

Review

Apoptosis in the Colonic Crypt, Colorectal Adenomata, and Manipulation by Chemoprevention

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Abstract

This review discusses the biology and the methods of assessment of apoptosis, of which, the monoclonal antibody M30 would seem to be the most useful; the role of apoptosis in the etiology of colorectal cancer; and its use

as a marker to monitor the beneficial effects of chemopreventative interventions to reduce the development of colorectal cancer within the context of clinical trials. (Cancer Epidemiol Biomarkers Prev 2009;18(6):1680–7)

Epidemiology of Colorectal Cancer

Worldwide diagnoses of bowel cancer approximate an estimated 1 million new cases per year, comprising 9% of all cancer cases, and this has continued to increase over the last 25 years.¹ In Europe alone in 2006 there were an estimated 412,800 cases of colorectal cancer diagnosed, and of the 1.7 million European cancer deaths, 207,400 were caused by colorectal cancer. This represents an estimated increase of 1.8% from 2004 (1).

There is a 25-fold variation in the incidence rates of colorectal cancer between different countries in the world, which is highly suggestive of underlying environmental influences in the etiology of this disease (2). It has been suggested that these underlying environmental factors are predominantly dietary and that up to 80% of sporadic colorectal cancers are therefore potentially preventable (3).

Apoptosis

The term apoptosis was first coined in 1972 by Kerr and colleagues (4), to describe a specific process of cell death in which a series of characteristic morphological changes are seen to occur. It is a naturally occurring phenomenon characterized by a genetically controlled mechanism and is fundamental to normal development and maintenance of tissue homeostasis (5). In contrast to necrosis in which several contiguous cells die, apoptosis is specific to individual cells, which neither then release their intracellular contents nor induce a subsequent inflammatory response (6).

Morphology of Apoptosis

Wyllie and colleagues (6) described the five cardinal features of apoptosis:

1. Loss of specialized surface structures. Loss of surface structures such as microvilli, and surface contact regions, results in the cell adopting a smooth contour, and becoming isolated from its viable neighbor cells.
2. Reduction in volume. The cell reduces in volume, primarily via a loss of water, with the increasing density associated with concentration of the cytoplasmic organelles and a resulting compaction of the cell. The cell may then split to form several apoptotic bodies at this point.
3. Conservation of cytoplasmic organelles. In contrast to necrosis, mitochondria and other organelles do not undergo swelling and subsequent membrane rupture.
4. Condensation of nuclear chromatin. The most striking feature of apoptosis is the change that results in the chromatin becoming condensed, more granular, and appearing as dense semilunar caps when viewed with electron microscopy. At the same time, the cell distorts and the nucleus breaks into several fragments giving a patchy appearance (7).
5. Phagocytosis. Following the above changes, particularly to the plasma membrane, the apoptotic cell is recognized by its viable neighbor cells (parenchymal cells or specialized phagocytes) as a target for phagocytosis. Within the phagosome of the ingesting cell, progressive degenerative changes of the apoptotic cell, or the apoptotic bodies into which it

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¹ Cancer Research UK [homepage on the Internet]. London: Cancer Research UK [cited 2007 October 15]. Cancer Worldwide. Available from: <http://info.cancerresearchuk.org/cancerstats/geographic/world/commoncancers/#source1>.

may have fragmented, take place, with disappearance of membranes, loss of any recognizable organelles, and ultimately the appearance of lysosomal residual bodies. Occasionally, apoptotic cells escape phagocytosis, for example apoptotic epithelial cells being lost into the duct lumen: these cells eventually degenerate, and the remnants are excreted via the gastrointestinal tract (7). Cytoplasmic organelles and lysosomes in apoptotic bodies remain intact, accounting for the absence of inflammation in apoptosis, in contrast to necrosis in which lysosomes leak their contents into the surrounding cytoplasm.

Mechanisms of Apoptosis

Apoptosis is an active process, under specific control mechanisms, and occurs physiologically during remodeling of tissues during fetal development. Apoptosis is also the method by which senescent cells are removed in differentiated tissues as well as cells that are irreparably damaged. A balance of cell proliferation and cell death normally maintains cellular homeostasis.

In basic terms, if apoptosis-inducing signals are selected in favor of anti-apoptotic signals, the cell will be selected for programmed death. However, clearly this has to be a tightly controlled process and involves a number of messengers across several steps.

Apoptosis can essentially be divided into four constituent stages:

1. Stimulus: The two primary stimuli for triggering apoptosis are physiological or pathological; first via the exposure to death promoting members of the Fas ligand (or CD95, APO-1), or tumor necrosis α family (8, 9), or second by lack of key survival or growth factors (10).
2. Signaling: Intracellular information is relayed to the cell, by another set of messengers in readiness for interpretation and regulation (step 3). The amount of information received depends on the type and duration of the stimulus and includes molecules from both gene expression (e.g., *p53*, see ref. 11, kinases, see ref. 12) and cellular metabolism (e.g., reactive oxygen species, see ref. 13).
3. Regulation: Once the cell has received all the necessary information, it is integrated and processed by the mitochondrion, which lies at the core of the regulatory pathways involved in apoptosis. If appropriate, the decision will then be made to terminate the cell, and this is mediated by the protein bcl-2 and its 30 family members, which are subclassified as either pro- (e.g., Bax, Bak, Bad, Bcl-XS, Bik, Bim) or anti-apoptotic (e.g., Bcl-2, Bcl-XL, Bcl-w, A1) (ref. 14).
4. Execution: Once the decision has been made, cell death ensues. The major pathway is via activation of a family of cysteine proteases, caspases, which ultimately kill the cell, which in turn occurs through release of cytochrome *c* from mitochondria (15) in response to the information received following regulation. Ultimately, DNA is cleaved into small packages by caspase-activated DNase (CAD) (16).

Caspases are named after the two main catalytic properties of the proteases: the "c" reflects a cysteine protease mechanism, and "aspase" refers to their ability to cleave after aspartic acid, the most distinctive catalytic feature of this protease family (17). Caspases are synthesized as proenzymes and are proteolytically activated. Caspases implicated in apoptosis are divided into initiators and executioners and are required for the accurate and limited proteolytic events that typify this type of programmed cell death (18). Initiator caspases link death signals to the cellular death program, and executioner caspases carry out a coordinated program of proteolysis resulting in the destruction of critical cell structures (19). It has been shown that when cells that have been induced to undergo apoptosis do so bathed in cell permeable caspase inhibitors, death is delayed but not abolished (20). Blocking caspase activity prevented cleavage of nuclear and cytosolic substrates and normal DNA degradation, whereas a decrease in mitochondrial membrane potential, production of reactive oxygen species, cytoplasmic vacuolation, and plasma membrane permeability still occurred (20). The resulting death morphology was not apoptotic but more reminiscent of necrosis.

This brief overview of apoptotic mechanisms is not exhaustive and two informative reviews exist that explain the process in more detail: one specific to colorectal carcinogenesis (21); the other relating to chemoprevention of colorectal cancer (22).

Methods of Determining Apoptosis in Histological Slides

Morphological Criteria in Hematoxylin-Eosin-Stained Sections. Apoptotic cells can be recognized by careful examination of hematoxylin-eosin-stained (H&E) sections, showing the morphological criteria defined by Kerr and colleagues (4). This examination is considered by some to be the reference standard technique for determining apoptotic cells in histological sections (23). However, criticisms of this technique are that apoptotic bodies can be easily missed and are the end result of the process, therefore potentially missing early apoptotic cells (24). For reasons such as this, a number of antibody based techniques have been developed in an attempt to overcome these problems.

Terminal Deoxyribonucleotidyl Transferase-Mediated Nick End Labeling and *In Situ* End Labeling. These two techniques rely on the presence of DNA strand breaks in apoptotic cells, caused by the activation of endogenous nuclease activity during the process of apoptotic cell death (25-27).

Terminal Deoxyribonucleotidyl Transferase-Mediated Nick End Labeling. In 1992, Gavrieli and colleagues (28) published details of a technique of *in situ* labeling of DNA breaks in nuclei, in routinely processed histopathological tissue sections. Terminal deoxyribonucleotidyl transferase-mediated nick end labeling (TUNEL) was based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, and subsequent TdT incorporation of biotinylated deoxyuridine at the site of DNA breaks. This signal is amplified by avidin-peroxidase,

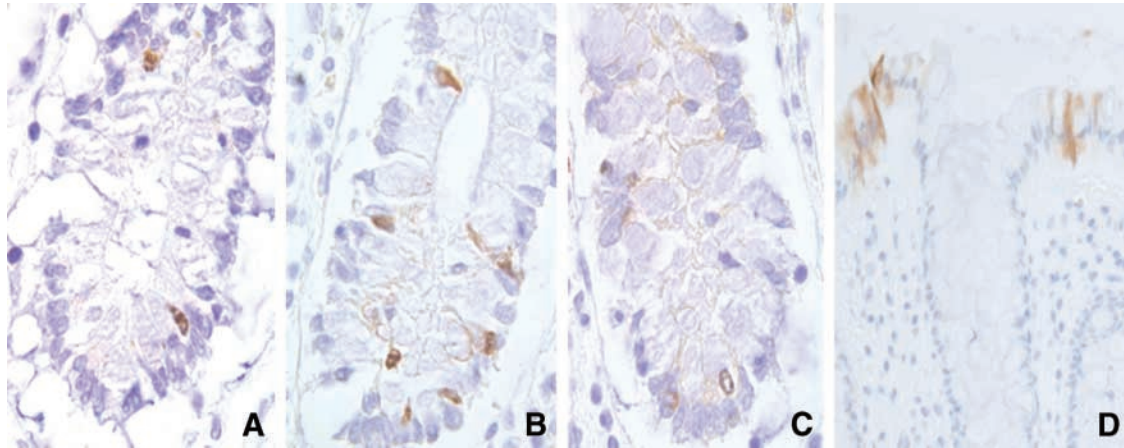


Figure 1. Illustrations of methods for demonstrating apoptosis in human colonic mucosal crypts. **A**, phosphorylated histone H2AX (basal crypt); **B**, cleaved caspase 3 (basal crypt); **C**, cleaved laminin A (basal crypt); **D**, M30 (luminal crypt). **A**, **B**, and **C** show human colonic crypts subjected to *ex vivo* deoxycholate induced stress to increase apoptosis, stained with three different immunohistochemical markers for apoptosis (indicated by brown staining). **D**, shows normal colonic mucosa ($\times 200$) stained with the M30 monoclonal antibody to show mucosal cells undergoing apoptosis (stained brown). Mucosal apoptosis has been upregulated in this individual by the administration of the omega₃ fatty acid EPA ref. 75.

enabling conventional histochemical identification by light microscopy.

In situ End Labeling. The *in situ* end labeling (ISEL) technique, described by Ansari and colleagues (29) in 1993, utilizes the same principles, but involves using the Klenow fragment of *Escherichia coli* DNA polymerase I, to add the biotin labeled deoxyuridine triphosphate to the 3'-OH end of fragmented single-stranded DNA.

The introduction of these two techniques has led to a number of publications utilizing both these methods to study apoptosis in a variety of tissues. However, there are a number of notable drawbacks with both: Most importantly, TUNEL and ISEL can lead to staining of necrotic and autolytic cells as well as apoptotic cells (29, 30). Additionally, false-positive staining in liver and intestine has been reported, because of the presence of endogenous endonucleases (31). Potten has questioned the reliability of these *in situ* labeling techniques, having found a false negative rate of 17% to 35% when identifying apoptotic cells in small intestinal mucosa, these cells showing morphological features of apoptosis but failing to stain by *in situ* labeling techniques (23). Finally, there is also evidence that the physical process of histological sectioning itself can produce TUNEL labeling (32). For reasons such as these, combined with the advent of new techniques, there is a move away from the TUNEL and ISEL methods for the detection of apoptosis in histological sections (24).

M30 Monoclonal Antibody. Prior to morphological nuclear disruption in apoptotic epithelial cells, a dramatic reorganization of the keratin cytoskeleton occurs, with degradation of simple epithelial keratins involving the specific cleavage of cytokeratin 18 by caspases (33). This caspase cleavage of cytokeratin 18 liberates a neo-epitope that is specifically recognized by the M30 monoclonal antibody, allowing the detection of apoptosis in epithelial cells at an early stage, and prior to the appearance of breaks in DNA (34). Furthermore,

the epitope is expressed in formalin-fixed, paraffin-embedded tissue, and viable and necrotic epithelial cells do not express this epitope and therefore show no immunoreactivity with the M30 antibody (34). In colorectal neoplasms, a strong positive correlation has been found between *in situ* end labeling and expression of the M30 antibody in formal-fixed, paraffin-embedded tissue (35). The procedure for M30 immunohistochemistry was found to be technically simpler and the immunoreactivity of the M30 antibody easier to interpret than *in situ* end labeling (35). More recently, apoptotic indices obtained by M30 immunoreactivity and morphological criteria were positively correlated ($r = 0.71$, $P < 0.01$) in normal colorectal mucosa, adenomas, and carcinomas (36). The M30 monoclonal antibody seems to be an accurate technique for demonstrating apoptotic epithelial cells in normal and neoplastic formalin-fixed, paraffin-embedded colorectal tissue.

Cleaved Caspase 3. Caspase 3 is one of the central effector caspases and mediates the cleavage of itself, as well as other downstream caspases. Normally caspase 3 exists as a procaspase in which the potential cleavage site is intact, but once cleaved through activation of the apoptotic cascade, the peptide end of this caspase represents a novel epitope not present in normal cells (37). Antibodies to cleaved caspase 3 have been developed and have been reported to be useful for detecting apoptotic cells in archival paraffin embedded tissue (37).

Other Markers of Apoptosis. A number of immunohistochemical markers of apoptosis, which can be applied to archival paraffin embedded tissue, have now been commercially developed. Recently, Holubec and colleagues (38) compared a number of these against morphological evaluation of apoptosis in colonic mucosa that had been stressed *ex vivo* with the bile acid deoxycholate to induce apoptosis. In addition to investigating the previously discussed antibodies to cleaved

caspase 3 and cytokeratin 18 (M30), they investigated antibodies to cleaved lamin A, cleaved poly(ADP-ribose) polymerase (PARP), apoptosis-inducing factor (AIF), and phosphorylated histone H2AX (γ H2AX). Lamin A is an intermediate filament protein and a major component of the nuclear lamina, which during apoptosis is cleaved by caspase 6 (39). AIF is released early on in apoptosis together with cytochrome-c and endonuclease G after alteration of the mitochondrial membrane (40). H2AX is one of the members of the H2A histone family that becomes rapidly phosphorylated at the sites of DNA double-strand breaks, and γ H2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis (41, 42). Finally, PARP-1 is cleaved by caspases 3 and 7 during apoptosis (43).

Strictly morphological assessment of colonic mucosa was the most sensitive method for detecting apoptosis within the crypts, whereas the M30 antibody was found to be almost as sensitive and about as specific for the identification of apoptotic colonic epithelial cells (38). Antibodies to cleaved caspase 3, cleaved lamin A, and γ H2AX, although specific for the identification of colonic apoptotic cells, were found to be less sensitive than either morphological criteria or M30. Examples of these four stains can be seen in Fig. 1. Antibodies to PARP and AIF were nonspecific for the identification of apoptotic cells. Therefore, of all these immunohistochemical methods of determining apoptosis, it would seem from this study that M30 is the most useful for distinguishing apoptotic cells in colonic epithelium.

Apoptosis and Colorectal Neoplasia

Apoptosis in Normal Colorectal Mucosa. Normal homeostasis of colonic mucosa relies on a balance between proliferation at the base of the crypt, and apoptosis at the surface epithelium. Apoptosis along the surface epithelium is therefore the result of physiological turnover of senescent cells, whereas apoptosis occurring in the lower proliferative zones of the crypt is an occasional phenomenon and thought to be in response to genetic damage (44, 45).

Koornstra reviewed studies assessing the changes in apoptosis from normal colorectal mucosa, through adenoma to carcinoma (44). In those studies that analyzed apoptosis in normal colorectal mucosa, which showed considerable variation (0.11–11% apoptotic index), it was noted that what many authors described as “normal,” was actually tissue taken adjacent to neoplasia, which could be interpreted as either a response to the carcinogenesis, or, representing the earliest stages of tumor formation (46). In addition, the majority of studies used TUNEL or ISEL, both of which have disadvantages, as discussed previously.

Although some authors recorded a difference in apoptosis in different parts of the colorectum, lower in the right colon when compared with the left colon (47), others did not (48). Although, Anti and colleagues (47) did show a gradient of apoptotic index in those patients with a history of adenomas, being higher in the right colon than the left (in contrast to Liu, ref. 48), which he concluded could explain the higher incidence of polyps and cancers in the left colon and rectum in the sporadic population.

Apoptosis in Adenomas. In adenomatous polyps, the pattern of apoptosis has been found to be reversed relative to normal mucosa, with reduced apoptosis in the surface epithelium, and increased levels of apoptosis at the base of the adenomatous crypts (49, 50). When the rates of apoptosis were assessed in the transition from normal mucosa to adenoma, most studies assessed by Koornstra showed an increase in apoptotic activity with a corresponding increase in apoptosis as the grade of dysplasia increased. However, no such correlation was found when comparing apoptotic indices with histological subtype of polyp (44).

Apoptosis in Colorectal Carcinoma. Apoptosis was noted to be higher in the majority of studies Koornstra reviewed, when carcinomatous tissue was compared with adenomata, including one study that compared both tissue types from the same tumor (51). However, others have either observed no change in apoptosis or a decrease (44).

Koornstra concluded that the accompanying increase in apoptosis was more obvious for the transition from normal mucosa to adenoma than from adenoma to carcinoma (44).

Apoptosis and a Field Defect for Colorectal Carcinogenesis. An important study that has suggested that higher levels of rectal apoptosis are associated with lower prevalence of colorectal adenomas, and in addition show a field effect for apoptosis, was published by Martin and colleagues in 2002 (52). Complete colonoscopy was done on 226 patients with adenomas and 493 adenoma-free controls with rectal biopsies in all, taken from grossly normal appearing mucosa, to assess the associations between nonsteroidal anti-inflammatory (NSAID) use, adenomatous polyps, and apoptosis. Apoptosis was assessed using morphology from H&E prepared tissue and showed that scores differed significantly between adenomas cases and controls (means = 2.46 versus 2.94, respectively) when compared in an analysis of variance procedure ($P < 0.0001$). In addition, compared with the lowest tertile, persons in the highest tertile of rectal mucosal apoptotic activity were much less likely to have adenomas [odds ratio (OR) 0.12; 95% confidence interval (CI) 0.07–0.20]. NSAID use and increased levels of apoptosis were independent for lower adenoma prevalence (52). Importantly, the authors concluded that the observed low levels of rectal apoptosis in association with the findings of adenomas elsewhere in the large bowel would be “consistent” with a field effect (52). Anti (48) also described the apoptotic index throughout the colon being lower in those patients with adenomas when compared with those controls without polyps, although it should be noted that fewer patients ($n = 15$) were included as compared with Martin and colleagues, and TUNEL was used to assess apoptosis.

More recently, Badvie and colleagues (53) measured labeling indices of the anti-apoptotic protein Bcl-xL in samples taken from the tumor, as well as normal mucosa 1 cm and 10 cm proximal to the tumor in 63 patients with colorectal carcinomas. Markedly increased Bcl-xL labeling indices were found in the tumor, as well as the normal mucosa 1 and 10 cm from the tumor when compared with colorectal tissue taken from controls ($n = 22$) with

benign disease. These findings are consistent with a field change of inhibited apoptosis in mucosa adjacent colorectal carcinomas. The effect was more marked with larger tumors and those with lymph node positivity (53). However, as the investigators did not biopsy normal mucosa farther away from the tumor than 10 cm, it is not possible to know if this perturbed Bcl-xL labeling persists throughout the colorectum in subjects with colorectal carcinomas.

Apoptosis to Predict Risk of Future Adenomas. A recent study having previously measured the levels of apoptosis (using morphology from H&E stained specimens) within the normal rectal mucosa of patients attending for colonoscopy for a variety of reasons, but without colitis, familial adenomatous polyposis (FAP), previous colorectal cancer resection, previous colon cancer or adenoma, then correlated this previously measured apoptosis score with the number, size, histological type, and degree of atypia of polyps found at a subsequent follow-up colonoscopy (54). The follow-up population included patients with polyps at baseline who returned for follow-up of these, and patients without polyps at baseline but who were scheduled for a clinically indicated colonoscopy. The findings were of an inverse relationship between apoptosis and the development of new adenomas, and subjects in the highest tertile of apoptosis were less likely to have adenoma recurrence. Because some patients who initially do not show adenomas will develop them in the future, but will be unlikely to enter surveillance programs because of the lack of an index polyp, it was suggested therefore by the authors, that apoptosis might be used to assess risk of future polyp development, but added that the current methods to assess apoptosis are too "tedious" to be clinically useful (54).

Human Intervention Studies Using Apoptosis as an Intermediate Biomarker. For colorectal cancer to develop via the adenoma-carcinoma sequence (55, 56), the colorectal mucosa becomes more susceptible to DNA insults by increased cell proliferation (57), DNA methylation (58), or inadequate apoptosis (4, 52). Deranged cell kinetics have been proposed as markers of colorectal carcinogenesis in animals (59), and increased cell proliferation labeling indices have been shown to be increased in patients with familial colorectal cancer syndromes (60-62), patients with sporadic colorectal adenomas or carcinomas (63, 64), and in those patients with a family history of sporadic colorectal cancer (65, 66). Patients with colorectal adenomas also show generalized decreased level of apoptosis throughout their colon (48, 52) compared with patients without.

Changes in the degree of apoptosis within normal colorectal mucosa need to be correlated with changes in the subsequent risk of colorectal neoplasia. Although no such evidence exists as yet in chemoprevention studies of sporadic colorectal adenomas, there is some evidence that polyp regression is associated with increased apoptosis in the luminal epithelium of patients with FAP. Keller and colleagues found that treatment with the NSAID sulindac caused significant regression of colorectal adenomas in FAP patients relative to placebo, and this was accompanied by significant alteration of the rectal epithelial apoptotic ratio with a relative increase in

apoptosis in surface epithelial cells compared with the deeper portion of the rectal crypts (67). On the basis of these findings, the authors suggested that the changes in the apoptotic ratio between luminal surface and the crypt base could be used as an intermediate biomarker to monitor response to sulindac treatment. However, in a subsequent study by the same investigators in subjects confirmed to have FAP by APC (adenomatous polyposis coli)-mutational analysis but without colorectal adenomas, the apoptotic ratio was not significantly different between subjects treated with sulindac or placebo after 4 months and 2 years of treatment (68). In addition, at 4 months and 2 years of evaluation, there were no statistically significant differences in either the apoptotic ratio, or the number of apoptotic cells in the surface epithelium or crypt base between subjects who developed colorectal adenomas or who remained free of adenomas (68). These results suggested that apoptosis indices in the normal appearing mucosa of presymptomatic subjects with FAP were not a useful biomarker in predicting the development of adenomas. More recently, Sinicrpe and colleagues (69) found that an increase in the ratio of superficial apoptotic to nonsuperficial apoptotic cells was found to be significantly correlated with polyp regression in FAP patients treated with celecoxib ($r = 0.71$, $P = 0.004$). The change represented an increase in apoptosis at or near the luminal surface and a concomitant decrease in the nonsuperficial region. In normal colorectal mucosa, the correlation between the change in number of superficial apoptotic cells and polyp regression was of borderline statistical significance ($r = 0.33$, $P = 0.053$), but this may relate to the reduced frequency of apoptosis in normal mucosa relative to adenomas and the small sample size of the study (69).

It has been proposed that diets enriched with omega-3 (or *n*-3) polyunsaturated fatty acids (PUFAs) may protect against colorectal carcinogenesis by reducing DNA adducts, aiding DNA repair, and so increasing apoptosis (70-72). Following initiation with a colorectal carcinogen, rats fed a *n*-3, as compared with an omega-6 (*n*-6) PUFA diet, exhibit suppressed cell proliferation and increased apoptosis indices (73). Beneficial effects on apoptotic indices, measured in normal sigmoid mucosa, have been described in human subjects with a history of colorectal adenomas given fish oil supplementation, in both the short- (74) and long term (75). These observations are suggestive that patients at risk for colorectal adenomas have disordered cell kinetics that can be beneficially modified with *n*-3 fatty acids.

In light of the increasing interest in the role of apoptosis in colorectal carcinogenesis, the two most recent intervention studies have also investigated the effect of *n*-3 fatty acids on mucosal apoptosis, as well as proliferation. Cheng and colleagues (75) undertook a 2-year investigation into the effect of an increased intake of *n*-3 fatty acids on colonic cell proliferation and apoptosis in those patients polypectomised for colorectal adenomas. The experimental group comprised 21 patients who were advised to decrease their intake of total fat from 30% to 20% of total energy intake, as well as reducing the intake of *n*-6 PUFAs and increasing their intake of *n*-3 fatty acids, which was aided by giving fish oil capsules providing 100 mg of EPA (eicosapentaenoic acid) and 400 mg of (docosahexaenoic acid) DHA per day. The comparison group comprised 20 patients who

were simply advised to decrease fat consumption from 30% to 20% of total energy intake. At the end of the study, proliferation rates in normal sigmoid mucosa were not significantly different between the experimental and comparison groups at 12 and 24 months (75). However, whereas not significantly different after 12 months intervention, the number of apoptotic cells was significantly greater in the crypts of normal sigmoid mucosa in the experimental group as compared with before the intervention and in the comparison group (75). This is an important study as it is the first to show that omega-3 fatty acid supplementation increases levels of mucosal apoptosis in humans. The apparent lack of any effect on colonic crypt cell proliferation may be related to the dosage of *n*-3 fatty acids received, as the daily dose of *n*-3 fatty acids provided by the capsules in the study by Cheng and colleagues was only one fifth that of the studies by Anti and colleagues (76, 77). Alternatively, it may be due to relatively few numbers of patients with hyperproliferation in baseline biopsies.

Courtney and colleagues investigated the effect of EPA in the free fatty acid form on proliferation and apoptosis in the normal mucosal of 30 patients with a history of colorectal adenomas (74). After 3 months supplementation, significant effects were seen in both proliferation (reduced) and apoptosis (increased) when compared with the control group. The results noted for this study were different than for Cheng and are perhaps explained by the higher dose of EPA used in the study by Courtney and colleagues, and there may be a threshold dose of EPA supplementation at which cytokinetics become significantly reduced. Also, whereas it took 24 months of supplementation using a relatively low dose of *n*-3 fatty acids to see a significant increase in mucosal apoptosis in the study of Cheng and colleagues, such an increase in apoptosis was seen after only 3 months of supplementation at 2 g/day in the study by Courtney and colleagues. The free fatty acid form of EPA has been shown to be more efficiently absorbed, and whereas there are no human studies comparing the biological activity of different EPA formulations, a study in colonic adenocarcinoma-bearing mice found that whereas the free fatty acid of EPA was effective in reversing host body weight and inhibiting tumor growth, the ethyl ester form of EPA was ineffective in either respect at the same dose level (78). In both these intervention studies, randomization produced diets with significantly increased amounts of *n*-3 fatty acids in the experimental (supplemented) groups. However, in the study of Cheng and colleagues, it took 12 months for the levels of *n*-3 fatty acids to be significantly elevated in the colonic mucosa, as opposed to only 3 months in the study by Courtney and colleagues. This difference is most likely due to the relatively higher dose of supplements (2 g versus 0.5 g of *n*-3 fatty acids) used in the study of Courtney and colleagues. Therefore it would seem, that short-term, high-dose, and long-term, low-dose supplementation of *n*-3 fatty acids can increase mucosal apoptosis in the colon of patients with a history of colorectal adenomas. Such changes may be beneficial, as two recent studies have described a generalized decreased level of mucosal apoptosis throughout the colon in subjects with colorectal adenomas (48, 52).

However, as previously stated, these biomarkers remain unvalidated, but both proliferation and apoptosis

were noted to be predictors for polyp regression in a population of FAP patients treated with celecoxib (69). The change in apoptosis represented an increase at or near the luminal surface and a concomitant decrease in the nonsuperficial region. In normal colorectal mucosa, the correlation between the change in number of superficial apoptotic cells and polyp regression was of borderline statistical significance ($r = 0.33$, $P = 0.053$), but this may relate to the reduced frequency of apoptosis in normal mucosa relative to adenomas and the small sample size of the study (69).

In summary, apoptosis is a specific process of cell death in which a series of characteristic morphological changes are seen to occur. A balance of cell proliferation and cell death normally maintains cellular homeostasis. The importance of apoptosis in the pathogenesis of colorectal cancer is increasingly being recognized. Several human observational studies have found that reduced rates of apoptosis within the colorectal mucosa are associated with increased risk of colorectal adenomas. Thus, a preventive strategy to reduce the risk of colorectal cancer may be to increase rates of apoptosis within the colorectal mucosa by dietary modulation or pharmaceutical agents. However, no human intervention studies as of yet have correlated changes in apoptosis within the normal colonic mucosa with risk of subsequent sporadic adenoma formation. Therefore, correlating the change in luminal apoptosis in normal mucosa with subsequent risk of adenomas in patients with sporadic colorectal adenomas would be advantageous to further investigate the validity of such an intermediate biomarker in chemoprevention studies.

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