Apoptosis and colorectal cancer. Studies on pathogenesis and potential therapeutic targets
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Chapter 2
Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature.

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Abstract

The development of colorectal cancer is characterised by an accumulation of molecular genetic alterations causing disorders in cell growth, differentiation and apoptosis. Although changes in apoptosis with colorectal cancer development have been studied extensively, a clear consensus of opinion has not yet emerged. In this review, the literature about changes in the frequency and distribution of apoptosis in tissue sections of normal and neoplastic colorectal tissues was systematically reviewed. Using a PUBMED search, 53 relevant articles were identified. Data from these studies are discussed with respect to the following aspects: methods used to detect apoptotic cell death; frequency and locoregional distribution of apoptosis in normal mucosa, adenomas and carcinomas; the correlation between levels of apoptosis and proliferation and the prognostic significance of the degree of apoptosis in colorectal cancer. Possible underlying mechanisms of dysregulation of apoptosis are briefly discussed. Finally, possible therapeutic implications of knowledge of the molecular regulation of apoptosis are discussed and potential options for further research are suggested.

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1. Introduction

Colorectal cancer is a major cause of cancer death and, in most cases, develops from a pre-existing adenoma: the adenoma-carcinoma sequence. This sequence is characterised by an accumulation of molecular genetic alterations causing disorders in cell growth, differentiation and apoptosis. It is generally believed that the balance between the rates of cell growth and apoptosis maintains intestinal epithelial cell homeostasis, and that during cancer development this balance gets progressively disturbed. Changes in cell proliferation have been well studied in the adenoma-carcinoma sequence and it is generally accepted that the number of proliferating cells increases in proportion to the severity of dysplasia. However, less is known about changes in the incidence and regulation of apoptosis during colorectal cancer development.

Apoptosis, or programmed cell death, plays an important role in many physiologic and pathologic processes. Amongst others, an important function of apoptosis lies in the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer and its poor response to chemotherapy and radiation (reviewed by Watson). Inhibition of apoptosis causes an imbalance in normal tissue homeostasis promoting cell growth and it also allows the survival of genetically damaged cells, both contributing to tumour development and progression. However, considerable controversy exists as to whether the frequency of apoptosis increases or decreases during the adenoma-carcinoma sequence.

A large number of studies have assessed the proportion of cells undergoing apoptosis, both in vitro and in human colorectal tissue sections. Several in vitro studies have shown changes in colorectal carcinogenesis affecting apoptosis (reviewed by Williams et al.). From these studies it appears that a gradual resistance to induction of apoptosis emerges during cancer development. Other evidence suggestive of the emergence of such a resistance comes from studies in which it was shown that bile salt-induced apoptosis was diminished in individuals with a history of colon cancer as compared to normal individuals. It was also found that adenoma cell lines were more sensitive than carcinoma cell lines to apoptosis induction by vitamin D3, its analogue EB1089 and sodium butyrate. Moreover, a decrease in the rate of spontaneous apoptosis was observed in the course of the adenoma-carcinoma sequence in colorectal epithelial cells in an ex-vivo culture experiment. Contradictory to the previous experiments, it was shown that colorectal carcinoma cells were more sensitive than colorectal adenoma cells to induction of apoptosis by the aspirin metabolite salicylate and the non-steroidal anti-inflammatory drug NS-398.

Apoptotic cell death has been extensively investigated in tissue sections of human colorectal adenomas and carcinomas. However, these studies have yielded confusing and controversial results and have hitherto not been systematically reviewed. In an attempt to clarify this subject, we performed a systematic search of the literature, focusing on the detection and quantification of apoptosis in benign and malignant colorectal tissue sections.
2. Methods

We searched PUBMED (www.ncbi.nlm.nih.gov/PubMed) from inception of the database to July 2001, with the MeSH headings “apoptosis”, “colorectal” or “colon”, “adenomas”, “carcinomas” or “cancer” including all subheadings. The resulting citations were examined on screen to identify possibly relevant studies and those thus identified were obtained in full. We included all studies in which apoptosis was studied in tissue sections of normal colonic mucosa, colorectal adenomas or carcinomas. Only papers published in English were included. The bibliographies of all included studies were carefully examined. In some studies, data on the frequency of apoptosis were not explicitly mentioned in the text but could be inferred from tables or figures. In some other studies, apoptosis was quantified and discussed in the article, without actual presentation of exact numbers.

3. Results and discussion

In total, 53 studies were considered eligible. Individual studies are summarised in table I. The first part of the table summarises those studies that assessed and compared apoptotic frequencies in different colonic tissue types (normal mucosa and/or hyperplastic polyps and/or adenomas and/or carcinomas). In the second section of the table, studies are depicted in which only one colonic tissue type was studied. Similarities and differences between the studies will be discussed below with respect to the following aspects: the methods used to detect apoptotic cells; the frequency of apoptosis in normal mucosa, adenomas and carcinomas; the locoregional distribution of apoptosis in benign and malignant colorectal tissues; the correlation between levels of apoptosis and proliferation and the correlation between levels of apoptosis and prognosis of colorectal cancer.

3.1 Methods of apoptosis detection

Several methods exist to examine apoptosis in tissue sections. The methods used in the reviewed studies were terminal deoxyribonucleotidyl transferase-mediated nick end labelling (TUNEL, n=29), in situ end labelling (ISEL, n=7), morphology by light microscopy (n=7), TUNEL or ISEL in conjunction with morphology by light microscopy (n=9), ISEL in conjunction with morphology by electron microscopy (n=1), morphology by light microscopy in conjunction with electron microscopy (n=2) and M30 immunoreactivity (n=2). All methods and their advantages and disadvantages will be briefly discussed below.

Apoptotic cells can be recognised by careful light-microscopic examination of hematoxylin-eosin-stained sections, showing morphological criteria defined by Kerr et al. These criteria include the identification of the formation of apoptotic bodies with nuclear condensation or nuclear fragmentation, with cytoplasmic shrinkage and loss of contact from surrounding cells. A more definitive method of morphologic identification of apoptotic cells is electron microscopy. Because of the brief duration of such morphologic changes and their seemingly low incidence, apoptosis has been difficult to detect in routine histologic sections. Therefore, several biochemical procedures have been developed to identify apoptotic cells in vivo; the methods most widely used are TUNEL and ISEL. These methods are based
Table 1. Overview of studies on the detection of apoptosis in colorectal tissues.

<table>
<thead>
<tr>
<th>Apoptosis assessed in different colon tissue types</th>
<th>Normal</th>
<th>Normal adjacent</th>
<th>Hyperplastic polyps</th>
<th>Adenomas</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>First author [ref]</td>
<td>Method(s) used</td>
<td>AI</td>
<td>n</td>
<td>AI</td>
<td>n</td>
</tr>
<tr>
<td>Sinicrope [4]</td>
<td>TUNEL &amp; morphology (HE)</td>
<td>-</td>
<td>16</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Bedi [6]</td>
<td>TUNEL</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>Hao [7]</td>
<td>ISEL</td>
<td>1.34</td>
<td>59</td>
<td>2.02</td>
<td>22</td>
</tr>
<tr>
<td>Hawkins [12]</td>
<td>ISEL</td>
<td>4.2 *</td>
<td>33</td>
<td>15.8 *</td>
<td>26</td>
</tr>
<tr>
<td>Carr [23]</td>
<td>ISEL</td>
<td>0.64</td>
<td>31</td>
<td>1.19</td>
<td>50</td>
</tr>
<tr>
<td>Hawkins [12]</td>
<td>M 30 reactivity</td>
<td>0.98</td>
<td>31</td>
<td>1.50</td>
<td>50</td>
</tr>
<tr>
<td>Aotake [36]</td>
<td>TUNEL</td>
<td>0.26</td>
<td>43</td>
<td>1.69</td>
<td>44</td>
</tr>
<tr>
<td>Ikenaga [37]</td>
<td>TUNEL</td>
<td>0.18</td>
<td>10</td>
<td>-</td>
<td>1.40</td>
</tr>
<tr>
<td>Ikenaga [38]</td>
<td>TUNEL</td>
<td>0.11</td>
<td>10</td>
<td>5.62</td>
<td>62**</td>
</tr>
<tr>
<td>Koike [39]</td>
<td>TUNEL</td>
<td>&lt;0.5</td>
<td>10</td>
<td>1.61</td>
<td>63</td>
</tr>
<tr>
<td>Moss [41]</td>
<td>TUNEL &amp; morphology (HE)</td>
<td>2.75</td>
<td>10</td>
<td>11</td>
<td>33</td>
</tr>
<tr>
<td>Moss [42]</td>
<td>TUNEL</td>
<td>2.75</td>
<td>10</td>
<td>-</td>
<td>1.23</td>
</tr>
<tr>
<td>Partik [43]</td>
<td>ISEL &amp; EM</td>
<td>0.28</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>Regitnig [44]</td>
<td>TUNEL</td>
<td>1.3</td>
<td>5</td>
<td>-</td>
<td>7.0</td>
</tr>
<tr>
<td>Takano [45]</td>
<td>TUNEL</td>
<td>0.18</td>
<td>10</td>
<td>0.55</td>
<td>10</td>
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<tr>
<td>Kawasaki [47]</td>
<td>TUNEL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>Nakamura [48]</td>
<td>TUNEL</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kikuchi [51]</td>
<td>TUNEL</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Nomura [52]</td>
<td>TUNEL</td>
<td>2.80</td>
<td>91</td>
<td>3.41</td>
<td>19</td>
</tr>
<tr>
<td>Yamamoto [53]</td>
<td>TUNEL</td>
<td>3.6</td>
<td>26</td>
<td>5.3</td>
<td>7</td>
</tr>
<tr>
<td>Baretton [54]</td>
<td>TUNEL</td>
<td>1.69</td>
<td>43</td>
<td>1.9</td>
<td>93</td>
</tr>
<tr>
<td>Valentini [55]</td>
<td>ISEL</td>
<td>9.8</td>
<td>51</td>
<td>6.3</td>
<td>34</td>
</tr>
<tr>
<td>Sinicrope [56]</td>
<td>TUNEL &amp; morphology (HE)</td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Strätter [71]</td>
<td>TUNEL</td>
<td>-</td>
<td>30</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>Kobayashi [128]</td>
<td>TUNEL</td>
<td>-</td>
<td>29</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td>Miki [195]</td>
<td>TUNEL</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Hao [196]</td>
<td>ISEL &amp; morphology (HE)</td>
<td>-</td>
<td>104</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>Schölzel [197]</td>
<td>TUNEL</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>16</td>
</tr>
</tbody>
</table>
on the assumption that genomic DNA is fragmented in a dying cell, producing fragments of consistent length in apoptotic cell death, as opposed to necrotic cell death where DNA is believed to be randomly degraded. Both the TUNEL and ISEL method detect the DNA strand breaks by adding nucleotides, including deoxyuridine triphosphate labelled with biotin, to the ends of DNA fragments. The only essential difference between the two is that TUNEL uses the enzyme terminal deoxynucleotidyl transferase using nick end labelling; ISEL: in situ end labelling; EM: electron microscopy; HE: hematoxylin-eosin; -: missing data; ** according to Japanese criteria.

Table legend: Normal adjacent: normal mucosa adjacent to adenoma or carcinoma; # AI: Apoptotic index (expressed as mean or median), defined as the percentage of apoptotic cells of the total number of epithelial cells counted or defined as arbitrary units (*); n: number of samples investigated; TUNEL: terminal deoxynucleotidyl transferase using nick end labelling; ISEL: in situ end labelling; EM: electron microscopy; HE: hematoxylin-eosin; -: missing data; ** according to Japanese criteria.
to apoptotic cells. Furthermore, they require pre-treatment steps that need careful optimisation, and the results depend on how these steps are performed. There is also evidence that the physical act of cutting tissue to create sections produces TUNEL activity. This resulted in serious reservations concerning the applicability of the TUNEL method to evaluate apoptotic cell death in the gastrointestinal tract. Results derived solely from TUNEL staining should therefore be considered with caution, unless supported by light microscopic analysis of cell morphology. Cell morphology by light microscopy alone is considered by some as superior to TUNEL or ISEL for the detection of apoptotic cells. The vast majority of the studies discussed in this review have utilised TUNEL or ISEL staining as the primary measure of apoptosis, and only a few confirmed their findings by morphological identification of apoptotic cells. When only these studies are considered, ranges in reported apoptotic indices, which will be discussed below, vary considerably, underlining the limitations of the currently used methods to identify apoptotic cell death.

In two studies, a new method of identifying apoptotic cells using the monoclonal antibody M30 was used. This method is based on the detection of a neo-epitope of cytokeratin 18, which is produced by caspase cleavage of cytokeratin 18 during apoptosis. As it remains immunoreactive in paraffin-embedded tissue and is not present in non-apoptotic cells, it has potential in the study of apoptosis in clinical and experimental material. A clear advantage over TUNEL and ISEL is that M30 is negative in necrotic cells. One disadvantage of the method might be that the time scale over which TUNEL or ISEL detects DNA fragmentation in the cell is considerably longer than the time during which cleaved cytokeratin 18 is expressed. Recently, results of M30 immunoexpression were compared with the ISEL method. In colorectal neoplasms, a strong positive correlation was found between in situ end labelling and M30 immunoreactivity. Immunoexpression of M30 was generally easier to interpret than in situ end labelling, and the procedure for M30 immunohistochemistry was technically simpler. It can be expected that in the near future new techniques with higher specificity for the detection of apoptotic cell death will be developed based on the detection of target proteins of caspase cleavage.

3.2 Changes in frequency of apoptosis in the adenoma-carcinoma sequence

In all but two studies, apoptosis was quantified in tissue sections as an apoptotic index, defined by the number of apoptotic cells as a percentage of the total number of epithelial cells counted. In the two exceptions, numbers of apoptotic cells were determined in 10 high power fields (light microscope; field diameter 490 µm), corrected for the fraction of neoplastic epithelium in those fields.

Normal mucosa. The proportion of epithelial cells undergoing spontaneous apoptosis in normal colonic mucosa appears to be highly variable and apoptotic indices of 0.11-11% have been reported. Two issues have to be considered here. First, in most studies sections of normal colonic mucosa were in fact retrieved from macroscopic normal tissue adjacent to adenomas or carcinomas. One could question whether this represents truly normal epithelium. For example, Moss et al. showed considerable differences in the degree of apoptosis between histologically normal adjacent epithelium in comparison to truly normal tissue, it being generally lower in the latter. Such alterations in normal tissue adjacent to colorectal neoplasms may reflect an early stage in the process of colorectal
carcinogenesis or a reaction to the adjacent tumour. Secondly, Liu et al. demonstrated significant differences between apoptotic indices in different parts of the colon. While the percentage of proliferating colonic epithelial cells was constant throughout the colon, apoptotic indices were found to be lower in the right colon in comparison to the left colon and rectum. However, Anti et al. could not show segmental differences in normal control subjects whereas in patients with a history of adenomas apoptotic indices were higher in the right colon compared to the left colon and rectosigmoid segment. The cause of these discrepancies is unclear and may be a result of the relatively small numbers of subjects studied. In most studies reviewed, the location from which normal mucosa was obtained was not stated.

Adenomas. There is virtually no debate as to whether the proportion of apoptotic cells increases or decreases during the transition from normal colonic mucosa to adenomas. Most studies report increased apoptotic indices in adenomatous tissue compared to normal mucosa, whereas only a few studies found the opposite. Reported apoptotic indices in adenomas vary between 0.50-9.4%. Several studies have assessed the degree of apoptotic cell death in different types of adenomas, i.e. low or high grade dysplasia and/or tubular or villous architecture of the adenoma. In reports where adenomas with different grades of dysplasia were studied, apoptotic indices increased in proportion with the severity of dysplasia in most studies, whereas others have found no such relationship, or the opposite. With respect to tubular and villous adenomas, some found higher apoptotic indices in tubulovillous and villous adenomas than in tubular ones, whereas others found the opposite. Taken together, these studies do not allow general conclusions of differences in apoptotic cell death between different types of adenomas as the numbers of adenomas studied were generally low and considerable differences in the methods to identify apoptotic cell death were present.

Carcinomas. Concerning the transition from adenoma to carcinoma, considerable controversy exists. Most researchers found that the frequency of apoptotic cell death in carcinomas was higher than in adenomas. Others observed a decrease, and some showed no differences. Tsujitani et al. compared carcinomatous with adenomatous components in the same tumours and found lower apoptotic rates in the carcinomatous components. Reported apoptotic indices in carcinomas vary between 0-11.4%. When considering only those studies in which TUNEL or ISEL staining was confirmed by morphological identification of apoptotic cells, and both adenomas and carcinomas were studied, no differences were found between adenomas and carcinomas. The one study not using TUNEL, ISEL or morphology, but the supposedly more specific method of M30 immunoreactivity, showed a higher apoptotic index in carcinomas than in adenomas. In summary, most studies indicate an increase in apoptotic frequency in the course of the adenoma-carcinoma sequence, in parallel with increasing dysplasia. The transition from normal mucosa to adenoma is more clearly accompanied by an increase in apoptotic frequency than that from adenoma to carcinoma.

How can these differences in apoptotic indices be explained? First, discrepancies in conclusions may reflect methodological differences in the detection of apoptosis and selection and preparation of tissue. Second, diagnostic criteria for carcinoma of the colorectum differ between Japanese and Western pathologists. Since intramucosal carcinomas in Japan...
are not accepted as carcinomas in the West, results from some Japanese studies 36,38,47,57,60,61 cannot readily be compared with the others.

Many authors of the reviewed articles refer to the study by Bedi et al. 6, in which a progressive decrease was reported of the proportion of apoptotic cells during the transformation of normal colonic epithelium to adenomas and adenocarcinomas, associated with enhanced ex vivo survival. As many other studies have shown a gradual increase in frequency of apoptotic cell death, the study has given rise to confusion and controversy, which, although being recognised by most authors, is usually not further addressed. We feel that a few remarks concerning this report should be made. In the study of Bedi, the TUNEL method was used to identify apoptotic cell death in different colonic tissue sections. In ex vivo cultures of normal, adenomatous and carcinomatous epithelial cells, apoptotic cell death was determined by flow cytometry analysis. It must first be noted that the TUNEL studies were performed on frozen tissue sections, unlike all of the other studies discussed in this review. Second, using this method, the authors observed no apoptosis in colorectal carcinomas, which is most unusual. As all other studies have shown apoptotic cells in carcinomas, the reported absence of apoptosis in the Bedi report is likely to reflect deficiencies in the TUNEL technique. Furthermore, it must be realised that the analysis by flow cytometry in the ex vivo cultures of colonic epithelial cells represents a rather artificial approach, with several limitations. For example, the ex vivo cell survival studies were assessed after collagenase digestion and the preparation of single-cell suspensions. By thus interfering with normal cell-cell contact, which is known to affect the degree of apoptosis 62,63, results from the ex vivo cultures cannot readily be compared with those of other studies.

As stated before, most studies show that the proportion of epithelial cells undergoing apoptotic cell death increases in the course of the adenoma-carcinoma sequence. At first glance, this seems contradictory to the general idea, mostly based on in vitro experiments, that tumour progression is associated with decreased sensitivity to apoptosis induction. This has led to some confusion in the literature, possibly because decreased sensitivity to apoptosis induction is confused with a decrease in apoptotic cell death. One should realize that all 53 reviewed studies concern spontaneous apoptotic cell death and not sensitivity to chemotherapy- or otherwise induced apoptosis. An increase in spontaneous apoptotic cell death in the course of the adenoma-carcinoma sequence is not necessarily contradictory to decreased sensitivity to apoptosis induction. First, higher rather than lower levels of apoptosis would provide a mechanism to keep the size of adenomatous polyps stationary for prolonged periods of time in spite of continuing proliferation and would explain the slow growth of adenomatous polyps 64. Second, it has become clear that cell proliferation and cell death are coupled processes in several respects 65. For example, there is evidence that the increased proliferative activity in tumours might directly activate the program of apoptosis due to lack of nutrients, competition for growth factors, or oxygen starvation 65. Furthermore, the oncogene c-myc, which is overexpressed early in the adenoma-carcinoma sequence, has been shown to be a potent inducer of not only cell proliferation but also cell death 66. Moreover, the higher the amount of c-Myc expressed in cells, the greater the tendency to undergo apoptosis 65,67. The regulation of the balance between cell proliferation, differentiation and apoptosis is particularly important in tissues with high cell turnover rates, such as the colon. Thus, it might well be that increased cell proliferation paralleled by an increased proportion of
apoptotic cell death during the adenoma-carcinoma sequence eventually may select for the survival of mutant cells or cells with a survival phenotype that are resistant to the induction of apoptosis. The selection of advantageous mutations may be as important as an increased mutation rate in carcinogenesis. Such a selective pressure to override apoptosis has indeed been suggested by others.

3.3 Changes in locoregional distribution of apoptosis in the adenoma-carcinoma sequence

Normal mucosa. The growth and differentiation of colonic epithelial cells proceeds through distinct phases, depending on the position of a particular cell in the crypt. Stem cells, located at the bottom of the crypts, divide to form daughter cells, which proliferate rapidly and migrate up the crypts, differentiating into columnar epithelium, goblet cells and enteroendocrine cells. In normal colonic mucosa the proliferative compartment is confined to the lower half to two thirds of the crypt. As cells move towards the upper segments, they differentiate, lose the capacity to divide, and die within several days. Until recently, it was assumed that the cells were then passively detached into the colonic lumen or mechanically shed by an exfoliating action of passage of the gut contents. While this may be the case for a proportion of cells, the main physiological mechanism by which enterocytes die is by apoptosis.

Apoptosis of colon epithelial cells has clearly been demonstrated predominantly in the upper levels of the crypts. Apoptotic rates, calculated from the number of cells undergoing apoptosis in each colonic crypt, approximately balance the rate of cell renewal which supports the idea that apoptosis is responsible for the homeostasis of the epithelial cell population. At the luminal surface, part of the apoptotic cells are actively extruded from the epithelial layer into the lumen, while others are engulfed by surrounding epithelial cells or lymphocytes. This engulfment pattern is predominantly observed in the basal parts of the crypt. The occasional apoptotic events that are found in the lower, proliferative zones of the crypts are thought to occur in response to genetic damage. A recent morphological study suggested that epithelial cells undergoing apoptosis may also pass through fenestrations in the basement membrane to the lamina propria where they are taken up by macrophages.

Adenomas. Several authors have observed that in adenomatous polyps proliferation predominates at the luminal surface and apoptosis at the base of the crypt, a complete reversal of the normal pattern. In all these studies, the lower portion of the adenomatous crypts showed more apoptotic cell death than normal tissue. This pattern was seen both in sporadic adenomas as well as those associated with familial adenomatous polyposis (FAP). Others have observed apoptotic cells along the whole length of the adenoma crypts. Thus, it is generally agreed that in adenomas apoptosis takes place mainly in the lower part of the crypt, and to a greater extent than in normal crypts. These findings can be interpreted in several ways. One view is that in adenomas genetic alterations occur preferentially in the cells at the base of the crypt and that these cells are eliminated by apoptosis. Diminished apoptosis at the luminal end of the crypt may mean that the effort to control the damage has failed there, so the adenoma grows. It supports the concept of a defensive function of apoptosis to eliminate immature cells carrying mutations before they have the chance to mature and migrate up the crypt. Alternatively, the adenomatous polyp may grow inward, i.e. proliferating cells migrate towards the base of the crypt, a concept first suggested by...
Moss et al. Support for the inward growth model comes from a kinetic study of colonocytic migration in a patient with Gardner syndrome. Distribution of thymidine labelled cells showed an abnormal retrograde migration away from the adenoma surface towards the crypt base. More support for an inward growth model comes from a recent study, showing that dysplastic cells in early adenomas were located at the luminal surface of the crypts whereas the cells at the base of the crypts appeared morphologically normal. Moreover, these dysplastic cells were found to be mutated in contrast to the cells at the base. The study strongly suggests that the dysplastic process is initiated at the luminal side of the crypt and that genetically altered cells spread laterally and downward from the surface to the base of the crypt rather than the other way around. From these data, it seems unlikely that the observed increased apoptotic rates at the base of the crypts in adenomas reflect the deletion of cells that have gained genetic damage, as in the normal mucosa.

Finally, in carcinomas, there is probably no specific distribution of apoptotic cells, as they were usually randomly spread throughout the tumour according to the reviewed studies.

3.4 The correlation between apoptosis and proliferation

The balance between cell production through proliferation and cell loss through apoptosis determines how fast a tumour grows and is an important determinant of tumour behaviour. Most colorectal adenomas are stable for a long time before, if ever, transforming to malignancies. For example, 3-5 years after the initial diagnosis of adenomas, 70% showed no change in size. In animal studies, adenoma and carcinoma cells had cell cycle times about half of that of normal epithelium, reflecting higher growth rates. As increased cell death may be an attempt to limit the expansion of the tumour cell population, several studies have linked the rate of apoptosis with the proliferative rate of adenomas and carcinomas, yielding conflicting results. Levels of proliferation were associated with those of apoptotic cell death in adenomas and carcinomas in a number of studies, whereas others found no correlation. The finding that apoptosis and proliferation are not correlated is often used as an argument to support the assumption that an imbalance between these processes emerges in the course of the adenoma-carcinoma sequence. Supportive for this assumption are results from a study in which proliferative activity was shown to be correlated with apoptotic index in adenomas with low-grade dysplasia whereas this correlation was lost in adenomas with high-grade dysplasia and carcinomas.

Some studies have focussed on the ratio of apoptotic cells to proliferating cells as a proposed measure of susceptibility to apoptosis. Koike et al. found that this ratio was higher in adenomas of small size and with low- and middle-grade dysplasia than in those of larger size and with high-grade dysplasia. Baretton et al. found that the ratio was higher in adenomatous components than in carcinomatous tissue components. Both studies thus support the hypothesis that in the course of the adenoma-carcinoma sequence the susceptibility to apoptosis induction gradually diminishes. In summary, most studies show a considerable increase in both apoptotic cell death and proliferation in colonic neoplasms, illustrating abnormally high cell turnover rates. However, the two processes do not seem to be correlated in individual tumours, providing further evidence for the concept of a progressively disturbed balance between apoptosis and proliferation in the course of the adenoma-carcinoma sequence.
3.5 Apoptosis and prognosis of colorectal cancer

Currently, the most important prognostic variables in colorectal cancer are tumour stage and preoperative elevation of carcinoembryonic antigen. Several reports indicate that tumour cell kinetics may be an important prognostic variable in colorectal cancer. Traditionally, an increase in cell proliferation, rather than a change in apoptosis, has been used to predict later tumour development and prognosis. However, in an animal model, the best predictor of tumour development was the degree of apoptosis. While there is general agreement that deregulation of apoptosis contributes to malignant transformation, the potential predictive or prognostic value of the degree of apoptosis in colorectal cancer is controversial. Several studies have examined the prognostic value of the apoptotic index in colorectal cancer, producing conflicting results. Schwandner et al. showed that the apoptotic index was not predictive of prognosis in a series of 160 cases of rectal cancer. In three other studies, no prognostic significance was found in large groups of carcinomas. However, stratification by tumour site revealed that the apoptotic index was an independent predictor of survival in a series of 82 distal tumours (distal to splenic flexure). In two studies, it was shown that a low apoptotic index in the tumour was associated with poor survival. Two reports showed that apoptotic indices were higher in tumours that were more highly differentiated and had not invaded or metastasised than in those that were poorly differentiated and invasive or metastasising. Tanaka et al. also found higher apoptotic indices in tumours without lymph node or distant metastases in comparison to tumours that had metastasised, but they found no correlation with the degree of tumour differentiation. On the other hand, Hawkins et al. demonstrated that Dukes A carcinomas had lower apoptotic indices than Dukes B to D carcinomas. Metastatic dissemination may depend upon the resistance of metastatic cells to apoptosis. Indeed, in several murine and human cancer cell lines a more aggressive metastatic phenotype was associated with increased resistance to apoptosis.

Two recent interesting studies should be mentioned in this respect. Most colorectal carcinomas in hereditary non-polyposis colorectal cancer (HNPCC) and a proportion of sporadic colorectal carcinomas show a particular form of genetic instability, termed microsatellite instability (MSI), which means an accumulation of deletion and insertion mutations in simple repeated sequences. Patients with colorectal cancers with the MSI phenotype are known for better survival rates than patients with tumours without this phenotype. It has been proposed that colorectal tumours with the MSI phenotype develop along a different pathway compared to colorectal tumours without this phenotype, and that these tumours have an increased mutation rate as a result of inactivation of a mismatch repair gene. It has also been shown that apoptosis can be induced by overexpression of the mismatch repair genes hMSH2 or hMLH1, suggesting that tumours with the MSI phenotype lose the ability to undergo efficient apoptosis. Surprisingly however, it was demonstrated by two groups that apoptotic cell death was actually more frequent in colorectal tumours with MSI than in those without MSI. Taken together, although most studies suggest that colorectal tumours with high apoptotic indices are associated with better prognosis and survival, we believe that more work is required before the apoptotic index can be considered as a potential prognostic marker or indicator for the choice of therapy in colorectal neoplasms.
4 Potential mechanisms of dysregulation of apoptosis

4.1 Abnormal expression of apoptosis-regulating genes in colorectal cancer

It has been firmly established that colorectal carcinogenesis is characterised by a stepwise accumulation of genetic alterations (for extensive recent reviews, see Chung et al. 107 or Ilyas et al. 108). The accumulation of genetic alterations during the adenoma-carcinoma sequence may provide the basis for changes in the degree of apoptotic cell death, as many of the genes involved have been shown to regulate apoptosis. Most studies have focused on the possible roles of the tumour suppressor genes adenomatous polyposis coli (APC) and p53 and the proto-oncogene bcl-2, which will be briefly discussed below.

4.1.1. APC

Mutations in the APC gene have been implicated in both sporadic and familial colorectal neoplasia 2,109. The frequency of APC mutations is similar in colonic adenomas and carcinomas (approximately 60%), suggesting that APC mutations may be an early or even the initiating event in the process of colorectal carcinogenesis 110. Immunohistochemical studies have shown that the APC protein is expressed in normal epithelial cells as they migrate toward the top of the crypt 111,112. Disruption of normal APC function presumably disturbs the equilibrium between new cell formation at the base of the crypts and cell death at the top of the crypts, leading to a relative expansion of the progeny of APC-mutant cells. An important role of the APC gene in regulating apoptosis is suggested by an experiment in which the expression of APC in human colorectal cancer cells containing endogenous inactive APC alleles resulted in the induction of cell death through apoptosis 113. The functional significance of the APC gene probably lies not only in the regulation of apoptosis, but also in control of cell cycle progression, migration and differentiation 114. It has been shown that in normal cells, the APC protein resides in a large complex with axin, glycogen synthase kinase 3ß (GSK3ß) and ß-catenin 114,115. Loss of APC protein function leads to ß-catenin accumulation in the nucleus where it binds to TCF/Lef transcription factors 116,117. This complex then activates transcription of TCF target genes 118. Thus far, genes including c-myc, cyclin-D1, matrilysin, peroxisome proliferator-activated receptor delta and MDR1 (multidrug resistance) have been reported to be the target genes of this complex, influencing cell proliferation and apoptosis 119,120. In the herein-reviewed studies, APC protein function was never assessed by either immunohistochemistry or by mutation analysis.

4.1.2. P53

Mutations of the p53 gene occur in various human tumours, including colorectal cancer 121,122. Deletions and mutations of the p53 gene can be detected in up to 85% of colorectal tumours and usually occur during the transition from adenoma to adenocarcinoma 123. Several functions have been ascribed to the p53 tumour suppressor gene, reviewed by Levine122 and Sigal 124. Its product, the p53 protein, may respond to DNA damage by triggering either growth arrest during G1 or G2 phase of the cell cycle or programmed cell death. In this manner, p53 may protect the normal cell from proceeding to replicate damaged DNA. The wild-type p53 protein, but not the mutant, can initiate apoptosis. Introduction of the wild-type p53 gene mediated apoptosis in human colon carcinoma cell lines and tumours in nude mice.
underwent regression if wild-type p53 expression was induced \(^{25}\). The mutated p53 protein may block the function of the wild-type p53 protein and thereby inhibit induction of apoptosis. It has also been found that p53 has profound effects on responses to chemotherapeutic drugs used in colorectal cancer, and that these effects vary considerably depending on the drug \(^{26}\).

Several studies have assessed the correlation between p53 protein expression and apoptosis in colorectal neoplasms. In a murine model, levels of spontaneous apoptosis were similar in both normal and p53 knockout mice in normal small intestine and colon epithelium \(^{27}\). In addition, homozygosity for an inactivating germ-line mutation of p53 did not affect the frequency of apoptosis in mouse adenomas \(^{50}\). Due to a germline APC mutation, these mice were strongly predisposed to adenoma formation. Moreover, p53 inactivation did not additionally enhance adenoma formation or adenoma progression. In the same study, the degree of apoptosis was not different in human colon adenomas between areas with high-grade dysplasia, associated with loss of p53 function, and areas with low-grade dysplasia, associated with intact p53 function. Some of the herein reviewed studies have indicated that adenomas and/or carcinomas with a high percentage of cells expressing the p53 protein were more likely to have a low apoptotic index \(^{4,6,19,45,128,129}\), whereas most studies did not show such a relationship \(^{7,12,33,35,36,39,45,48,51,52,54,57,81,92,130,131}\).

In summary, the fact that neither immunohistochemical overexpression of p53 nor p53 mutations correlated to the frequency of apoptotic cell death in the majority of studies does not support a major role for mutant p53 protein as an inhibitor of apoptosis in the development of colorectal cancer.

4.1.3. Bcl-2

At least 15 bcl-2 family member proteins have been identified in mammalian cells, including proteins that promote apoptosis and those that prevent it \(^{132}\). The oncogene bcl-2 promotes cell survival by blocking apoptosis \(^{133}\). Bcl-2 protein is normally expressed only in the lower half of the crypts of the colon, corresponding to the stem cell compartment, where bcl-2 is believed to protect stem cells from apoptosis \(^{134}\). Most colonic adenomas express bcl-2 protein at high levels throughout the neoplastic epithelium \(^{4,48,51,133,135-139}\), while non-neoplastic polyps have a normal pattern of bcl-2 expression \(^{48,136,137}\). Overexpression of bcl-2 may therefore contribute to the transition between hyperplastic epithelium and adenomas. Bcl-2 protein expression in colorectal carcinomas is higher than in normal mucosa, but lower than in adenomas \(^{12,48,51,70,135-139}\). In a murine model, bcl-2-null mice have increased levels of spontaneous apoptosis in colonic crypts compared with wild-type mice \(^{131}\). An inverse correlation has indeed been reported between bcl-2 expression and the apoptotic index of colonic tissues \(^{4,7,12,36,54,90}\), whereas others have found no such correlation \(^{33,55,41,48,51,92,61,139,94,95}\). With respect to the correlation between bcl-2 expression and prognosis in colorectal cancer, reports are conflicting \(^{91,135,140-144}\). Considering the relationship between bcl-2 and p53 overexpression, some have found an inverse correlation in adenomas \(^{7,55,131,139}\).

Taken together, bcl-2 expression seems to be gradually reduced in the course of the adenoma-carcinoma sequence and inversely related to p53 overexpression. As most studies show a gradual increase in frequency of apoptotic cell death, a possible relationship with downregulation of bcl-2 can be hypothesised. However, bcl-2 is probably only one of the genes that determine the incidence of apoptotic cell death in colorectal neoplasms. Indeed,
changes in the expression of other members of the bcl-2 family have been shown during progression of colorectal tumors, such as the anti-apoptotic proteins bcl-X<sub>L</sub>, mcl-1 and the pro-apoptotic protein bak, which may be more important than bcl-2<sup>145</sup>.

4.2 The role of apoptosis induction by the TNF ligand family

Apoptotic cell death can be initiated by endogenous stimuli, such as the absence of oxygen, nutrients or growth/survival factors, the presence of DNA damage or the action of cytokines as well as by exogenous stimuli such as ionising radiation and chemotherapeutic drugs<sup>69</sup>. It has been suggested that alterations in frequency of apoptosis and the development of resistance to apoptosis induction may be explained by changes in apoptosis-regulating cytokines<sup>146,147</sup>. The tumour necrosis factor (TNF) ligand family comprises cytokines that serve important functions as mediators of immune regulation, inflammation and apoptosis<sup>148</sup>. Members of the TNF ligand family bind and interact with specific receptors of the TNF receptor (TNF-R) family. Binding of the ligands to their respective receptors triggers diverse intracellular signalling pathways, promoting cell differentiation, apoptosis, but also cell proliferation<sup>149</sup>. Three major apoptosis-inducing cytokines have been identified so far<sup>150</sup>. These are TNF-α, Fas ligand (FasL, also known as CD95L) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), all of which induce apoptosis by binding to their respective receptors<sup>151</sup>. Current knowledge on the possible roles of these cytokines in the development of colorectal cancer is briefly discussed below.

The most important physiological function of TNF-α probably lies in the regulation of inflammation rather than apoptosis-induction and its role in colorectal cancer development seems limited<sup>152</sup>. Fas mediates apoptotic cell death upon engagement by its natural ligand, FasL<sup>153</sup>. Fas-mediated apoptosis is involved in several regulatory functions within the immune system (for recent reviews see Walczak<sup>148</sup> or Krammer<sup>154</sup>). Human colonic epithelial cells constitutively express Fas and an intact Fas signalling pathway has been shown to be capable of regulating apoptosis in human colon carcinoma cell lines<sup>146,147,155</sup>. It is known that many tumour cells escape from immune cytolysis and become resistant to FasL from anti-tumour immune effector cells<sup>156</sup>. The mechanisms responsible for this resistance are complex, and several possibilities have been proposed, including downregulation of the Fas receptor and upregulation of FasL in tumour cells<sup>157,159</sup>. p53 mutations in colon carcinomas may also directly inhibit Fas signalling<sup>156,160</sup>. A concept that has become known as the Fas-counterattack has gained wide attention<sup>156,161,162</sup>. It is based on the observations that tumour cells from various origins, including colon cancer, expressed FasL and induced apoptosis in Fas-expressing tumour-infiltrating lymphocytes and/or peripheral T cells<sup>163</sup>. However, recent experiments could not confirm these results in several colon cancer cell lines<sup>163</sup>. In summary, although the Fas/FasL pathway has been extensively studied in colorectal cancer and several experiments have suggested a possible role for disturbances in Fas/FasL-mediated apoptosis in colorectal carcinogenesis, its precise impact still has to be defined and the counterattack hypothesis is seriously debated<sup>164</sup>.

TRAIL has been shown to induce apoptosis in a wide variety of tumour cells in vitro. Approximately 80% of human cancer cell lines, representing colon, lung, breast, skin, kidney and brain tumours were sensitive at least to some extent to TRAIL, whereas most normal cell types were relatively resistant<sup>155-167</sup>. Yet, little is known about the physiological
role of TRAIL mediated apoptosis. In a murine model, it was recently shown that TRAIL was partly responsible for surveillance of tumour metastasis by liver natural killer cells, suggesting a physiological role as a tumour suppressor \(^{168}\). For TRAIL, five different receptors have been identified: two cell death-inducing receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2), two non-cell-death-inducing receptors (DcR1/TRAIL-R3 and DcR2/TRAIL-R4) and osteoprotegerin \(^{169,170}\). Until recently, levels of TRAIL-receptors were known only from mRNA expression. TRAIL mRNA and DR4 and DR5 transcripts are expressed in several tissues, including normal colon epithelium and colon adenocarcinomas \(^{171}\). Future studies aimed at elucidating the expression of TRAIL and its receptors in the adenoma-carcinoma sequence will not only contribute to our general understanding of colorectal carcinogenesis but may also serve to design new therapeutic approaches aimed at stimulating apoptosis. TRAIL specifically appears to be a promising new anti-cancer agent which, unlike TNF-\(\alpha\) and FasL, seems to induce tumour-selective apoptosis, while sparing normal cells \(^{165,172}\). With respect to differences in sensitivity towards TRAIL-induced apoptosis between normal and tumour cells, several theories exist, including the decoy hypothesis. This hypothesis is based on the assumption that cellular sensitivity or resistance to TRAIL-induced apoptosis is dependent on the presence or absence of specific receptors \(^{173}\). It was supposed that TRAIL does not induce apoptosis in normal cells because these are protected by the presence of the non-signaling receptors DcR1 and DcR2. However, examination of TRAIL receptors in large panels of cultured tumor cells showed no correlation between the expression of DcR1 and DcR2 mRNA and the resistance or sensitivity to TRAIL treatment \(^{174}\). In addition, it has been shown that the expression and localisation of TRAIL receptors varies between different cells and that resistance to TRAIL is mediated by different mechanisms such as the differential subcellular localisation of decoy receptors and intracellular inhibitors of apoptosis \(^{175}\).

Although the precise mechanism of TRAIL-mediated apoptosis in normal and tumour tissues as well as the clinical significance and the molecular biologic regulatory mechanisms have yet to be elucidated, the cell killing properties of this cytokine has made it an exciting target for drug development \(^{176}\). Concerns of hepatotoxicity or brain toxicity associated with the use of TRAIL have been expressed following experiments where polyhistidine- and FLAG-tagged recombinant versions of human TRAIL were found to induce apoptosis in vitro in isolated human hepatocytes and brain tissue slices, respectively \(^{177,178}\). However, in contrast to polyhistidine-tagged TRAIL, native rhTRAIL was nontoxic to cultured hepatocytes \(^{179}\). In addition, two relevant non-human primate models have indicated that systemic administration of native rhTRAIL is unlikely to cause major toxicity to the liver or other organs \(^{179,180}\).
5. Possibilities for intervention: Apoptosis and chemoprevention

Therapies designed to stimulate apoptosis in target cells play an increasingly central role in the prevention and treatment of both hereditary and non-hereditary colorectal cancer. Chemoprevention of colorectal cancer is defined as the use of pharmacological agents to prevent or reverse the development of adenomatous polyps and subsequent progression to colorectal cancer. The importance to identify drugs that can prevent disease development and progression, particularly in high-risk populations is generally recognised. Several agents have been studied for chemoprevention for colorectal cancer (for recent reviews, see Sharma or Krishnan). Of these agents, non-steroidal anti-inflammatory drugs (NSAIDs) are currently most widely used. It has been shown that NSAIDs inhibit cell growth in culture, decrease the incidence of colon tumours in carcinogen induced murine models and decrease the relative risk of incidence and mortality of colorectal cancer in human epidemiological studies. Although NSAIDs may exert effects through a number of potential mechanisms, accumulating evidence suggests an effect of these drugs on apoptotic cell death. For example, numerous reports have shown that the NSAID sulindac induces apoptosis in epithelial cells in normal colonic mucosa and adenomas in familial adenomatous polyposis (FAP) patients and in a murine model of FAP. Several animal studies and cell culture experiments have confirmed the ability of sulindac to induce apoptosis. Unfortunately, there is significant toxicity associated with long-term NSAID use, in particular gastrointestinal bleeding. Such toxicity seriously compromises the overall value of NSAID-mediated chemoprevention in at-risk individuals and necessitates the development of safer agents. Agents that directly induce apoptosis may reduce the risk of toxicity and reduce the opportunity for acquired drug resistance. A critical issue in chemoprevention is the relative sensitivity of cells at different stages of neoplastic progression. The molecular elements determining the sensitivity remain to be determined. Nevertheless, the identification of other biochemical targets for chemoprevention that specifically induce apoptosis would provide new strategies to prevent colonic neoplasms. As death receptor-mediated apoptosis is predicted to be independent of p53 status, application in treatment or prevention of colorectal cancer seems promising. In the coming years, it seems likely that rational strategies to manipulate apoptotic programs will produce new therapies that are less toxic than current chemoprevention strategies.

6. Conclusions

The ability to undergo apoptosis is an important mechanism for maintaining control over a population of cells under continuous renewal, as in the colonic epithelium. Furthermore, apoptosis is particularly important for the elimination of cells with unrepaired DNA damage. A reduction of this ability would result in the retention of cells with DNA damage and a consequent increased risk of mutations, including those that are carcinogenic. Several genes that regulate apoptosis are inappropriately expressed or mutated in colorectal cancer. Although the idea of decreased apoptosis during colorectal cancer development is conceptually attractive, the majority of studies show that the proportion of cells undergoing apoptosis increases in the course of the adenoma-carcinoma sequence, possibly as a
mechanism to delete cells with sustained DNA damage. As most in vitro studies indicate the emergence of gradual resistance to apoptosis-induction towards tumour progression, it might be that the increased cell turnover rates together with increase in apoptotic cell death selects for the survival of cells resistant to induction of apoptosis. Considering changes in the locoregional distribution of apoptotic cells, consensus emerges from the reviewed studies that in adenomas a reversal of the normal pattern of proliferation and apoptosis in colonic crypts is observed. Evidence is accumulating supporting an ‘inward’ or ‘top-down’ growth model of adenomas. The degree of apoptosis in colorectal tumours is not related to the degree of proliferation in most studies. The use of the apoptotic index in a tumour as a potential prognostic marker or indicator for the choice of therapy, as suggested by some, is currently not supported by the available data. In the reviewed studies, the methods used to identify apoptotic cell death varied widely, with in situ end labelling methods prevailing. As a consequence, reported apoptotic indices varied considerably. Considering the limitations of the methods of in situ end labelling of DNA fragments, used in the vast majority of studies, improved diagnostic criteria for the identification of apoptotic cells are needed and can be expected in the near future. Further identification of the pathways controlling apoptosis including the role of TNF ligand family members will provide opportunities to understand the homeostatic control of normal colon tissue and reveal new targets for prevention and therapy of colorectal cancer.
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