Apoptosis, Cell Replication, and Western-style Diet-induced Tumorigenesis in Mouse Colon

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ABSTRACT

In this study, feeding Western-style diets (WDs) to mice for a duration of two years without any chemical carcinogen led to the development of gross colonic lesions that were histologically classified as dysplastic crypts and focal hyperplasias with or without atypical nuclei. To better understand early biological events contributing to the development of colonic neoplasia, grossly normal colonic mucosa was investigated mitotic and apoptotic colonic epithelial cells, atypical mitosis, and atypical nuclei were studied. A significant and transient increase of mitotic activity in the basal and intermediate portions of the colonic crypts was seen in young mice after feeding them the WDs. This was accompanied by diffuse activation of apoptosis of the colonic epithelial cells.

In the middle of the rodents' life span, after administration of both the WDs and control diet, the rodents developed a marked depletion of apoptotic epithelial cells in the mid-region of the colonic crypts; this was followed by the expansion of an epithelial cell population containing atypical nuclei, and the emergence of the gross lesions noted above. With this sequence of events, prolonged feeding of WDs to mice produced single-crypt dysplastic lesions and focal hyperplasias indicative of tumorigenesis.

INTRODUCTION

Histological, clinical, and molecular evidence supports one of the most important models of human carcinogenesis, the adenoma-carcinoma sequence in the large intestine (1). This sequence involves the development of cancer from a dysplastic, polypoid, focal precursor: the adenoma. Adenomas are noninvasive glandular neoplasms characterized by the presence of dysplastic epithelium, originating from a single crypt in the colonic mucosa. The basic steps of the sequence are the microadenomas and the unicrypt adenomas, in which few crypts or one crypt are lined by dysplastic epithelium (1).

Recent studies have demonstrated that high dietary fat can act as a promoter of experimentally induced colonic carcinogenesis, and a WD containing four risk factors of the human WD (i.e., increased fat and phosphate and decreased calcium and vitamin D) induced hyperplasia and hyperproliferation in the colonic mucosa of rodents (2, 3). In a previous short-term study (4), a WD also induced changes of epithelial cell nuclei in the colonic crypts.

In this study, two WDs were fed to the rodents for a 2-year duration, and a step-by-step analysis was carried out of changes induced in the mouse colonic mucosa during this long-term feeding period. Morphological features known to be related to tumor development and progression (mitosis and atypical mitosis, cells with atypical nuclei, and apoptotic cells) were quantified to attempt to elucidate early biological events, temporal relationships, and mechanisms involved in the evolution of abnormal epithelial cells and neoplasia.

A major focus of this analysis was the identification of apoptosis in the lower two-thirds of the colonic crypts, where colonic epithelial cells rapidly proliferate and differentiate normally into mature nonproliferative cells. Current biological models envisage homeostasis of normal renewal of epithelial cells and the evolution, progression, and regression of neoplasms as resulting from differences in the modulation and balancing of interactions between cell proliferation and PCD (5), with this modulation leading to the evolution of either normal terminally differentiated cells or cells capable of further abnormal proliferation, growth, and development.

PCD is a selective process of cell deletion designed to ensure genetic fidelity through the elimination of genotypic alterations and minimization of phenotypic variations that contribute to controlling the size of a cell population (6). Apoptosis is, mainly, the morphological manifestation of PCD, and apoptotic cells can be considered the inverse counterparts of mitotic cells. PCD has been proposed as a regulator of the growth of advanced tumors, but at present, little is known about its role in the early stages of tumor development.

MATERIALS AND METHODS

Animals. A total of 180 animals (90 males and 90 females; C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME) at 21 days of age (after weaning). The mice were placed on ad libitum control feed (AIN-76A) for 3 weeks during acclimatization and then randomly divided to ad libitum feeding of CD or experimental diets (time 0). They were housed in plastic cages with wire tops and sawdust bedding and kept on a 12-h light/12-h dark cycle in a temperature-controlled environment. Throughout the experiment, the animals had free access to water. The mice were weighed weekly, and abnormal signs were recorded. At 12, 19, 27, 52, 72, and 104 weeks after the start of the experimental feeding, 10 animals (5 males and 5 females) in each experimental group were sacrificed.

Diet. The diets used were based on a semisynthetic rat and mouse diet, AIN-76A, developed by the American Institute of Nutrition (7) and designed for optimum growth of rodents. The complete form of the diet was used as the CD (60 animals). The AIN-76A diet was modified (high fat and phosphate, low calcium and vitamin D) to simulate the mixed lipid intake of the average American diet, with a polyunsaturated:saturated ratio of 0.55. It contains: 27% beef tallow, 15% butter fat, 13% lard, 27% partially hydrogenated soybean and other vegetable oil, 12% partially hydrogenated soybean, 5% peanut oil, and 1% corn oil. In WDI (60 animals), corn oil, a polyunsaturated vegetable oil (2), was used in place of American Blend Fat. The components of the three diets are described in Table 1.

Histology. At the time of sacrifice, the mice were subjected to a complete autopsy, and the gross appearance of the colon and other abnormal findings were noted. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PCD, programmed cell death; WD, Western-style diet; WDI, Western-style diet 1; WD2, Western-style diet 2; CD, control diet.

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3 The abbreviations used are: PCD, programmed cell death; WD, Western-style diet; WDI, Western-style diet 1; WD2, Western-style diet 2; CD, control diet.

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were noted. The colons were removed and fixed in 10% phosphate-buffered formalin. Four to six representative cross-sections of the descending distal colon with grossly normal-appearing mucosa were obtained between 1 and 4 cm from the anus. In our previous short-term studies of the effects of these diets, hyperproliferation and hyperplasia occurred and were most pronounced in the distal colon (2).

Sections (3—4 μm) were prepared and stained with H&E or Feulgen stain. One thousand longitudinally oriented crypt columns were examined in each animal. The number and distribution of the findings noted below were evaluated by dividing each crypt column into three equal longitudinal compartments from the basal third of the colonic crypts (Compartment 1) to the middle third (Compartment 2) and the upper third reaching the mouth of the crypt (Compartment 3).

All mitotic figures present in the sections were classified as normal or atypical mitoses, according to Rubio’s criteria (8). The spatial position of metaphase plates was regarded as an important parameter to distinguish normal metaphases (“equatorial plate vertical to the luminal-cytoplasmic interphase”, Ref. 8) from the atypical ones (“oblique or horizontal equatorial plate”, Ref. 8; Fig. 1, A and B).

Apoptotic cells were identified by the following morphological features (Fig. 2A): cell shrinkage and loss of normal contact, condensation and margination of chromatin, convolution of the nuclear and cell outline, and cellular budding and fragmentation (9, 10). A single, huge, dense, rounded apoptotic body as well as a cluster of small apoptotic bodies (Fig. 2B) were recorded as one apoptotic cell. To avoid possible interference of lymphoid cell apoptosis, crypts next to lymphoid follicles were disregarded.

Nuclei were classified as atypical when they showed an increase in size and stainability and/or significant differences in shape and polar orientation with respect to the surrounding ones. Mitoses, atypical nuclei, and apoptotic cells were expressed as the number per 1000 crypt columns (previously normalized in their height). Atypical mitoses were expressed as the percentage ratio between the number of atypical and total mitoses.

A total of 10—20 serial 4-μm sections were cut from all grossly identifiable lesions and stained with H&E. The findings were histologically classified in accordance with well-established categories: focal hyperplasia (Ref. 11; Fig. 3), focal hyperplasia with scattered atypical nuclei (Fig. 4), dysplastic crypts (Fig. 5), and early neoplastic lesions (12).

Statistical Analysis. Results were expressed as means ± SEM. The Mann-Whitney nonparametric u test was used to assess the significance of differences between the morphological indices for the three groups of animals. The χ² test and Fisher’s exact test (when appropriate) were used for inference on proportions in assessing the incidence of gross lesions. Statistical significance was assumed with P < 0.05.

RESULTS

Grossly Normal Mucosa. The mean crypt height, expressed as the number of cells in each hemi-crypt, is shown in Table 2. Hyperplasia, i.e., the elongation of the crypt by increased number of epithelial cells in crypt columns, was observed after administration of the WDs in both WD1- and WD2-treated animals, although significant differences from controls were found only at 12, 52, and 104 weeks.

At the base of the crypt (Compartment 1), the main results were clustered at the beginning (12—27 weeks) and the end (104 weeks) of the nutritional experiment (Fig. 6A). A significant but transient increase of mitotic activity was associated with WD1 feeding. At 104 weeks, a highly significant increase of the atypical nuclei was seen in both WD1- and WD2-treated animals.

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increases in mitosis, atypical mitosis, apoptosis, and atypical nuclei were similar to those occurring in Compartment 1, with an earlier increase in atypical mitosis occurring in WD1. Mitosis continued to be higher in the WD groups than in the controls throughout the rodents' life span. This increase was accompanied by a progressive increase in the development of cells having abnormal nuclei, which was higher in both WD groups than the controls at later periods. The most notable event occurring in compartment 2, however, seemed to be the depletion of apoptotic cells in both control and treated animals at 52 weeks, followed at later times by a marked increase in cells having abnormal nuclei in both WD groups, and the emergence of polypoid lesions and dysplastic colonic crypts in both WD groups. By 104 weeks, apoptosis had returned to higher values in all groups.

In Compartment 3 (Fig. 6C), increased apoptotic activity extending to the surface epithelium occurred in the early and late periods, corresponding to compartment 2. By 52 weeks, apoptosis had declined in WD1 to the levels present in the other groups. The main depletion of apoptotic cells, however, occurred in Compartment 2, the major region in the colonic crypts where epithelial cells differentiate and normally become nonproliferative cells or continue to replicate as cells undergoing abnormal development.

**Development of Gross Lesions in the Colon.** Starting at 52 weeks, polypoid excrescences ranging 0.2—2 mm in size were found, mostly in the distal 5 cm of the colon and less frequently in the ascending colon (65% versus 35%, respectively).

Table 3 compares the histologically categorized lesions of the distal colon in the three diet groups. The incidence of both focal hyperplasias (with and without atypical nuclei) and dysplastic colonic crypts was significantly higher in the WD1 and WD2 groups than in the controls in the majority of time periods. No statistically significant differences were found in the incidence of lesions between WD1- and WD2-treated animals.

**DISCUSSION**

In accordance with theoretical expectations (2), these findings show that WDs, when continued throughout the rodents' life span, induce colonic neoplasia without any chemical carcinogen. The first specific morphologically identifiable step for colonic carcinogenesis, i.e., "the repopulation of the crypt by atypical, neoplastically transformed cells" (12) (dysplastic crypts, Ref. 12; focal atypia in a single crypt, Ref. 13; basophilic crypts, Ref. 14) was found in 20, 40, and 40% of animals at 52, 72, and 104 weeks after the start of the WDs (Table 3). The dysplastic crypts were observed only within the grossly evident lesions and were irregularly intermingled with hyperplastic crypts. Because neither polypoid adenomas nor adenocarcinomas were found,
dysplastic crypts should be considered the end point of tumor progression in this model. With respect to the dysplastic aberrant crypt foci as described in animal (15) and human (16) colon, the dysplastic crypts did not show any significant architectural disturbances (i.e., loss of mucosal polarity, elongation, or increased size; Ref. 16). Such architectural changes are likely to be subsequent stages of colonic tumor morphogenesis and the result of multiple cycles of crypt fission of a parent dysplastic crypt. Therefore, WDs seem to trigger and sustain tumorigenesis of colonic mucosa, but their effect is clearly delayed and less powerful than that exerted by chemical carcinogens.

Hyperproliferation induced by the WDs in short-term studies has been interpreted as affecting mechanisms by which DNA damage caused by genotoxic substances is fixed and amplified (2). After the administration of chemical carcinogens that have activity in initiation and tumor promotion in rodent models, hyperplasia and hyperproliferation also occur in colonic epithelium. Most chemical carcinogens are in effect both mutagenic and cytotoxic to target tissues, resulting in regeneration and a sustained increase in cell proliferation (17). Findings in this long-term dietary study suggest that WDs affect a more complex series of events in which cell proliferation, apoptosis, and genomic protecting mechanisms are selectively and sequentially triggered and down-regulated. In this study, increased cell division occurred in the first 12 weeks of feeding, induced most by the American Blend Fat together with low calcium and vitamin D (WD1). Throughout the remaining period (19–104 weeks), mitotic activity in WD-treated animals roughly paralleled but remained at a higher level than that observed in AIN-76A controls in the lower crypts.

Atypical mitosis, indicative of genomic instability, peaked early in the WD groups, and this was followed by a major expansion of an epithelial cell population with atypical nuclei, higher in the WD groups in the late phase of the study. Thus, these findings indicate that both a transient induction of colonic epithelial cell hyperproliferation and a mild clastogenic effect were exerted by the WDs.

Subtle morphological changes, however, distinguish genotoxic damage occurring in this nutritionally induced tumorigenesis model from chemically induced carcinogenesis. In the latter, prominent nucleoli develop in the atypical cells (12), whereas nucleoli were not prominent in the present nutritional model. It is well known that the size and number of nucleoli reflect the level of RNA synthesis, and nucleoli may be small or absent in cells with low levels of protein synthesis. Therefore, the lack of huge nucleoli observed in this nutritional model can be related to a lower and more transient replicative activity, although hypotheses could be proposed based on mutagenic pathways involved in the WD model.

The most significant result emerging from this study is that apoptosis seems to have a key role in the early phases of tumorigenesis induced by WDs. Misregulation of apoptosis can promote cancer development by two distinct mechanisms: (a) by allowing the accumulation of proliferating cells in the colonic crypts; and (b) by reducing the deletion of genetic variants in the genome that have enhanced malignant potential (18). In previous studies, the investigation of alterations in cell proliferation that occur during tumorigenesis has been the predominant focus of most investigations, whereas the role of cell loss in the formation of tumors has often been neglected.

Changes in cell proliferation in the colonic mucosa have been exhaustively described throughout the course of chemical-induced carcinogenesis in rodents (19) and in high-risk human subjects (20) and represent one of the most reliable premorphological intermediate biomarkers of colorectal tumor development (21). In renewing tissues, the maintenance of homeostasis of normal tissue development and size is assured by an adequate balance and active interplay between cell proliferation and cell death, i.e., between mitotic and apoptotic cells (22). Conversely, loss of homeostasis, as found in unregulated tumor growth, can occur by an increase in cell proliferation, a reduction in the rate of cell death, or by both mechanisms (23).

In this study, a significant increase of apoptotic cells in all colonic crypt compartments was observed during the first four months of feeding in WD1-treated animals. Such a diffuse and transient activation of apoptosis can be interpreted as a homeostatic response (so-called compensatory cell death; Ref. 24) to the hyperproliferative wave that was induced during the same period. Therefore, apoptosis did not seem to be misregulated in this early stage of WD feeding. Apoptosis is also an efficient process in preventing the malignant transformation of cells because it is responsible for the deletion of genetically aberrant cells from the population. In effect, the striking fall of apoptotic cells in the middle third of the colonic crypts in the

![Fig. 4. Focal hyperplasia with scattered atypical nuclei (arrows). A, ×50; B, ×100; H&E.](image-url)
middle of the rodents' life span could be attributed to an impairment of this apoptotic process. Radiation-induced apoptotic cells with wild-type p53 expression protecting against genetic damage are located in the same region of the mouse colonic crypts (25). Data are now available showing that in human colonic mucosa, cells engaged in apoptosis and abnormally located in the intermediate cryptal segments are specifically assigned to control genetic fidelity (26).

According to this interpretation of the role of apoptosis in colonic crypts, a decrease in apoptotic surveillance observed in this study would allow the clonal expansion of DNA-altered cells, morphologically identifiable as atypical mitotic figures accumulating in compartment 2 during the period of 19–27 weeks. The onset of grossly evident lesions starting at the 52nd week also supports this interpretation.

Interestingly, in this study, we found a depletion of apoptotic activity in the middle third of the colonic crypts (the area where colonic epithelial cells undergo rapid differentiation) in both control and treated animals, suggesting that this phenomenon may be unrelated to the nutritional effects and suggesting a link with physiological events occurring in the middle of the rodents' life span.

### Table 2 Height of colonic crypts

<table>
<thead>
<tr>
<th></th>
<th>12 wk</th>
<th>19 wk</th>
<th>27 wk</th>
<th>52 wk</th>
<th>72 wk</th>
<th>104 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>23.3 ± 0.2</td>
<td>22.9 ± 0.4</td>
<td>23.1 ± 0.2</td>
<td>23.1 ± 0.1</td>
<td>24.8 ± 0.8</td>
<td>23.6 ± 0.6</td>
</tr>
<tr>
<td>WD1</td>
<td>24.3 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.1 ± 0.5</td>
<td>24.3 ± 0.5</td>
<td>26.9 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.6 ± 0.3</td>
<td>25.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WD2</td>
<td>25.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.2 ± 0.7</td>
<td>24.9 ± 0.3</td>
<td>24.5 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.2 ± 0.2</td>
<td>26.0 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Mean number of epithelial cells/crypt column ± SEM.
<sup>b</sup> p < 0.05 versus controls.
<sup>c</sup> p < 0.001 versus controls.
<sup>d</sup> p < 0.01 versus controls.

Fig. 5. Polypoid lesions composed of a group of dysplastic crypts lined by atypical cells along their entire length. A, ×50; B, ×100; C, ×250; D, ×400; H&E.
In conclusion, the present data indicate that WDs can trigger and sustain the early phases of tumorigenesis in the colonic mucosa, inducing significant changes in cell renewal, apoptosis, and genetic instability in the epithelium.

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