Chapter 3

Assessment of apoptosis by M30 immunoreactivity and the correlation with morphological criteria in normal colorectal mucosa, adenomas and carcinomas.


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Abstract

Background: Although TUNEL and ISEL are the methods most often used to demonstrate and quantify apoptosis in histological tissue sections, the interpretation and specificity of these techniques have been controversial. Immunohistochemistry using the monoclonal antibody M30 that recognises caspase-cleaved cytokeratin 18 is considered a promising alternative but has yet to be validated against a generally accepted standard.

Methods: Paraffin sections of normal colonic mucosa (n=30), normal mucosa obtained from resection margins from carcinomas (n=30), colorectal adenomas (n=84) and carcinomas (n=40) were studied. Apoptosis of epithelial cells was assessed by M30 immunoreactivity and morphological criteria and expressed as a proportion of the total number of cells counted (apoptotic index).

Results: Mean apoptotic indices assessed by M30 immunoreactivity were 0.18 ± 0.04 % in normal mucosa, 0.42 ± 0.04 % in adenomas and 1.97 ± 0.24 % in carcinomas. Apoptotic indices using morphological criteria were 0.23 ± 0.03 %, 0.62 ± 0.06 % and 1.78 ± 0.19 %, respectively. Apoptotic counts were higher in normal mucosa obtained from resection margins than in genuinely normal mucosa using the M30 antibody. Apoptotic indices obtained by M30 immunoreactivity and morphological criteria were positively correlated (r =0.71, p <0.01).

Conclusion: Assessment of apoptotic cells by M30 immunoreactivity correlates well with morphological criteria. Apoptotic indices increase in the course of the adenoma-carcinoma sequence. Apoptosis in normal mucosa obtained from resection margins differs from genuinely normal mucosa that warrants caution when interpreting studies on apoptosis in normal colonic mucosa. Our findings support the use of M30 immunoreactivity in the study of apoptosis in colorectal tissues.

Introduction

The adenoma-carcinoma sequence of the colon represents one of the most well studied and characterised models of tumour progression. The gradual progression from normal mucosa through adenoma to colorectal cancer is accompanied by genetic alterations that affect normal tissue homeostasis. Alterations in apoptosis and proliferation contribute to carcinogenesis. Indeed, many studies indicate that both proliferative activity and apoptosis are different in various stages of colorectal cancer development. Although it is generally accepted that proliferative activity gradually increases with tumour progression, considerable controversy exists about whether the frequency of apoptosis increases or decreases in the course of the adenoma-carcinoma sequence. In most studies, mainly in-situ end-labelling techniques of DNA fragments (TUNEL and ISEL) have been used to obtain data concerning apoptotic cell death in human colorectal mucosa. However, serious reservations have been expressed concerning the applicability of these methods to evaluate apoptosis in the gastrointestinal tract.

Recently, several antibodies have been introduced that specifically identify apoptotic cells in different human cell types. One of these is the murine monoclonal antibody...
M30, which reacts with a caspase-cleaved product of cytokeratin. Immunoreactivity of M30 is confined to the cytoplasm of apoptotic epithelial cells and is expressed during early apoptosis. It has been suggested that this antibody is a promising alternative for TUNEL to detect apoptotic cells. The identification of apoptotic cells by morphological criteria with light-microscopic examination of hematoxylin-eosin stained sections is generally considered as the reference standard. However, no studies are available that have validated the M30 method against this reference.

Another limitation of previous studies on apoptosis in normal colon epithelium is that in most studies normal mucosa had been obtained from resection margins from surgically removed carcinomas. One could argue whether this tissue represents genuinely normal mucosa.

The primary aim of this study was to assess the frequency of apoptosis in different stages in colon cancer development by M30 immunoreactivity and compare it with morphological identification of apoptotic cells. Second, possible correlations between the degree of apoptosis, proliferative activity and histopathological characteristics were explored. Finally, we compared the frequency and localisation of apoptotic cells in normal mucosa obtained from resection margins from surgical specimens with that in genuinely normal mucosa.

Materials and methods

**Patients and tissue samples**

Paraffin-embedded specimens from 84 colorectal adenomas and 40 adenocarcinomas were retrieved from the files of the Department of Pathology. The adenomas were of at least 3-mm circumferential size and had been consecutively removed by endoscopy in 1997 at the Department of Gastroenterology. Cases of primary adenocarcinomas had been consecutively removed in 1999 at the Department of Surgery. Samples from patients with familial adenomatous polyposis or hereditary non-polyposis colorectal cancer were excluded. None of the patients had received chemo- or radiotherapy prior to resection or removal of the tumour. In addition, tissue sections were selected from the 40 patients with carcinomas from the non-involved resection margins with normal macroscopic appearance. From these cases, 30 samples of resection margins were available. For comparison, 30 samples of genuinely normal colonic mucosa were selected from archival materials, which had been consecutively obtained in 1999. These samples were from patients with normal macroscopic findings at colonoscopy and from whom random biopsies had been taken which had not shown any abnormalities at histologic examination. If the biopsies had been taken from normal mucosa from patients with a history of adenomas or carcinomas, they were excluded.

Histologic classifications were carried out on hematoxylin-eosin (HE) stained slides. The morphological classification of the carcinomas and adenomas was conducted according to the World Health Organisation (WHO) criteria. From adenomas, the circumferential size was measured, and the severity of dysplasia expressed as low- or high-grade dysplasia. Adenomas were classified as tubular, tubulovillous or villous. For statistical purposes, adenomas with tubulovillous and villous architecture were taken together. Adenocarcinomas
were graded into well, moderately and poorly differentiated. For statistical purposes, tumours with Dukes stages A and B were compared with those with stages C and D. The location of adenomas and carcinomas was retrieved from endoscopy, surgical or pathology reports. The caecum, ascending colon and transverse colon were regarded as the proximal colon, while the descending colon and sigmoid were referred to as the distal colon. Rectal localisation was recorded separately.

**Immunohistochemistry**

For immunohistochemical staining, 3 µm-thick sections were cut from paraffin blocks. After deparaffinisation, endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. The primary antibodies M30 (Boehringer Mannheim, GmbH, Mannheim, Germany) and MIB-1 (against Ki-67, Immunotech, Marseilles, France) were applied in a 1:50 and 1:400 solution, respectively. Antigen retrieval was performed, for MIB-1 in a pressure cooker, for M30 in a microwave oven for 8 min in 0.01 M citrate buffer (pH 6.0). After 1 h incubation with the primary antibody, samples were incubated with a secondary rabbit-anti-mouse antibody conjugated with peroxidase (DAKO, Glostrup, Denmark) and a tertiary goat-anti-rabbit peroxidase-conjugated antibody (DAKO) to intensify the staining reaction. Counterstaining was performed with hematoxylin. As negative controls, slides were immunostained in the absence of the primary antibody.

**Evaluation of staining results**

Evaluation of staining was performed by two investigators using light microscopy in serial tissue sections. Quantitative analysis was performed in complete crypts in normal mucosa and adenomas, and at random in carcinomas. M30 positivity was identified as brown cytoplasmic staining. Morphological characteristics including the presence of apoptotic bodies, nuclear condensation, cytoplasmic shrinkage and membrane blebbing were assessed in HE stained tissue sections. In all cases, at least 1000 epithelial cells were counted and morphological apoptotic and M30 positive cells were expressed as a percentage of the total number of cells counted (apoptotic index). In normal mucosa and adenomas, the localisation of M30 positive cells was recorded in the upper and lower half of the crypt separately as positive or negative. Apoptotic cells located in the lumen, assessed either by morphological criteria or M30 positivity, were excluded. For evaluation of MIB-1 staining in normal mucosa and adenomas, complete crypts were counted. In carcinomas, counts were made randomly. In all samples, at least 1000 cells were counted and MIB-1 positive cells were expressed as a percentage of the total number of epithelial cells counted.

**Statistical analysis**

The correlation between the identification of apoptotic cells by morphological criteria and the M30 method was assessed using the non-parametric Spearman test. Mean M30 and MIB-1 counts were compared across different types of colon tissue and different histopathological characteristics, using the Mann-Whitney test. Multiple linear regression analysis was used to compare apoptotic and proliferative indices across different types of colon tissue and different histopathological characteristics after adjusting for potential confounding by other variables such as size, tumour type, stage and grade. Differences in distribution of apoptotic cells
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between genuinely normal mucosa and normal mucosa obtained from resection margins were assessed using chi-square tests. Correlations between apoptosis and proliferative activity and continuous histopathological variables were assessed using the Spearman test. P-values below 0.05 were considered significant. SPSS for Windows software (SPSS Inc., Chicago, IL, USA) was used in all statistical analyses.

Results

**Patient and histological characteristics**

The mean age of patients at the time of adenoma removal was 64.2 ± 13.4 years (range 28-89), and the male/female ratio was 1.8. The mean age at time of cancer diagnosis was 65.2 ± 12.4 years (range 40-88), with a male/female ratio of 1.1. Histopathological characteristics of the adenomas and carcinomas are depicted in table 1. Mean adenoma size was 9.6 mm (range 3-42).

**Apoptosis and proliferative activity in the adenoma-carcinoma sequence**

Figure 1 summarises the apoptotic indices as assessed by M30 immunoreactivity (A) and proliferative indices (B) in different colon tissues. The mean apoptotic index in genuinely normal epithelial mucosa was 0.18 ± 0.04 %, which was lower than in normal mucosa adjacent to carcinomas: 0.41 ± 0.03 %. The mean apoptotic index in adenomas...
Figure 1. Apoptotic indices assessed by M30 immunoreactivity (A) and proliferative indices (B) in normal mucosa, normal mucosa from resection margins ('adjacent'), adenomas and carcinomas. Box plots show median value, interquartile ranges and 90% ranges (extremes not shown). ns: not significant.
was 0.42 ± 0.04 %, lower than the apoptotic index of 1.97 ± 0.24 % in carcinomas. Using morphological criteria, these indices were 0.23 ± 0.03 %, 0.58 ± 0.05 %, 0.62 ± 0.06 % and 1.78 ± 0.19 %, respectively. There was a positive correlation between the frequency of apoptosis as assessed by M30 immunoreactivity and morphological criteria (Spearman correlation coefficient 0.71, p < 0.01).

In genuinely normal epithelial mucosa, the mean percentage of proliferating cells was 27.4 ± 16 %, comparable with that in normal mucosa from resection margins with a mean of 27.6 ± 2.3 %. Mean proliferative activity in adenomas was 35.3 ± 193 %, higher than in normal mucosa and normal mucosa from resection margins but lower than that in carcinomas: 56.5 ± 37 %.

Figure 2. Examples of M30 immunoreactivity (A-B; magnification x 100), Ki-67 staining (C-D; magnification x 100) and HE-staining (E; magnification x 400) in normal mucosa. (A) Genuinely normal colon mucosa, M30 labelling is predominantly limited to cells at the mucosal surface (arrow). (B) Normal mucosa obtained from a resection margin, M30 labelling is seen along the crypt axis (arrows). (C) Normal mucosa and (D) normal mucosa obtained from resection margins show Ki-67 positive cells limited to the proliferation zone in both cases with comparable proliferative indices. (E) High power view of normal mucosa obtained from a resection margin, showing the presence of apoptotic cells (arrows) in the crypt base. See appendix for colour pictures.
Apoptosis assessed by M30 immunoreactivity in relation to histopathological characteristics and proliferative activity

Results are summarised in table 1. In adenomas, the degree of apoptosis was higher in adenomas ≥ 10 mm than in smaller ones and higher in proximal than rectal adenomas. There was no difference between adenomas with different grades of dysplasia or growth type. When multiple regression analysis was performed adjusting for all variables (size, localisation, dysplasia and growth type), only size larger than 10 mm remained significant (p=0.04), indicating that size was the only variable independently associated with a high apoptotic index. There was also a significant but weak linear correlation between the apoptotic index and adenoma size (r = 0.235, p < 0.05) but no correlation with proliferative activity (r = -0.15, p = 0.89). Proliferative activity in adenomas was higher in lesions with high-grade dysplasia compared to those with low-grade dysplasia.

In carcinomas, the degree of apoptosis was higher in Dukes stages C and D compared to A and B and higher in rectal than in distal tumours. However, after multiple regression analysis only the difference between stages remained significant (p = 0.02), indicating that a higher Dukes stage was the only variable independently associated with a high apoptotic index. As in adenomas, the degree of apoptosis was not correlated with proliferative activity (r = 0.22, p = 0.16) in carcinomas.

Distribution of apoptotic cells assessed by M30 immunoreactivity in normal mucosa, adenomas and carcinomas

Examples of M30 immunostaining are shown in figures 2 and 3. In genuinely normal mucosa, apoptotic cells were exclusively seen in the upper (luminal) half of the crypt and the luminal surface (figure 2A). In contrast, in normal mucosa obtained from resection margins, apoptotic cells were not only observed in these regions but also in the lower half of the crypts in 18 of 30 cases (figure 2B; p < 0.01 for the difference with genuinely normal mucosa). Similar results were obtained when morphological criteria were used (figure 2E). Proliferating cells in both types of normal mucosa were exclusively located in the lower half of the crypt (figure 2 C-D). In adenomas, apoptotic cells were seen in both the upper and lower half of the crypts in all cases (figure 3, A-B). In carcinomas, apoptotic cells were randomly distributed throughout the tumour (figure 3 C-D).

Discussion

The main physiological mechanism by which colon epithelial cells die is by apoptosis. Apoptosis has been studied extensively in the course of the adenoma-carcinoma sequence. In a recent systematic review of these studies, a wide variety in reported apoptotic indices was found, differing up to a 100-fold. This variety can be partly attributed to drawbacks of TUNEL and ISEL, the methods that were most often used to demonstrate apoptosis. Although the morphological changes that are seen in hematoxylin-eosin stained tissue sections are considered the reference standard for recognising apoptotic cells, neither TUNEL nor morphological investigation discriminates between various cell types, e.g. apoptotic epithelial cells cannot be differentiated from apoptotic lymphocytes or apoptotic...
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Mesenchymal cells. A clear advantage of the monoclonal antibody M30 over in situ end-labelling techniques and morphological methods is that M30 is exclusively expressed in apoptotic epithelial cells. M30 immunoreactivity was shown to be positive in epithelial cells with apoptotic characteristics such as chromatin condensation, nuclear fragmentation and detachment of cytoplasm from the environment, whereas cells with normal morphology and end-stage apoptotic cells were negative. In the present study, M30 staining and morphological identification of apoptotic epithelial cells were well correlated. In most cases, apoptotic counts were slightly higher using morphological criteria compared to M30 immunostaining. This may suggest false-negative staining results with the M30 method. However, it must be noted that cells with morphological criteria of apoptosis are of various, including epithelial, origin, whereas M30 positivity is limited exclusively to epithelial cells. Second, M30 immunoreactivity eventually disappears in end-stage apoptotic cells.

In a previous study, M30 immunoreactivity was compared with in situ end-labelling in colorectal neoplasms, showing a strong positive correlation between the two methods. Hence, the current validation of M30 immunostaining against two reference methods supports the application of this technique in the study of apoptosis in colorectal epithelial cells.

Using M30 immunohistochemistry, we found a higher apoptotic index in carcinomas than in adenomas and the lowest counts in normal mucosa. The mean apoptotic index of 1.97% in our series of carcinomas is in accordance with the other studies in which apoptotic cell death was assessed using M30 immunoreactivity. In these studies, apoptotic indices of 1.50% and 2.46% were found respectively. Although this may suggest some

Figure 3. Examples of M30 immunostaining in an adenoma (A-B) and a carcinoma (C-D).
(A) M30 positive cells are seen along the entire crypt (magnification x 100). (B) Detail of a crypt containing numerous M30 positive cells with strong cytoplasmic staining (magnification x 400). (C) M30 positive cells are heterogeneously distributed throughout the carcinoma (magnification x 100). (D) High power view of the carcinoma shown in C (magnification x 400). See appendix for colour pictures.
inconsistency in the results of M30 immunostaining, the variation in apoptotic indices with M30 is considerably less compared to the range of 0-11.4% reported using in-situ end-labelling methods. We found a lower degree of apoptosis in adenomas using M30 immunoreactivity as compared to a study in which 31 adenomas were included showing a mean apoptotic index of 0.98%. This difference may be attributable to differences in adenoma characteristics.

Using two different methods, we found that the proportion of epithelial cells undergoing apoptotic cell death increases in the course of the adenoma-carcinoma sequence, in accordance with the majority of studies on this subject. At first glance, this may seem contradictory to the general idea, mostly based on in vitro experiments, that tumour progression is associated with a decrease in apoptotic cell death. On the other hand, it is well known that colorectal epithelial cells accumulate genetic alterations in the course of the adenoma-carcinoma sequence. As DNA damage can be a trigger for the induction of apoptosis, one could well expect higher rates of spontaneous apoptotic cell death towards carcinoma development. It has been shown that the presence of aneuploidy in adenomas is associated with adenoma size, architecture and grade of dysplasia, but that only adenoma size is correlated to the presence of specific gene mutations. Interestingly, the only variable independently associated with a high apoptotic index in adenomas in our study was size larger than 10 mm. The absence of a correlation between apoptosis and proliferation in colorectal neoplasms, as found in this and other studies, suggests that apoptotic cell death is not primarily driven by increasing proliferative activity. Alternatively, apoptotic cell death may be induced by accumulating genetic alterations.

It must be noted that in the vast majority of studies on apoptosis in normal mucosa, histologically normal mucosa was investigated adjacent to carcinomas, usually obtained from resection margins. One could argue whether this represents genuinely normal mucosa. Indeed, we found a higher degree of apoptosis in normal mucosa obtained from resection margins than in genuinely normal mucosa. This is in accordance with the only other study in which this was investigated. In that study, in which TUNEL was used, apoptotic indices were 11% and 3% in adjacent normal mucosa and genuinely normal mucosa, respectively. The discrepancy in apoptotic indices between this study and ours may be explained by the fact that the time scale over which TUNEL detects DNA fragmentation in the cell can be assumed to be longer than the time during which cleaved cytokeratin 18 is expressed in an apoptotic cell.

Not only the degree of apoptosis, but also the distribution of apoptotic cells was found in our study to be different between genuinely normal mucosa and mucosa obtained from resection margins. Our finding of apoptotic cells in the lower half of the crypts in normal mucosa from resection margins, a pattern similar to that in adenomas, supports the assumption that this type of mucosa does not represent genuinely normal mucosa. We found no difference in proliferative activity between genuinely normal mucosa and normal mucosa obtained from resection margins, suggesting that the difference in degree of apoptosis is probably not driven by different proliferative activities. The higher degree of apoptosis may be explained on technical grounds, for example hypoxia-induced apoptosis, occurring during the surgical procedure or delayed fixation procedures. Irrespective of the underlying mechanism, the fact that the degree and distribution of apoptotic cells differ...
between normal mucosa from resection margins and genuinely normal mucosa must be kept in mind when assessing studies on apoptosis in normal colon mucosa.

In conclusion, M30 immunohistochemistry is well suited for the demonstration of apoptotic cells in benign and malignant colorectal tissues. Our results confirm previous studies that the degree of apoptosis increases in the course of the adenoma-carcinoma sequence.
Chapter 3

References


