically confirmed [12, 21, 23]. In colonic adenomas and carcinomas, the basement membrane is altered, at least with respect to its content in collagen IV and VII and some other components [13, 20]. Alterations of the basement membrane in neoplasia may ensue from disturbed interaction between epithelial cells and fibroblasts, which are responsible for basement membrane synthesis [17]. In addition to altered composition, the basement membrane in adenomas might also differ in structure or shape from its normal counterpart, e.g., by containing fewer fenestrae. This, however, requires further studies. An additional possibility for the retention of ABs in the epithelial layer is that, in highly proliferating epithelium, some epithelial cells lose contact with the basement membrane and, thus, with the macrophages in the lamina propria.

In pronounced *Pseudomelanosis coli*, we observed "empty" (not pigmented) macrophages predominantly in close proximity to the crypt epithelium, whereas pigment-containing macrophages were mainly found in the central space of the lamina propria between crypts. Moreover, in *Pseudomelanosis coli*, macrophage-associated pigment increased in the lamina propria toward the lamina muscularis mucosae. We interpret these findings as follows: (1) macrophages permeate the walls of blood vessels and emigrate from the bloodstream into the lamina propria; (2) macrophages approach the epithelium and accompany the epithelial cells during their migration to the top of the crypt, thereby taking up apoptotic material (Fig. 3a, b); and (3) pigment-laden macrophages migrate through the central space of the lamina propria between the crypts toward the lamina muscularis mucosae, reach

the lymph vessels (Fig. 3c) and are finally transported to the lymph nodes (where pigment-laden macrophages can be found; Fig. 3d). Therefore, according to our hypothesis, phagocytosis of apoptotic fragments occurs during upward movement, whereas the process of degradation and pigment formation starts in the superficial intercrypt space and increases during the downward course of macrophages. In neoplastic colonic lesions, this pathway seems to be disturbed.

The hypothesis that adenomas lack macrophages in their lamina propria as an explanation for the lack of pseudomelanosis is refuted by our results obtained with CD68 antibodies. CD68-stained macrophages were reduced but not absent in adenomas. Moreover, the rarity of intraepithelial macrophages within the epithelial layer as revealed by CD68 staining is not in line with the hypothesis of Walker et al. [21] that intraepithelial macrophages degrade ABs.

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