Modulation of Apoptosis by Sulindac, Curcumin, Phenylethyl-3-methylcaffeate, and 6-Phenylhexyl Isothiocyanate: Apoptotic Index as a Biomarker in Colon Cancer Chemoprevention and Promotion

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ABSTRACT

Recent evidence supports the theory that tumor growth in vivo depends on evasion of normal homeostatic control mechanisms that operate through induction of cell death by apoptosis. This study tested the hypothesis that several potential chemopreventive agents share the ability to induce apoptosis and that inhibition of apoptosis is a mechanism of tumor promoters. The present study was designed to investigate whether the chemopreventive properties of sulindac, curcumin, and phenylethyl-3-methylcaffeate (PEMC) and the tumor-promoting activity of 6-phenylhexyl isothiocyanate (PHITC) that were observed in our previous studies are associated with the induction or inhibition of apoptosis in azoxymethane (AOM)-Induced colon tumors in male F344 rats. At 5 weeks of age, groups of rats were fed control or modified AIN-76A diet or diets containing 320 ppm of sulindac, 2000 ppm of curcumin, 750 ppm of PEMC, or 640 ppm of PHITC. At 7 weeks of age, all rats except those intended for vehicle (normal saline) treatment were given AOM (15 mg/kg body weight) once weekly for 2 weeks. To study the effect of sulindac administered during promotion/progression stage, the rats were fed the control diet initially and then fed the experimental diet containing 320 ppm of sulindac 14 weeks after the second AOM treatment. The rats were sacrificed 52 weeks after carcinogen treatment, and their colonic tumors were subjected to histopathological evaluation and the appearance of apoptosis. In the current study, chronic administration of sulindac, curcumin, and PEMC or sulindac given only during promotion/progression significantly increased the apoptotic index (percentage of apoptosis) as compared to administration of the control diet; the apoptotic indices in the control, sulindac, curcumin, and PEMC diets were 8.3, 17.6, 17.7, and 18.5%, respectively, and in sulindac administered during promotion/progression stage, the apoptotic index was 19.1%. However, dietary PHITC blocked the process of apoptosis during colon carcinogenesis. The apoptotic index in PHITC diet was 7.9%. Taken together, our data show that chemopreventive properties of agents are correlated with the degree of apoptosis. Therefore apoptosis seems to be a reliable biomarker for the evaluation of potential agents for cancer prevention.

INTRODUCTION

Colorectal cancer is one of the leading types of cancer in both men and women in the United States (1). Chemoprevention is a promising tool for cancer prevention and control because therapy alone has not been fully effective in combating even the high incidence of low survival rate of several cancers (2, 3). Evaluation of naturally occurring as well as synthetic agents that can modulate colon cancer should lead to new strategies for colon cancer chemoprevention. Efficacy studies in laboratory animals and subsequent human clinical trials have identified several agents with chemopreventive potential in colon cancer (2–6). In this context, epidemiological studies have revealed an association between the long-term consumption of non-steroidal anti-inflammatory drugs, such as aspirin, and reduced incidence of mortality from colon cancer (7). Sulindac has been shown to reduce the number and size of colonic and rectal adenomatous polyps in patients with familial adenomatous polyposis (8), although this effect may not be permanent (9). Animal studies also have shown that sulindac reduces the number and size of chemically induced primary colonic tumors in rats and mice (6, 10). Curcumin, a phenolic compound that has been identified as the major pigment in turmeric, possesses both anti-inflammatory (11) and antioxidant properties (12). Studies from our laboratory had shown that 2000 ppm of curcumin in the diet significantly suppressed the AOM-induced colon aberrant crypt foci formation, which are early preneoplastic lesions, and colon tumor incidence and tumor multiplicity in male F344 rats (9, 13). Dietary administration of 2% turmeric inhibited chemically induced tumors of the skin and gastrointestinal tract in mice (14, 15). Propolis, a product derived from bee hives, exhibited a broad spectrum of activities including antibacterial, antifungal, cytostatic, and anti-inflammatory properties (16, 17). Caffeic acid and its esters inhibited cell growth of colon adenocarcinoma HT-29 and HCT-116 (18) and exhibited differential toxicity to cancer cells versus normal cells (19). Dietary administration of PEMC significantly inhibited both the incidence and multiplicity of AOM-induced colon adenocarcinoma in rats (4). Aryl alkyl isothiocyanates such as benzyl isothiocyanate, phenylethyl isothiocyanate, and PHITC, which have been shown to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung adenomas in A/J mice (20), enhanced chemically induced colon carcinogenesis in rats during postinitiation (21, 22). Long-term administration of alkyl isothiocyanate elicited hyperplasia and papillomas of the urinary bladder and leukemia as well as histopathological changes in the livers of male and female rats (23). PHITC also enhanced chemically induced skin tumor multiplicity in A/J mice (24). The above studies suggest that several nonsteroidal and naturally occurring anti-inflammatory agents can effectively inhibit colon carcinogenesis, whereas some isothiocyanates enhance colon carcinogenesis.

Chemoprevention trials in colorectal cancer patients would involve excessively long follow-up if traditional end points of efficacy are used. Prevention of colon cancer demands the development of biomarkers that can assist in the rapid evaluation of potential chemopreventive agents and of nutritional agents for their utility in clinical chemoprevention trials. The intermediate biomarker should be closely associated with the causal pathway for carcinogenesis (3). In this context, it is noteworthy that several chemopreventive agents have induced apoptosis, and several tumor promoters have inhibited apoptosis (25–29). Dive and Hickman (30) have implied that the efficacy of various antitumor agents is related to the intrinsic ability of the target tumors to respond to these agents with induction of apoptosis. Transformation of colorectal epithelia to adenomas and adenocarcinomas has been shown to be associated with a progressive inhibition of apoptosis (31). Apoptosis is an orderly process of inter-

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3 The abbreviations used are: AOM, azoxymethane; COX, cyclooxygenase; PEMC, phenylethyl-3-methylcaffeate; PHITC, 6-phenylhexyl isothiocyanate.
nal cellular disintegration (32), which is associated with membrane blebbing, structural condensation, and the maintenance of some organelle integrity (33). In view of the above discussion concerning the colon tumor-inhibitory or tumor-promoting properties of certain agents and the induction of apoptosis as a biomarker for monitoring colon tumorigenesis, the present study was designed to examine whether the observed chemopreventive properties of sulindac, curcumin, and PEMC and the tumor-promoting activity of PHITC are associated with induction or inhibition of apoptosis in AOM-induced colon tumors in male F344 rats. The major goal of this study was to determine whether inhibitors and promoters of colon tumors can enhance or suppress apoptosis in this model system.

MATERIALS AND METHODS

Animal Diets and Chemopreventive Agents. The experimental protocols were described in detail in our previous publications (4—6, 22). Briefly, male F344 rats received at weaning had free access to the modified AIN-76A control diet. Experimental diets were prepared by adding 640 ppm of PHITC, 2000 ppm of curcumin, 750 ppm of PEMC, or 320 ppm of sulindac to the control diet. When the rats were 5 weeks old, groups of animals were fed the control diet or one of the experimental diets containing the chemopreventive agents. At 7 weeks of age, all rats, except the vehicle-treated groups, received s.c. injection of 15 mg/kg body weight of AOM once weekly for 2 weeks. Vehicle-treated animals received an equal volume of normal saline. Additional subgroups, which were intended to investigate the effect of sulindac administered only during promotion/progression, were switched over from the control diet to the 320 ppm of sulindac diet starting 14 weeks after the last carcinogen treatment. The rats continued to receive this diet until the termination of the study 52 weeks after the AOM treatment. The rats were killed by CO2 euthanasia. Colonic tumors were excised, fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with H&E stains. The histological criteria used for intestinal tumor classification were described previously (34).

Detection of Apoptosis. Previously described methods were used for the detection of apoptosis (29, 35, 36). Microscopic studies of apoptosis occurring in vitro have shown that shrinkage and breaking cells to form apoptotic bodies are completed within several minutes (37). Moreover, both in vitro and in vivo studies have demonstrated that the onset of the apoptotic process in the individual members of a cell population is never synchronous (33). Therefore, the most commonly encountered histological manifestation of apoptosis is the presence of apoptotic bodies. Apoptosis is also characterized by DNA fragmentation and cleavage into 180—200-bp internucleosomal-sized fragments. The appearance of a "ladder" of nucleosomal-sized fragments on agarose gel electrophoresis has been used as a hallmark of apoptosis (38). However, it should be noted that DNA cleavage is not universally found in apoptosis (36, 39, 40). A ladder of DNA fragments is also associated with necrosis in some types of cells (41, 42). In this study, apoptotic cells were evaluated by using paraffin-embedded sections stained with H&E using light microscopy. Apoptotic cells are identified by cell shrinkage, nuclear condensation, and formation of apoptotic bodies (33). The light microscopic appearances of apoptotic bodies are quite diverse; most are round or roughly oval in shape. Apoptotic bodies vary in size, but they are a little smaller than the parent cells. Some apoptotic cells contain pyknotic chromatin, and some are devoid of a nuclear component (33). The number of cells per microscopic field ranged from 15 to 40. Crypts were chosen randomly, and about 200 cells were counted by an observer blinded to the animal treatment group.

Statistical Analysis. Student's t test was used to determine whether mean values were significantly different among the dietary groups. Values given in Fig. 1 are mean ± SE.

RESULTS

The results, which were published previously and are summarized in Table 1, demonstrate that dietary supplementation with curcumin, sulindac, and PEMC significantly inhibited the colon tumor incidence (percentage of animals with tumors), whereas PHITC enhanced the tumor multiplicity as compared to effects with the control diet (4—6, 22). Administration of sulindac during the promotion/progression stage also significantly decreased colon tumor incidence as compared to that of controls (6).

Induction of Apoptosis by Curcumin, Sulindac, and PEMC. On the basis of cytological and architectural features, lesions can be graded from well-differentiated to poorly differentiated adenocarcinomas. In poorly differentiated lesions, cells are not differentiated into mucus-secreting epithelial cells known as goblet cells. Most of the tumors that showed induction of apoptosis by curcumin, PEMC, and sulindac as dietary supplements were poorly differentiated adenocarcinomas. Mucinous carcinoma, which is a poorly differentiated tumor, consists of large masses of mucus-containing complexes and also shows induction of apoptosis. Well-differentiated adenocarcinomas with well-differentiated goblet cells are completely devoid of any apoptotic cells. In this study we tested whether colon tumor inhibition by sulindac, curcumin, and PEMC modulates apoptosis. As summarized in Fig. 1, administration of diets containing curcumin, sulindac, and PEMC during the initiation and postinitiation significantly increased apoptotic indices (percentage of apoptotic cells:total number of cells) as compared to the control diet alone (P < 0.0001); the apoptotic indices for curcumin, sulindac, PEMC, and control diets were 17.7, 17.6, 18.5, and 8.3%, respectively. In addition, administration of sulindac during the promotion/progression stage significantly induced apoptosis (19.1%) as compared to the control diet alone (8.3%, P < 0.0001). As shown in Fig. 2, sulindac (B), curcumin (C), and PEMC (D) induced apoptosis when compared with the effects of the control diet (A). The apoptotic cells lose surface junctions and shrink in size, and the nuclear chromatin condenses beneath the nuclear membrane. There is also splitting of the cell into several fragments known as apoptotic bodies. These findings suggest that one

<table>
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<th>Table 1: Effect of dietary sulindac, curcumin, PEMC, and PHITC on colon tumor incidence in male F344 rats</th>
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<td><strong>Experimental group</strong></td>
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* Summarized from published results (4—6, 22).
* Significantly different from control group by χ² test, P < 0.0001.
* Animals fed 320 ppm of sulindac diet 14 weeks after carcinogen treatment.
* Significantly different from control diet groups by χ² test, P < 0.004.
* Significantly different from control diet by χ² test using Z-probability, P < 0.002.
common property of this diverse panel of chemopreventive agents is their ability to induce apoptosis.

Resistance to Apoptosis after Treatment with PHITC. In our laboratory, PHITC has been shown to be a tumor promoter based on a recent study (22). In the current study we examined whether the colon tumor promotion by PHITC modulates apoptosis. Microscopic evaluation of colonic tumors of animals fed PHITC diet during the initiation phase and postinitiation revealed resistance toward undergoing apoptosis as shown in Fig. 3. The apoptotic index with PHITC was 7.0%, which is somewhat similar to the control value of 8.3% (Fig. 1). This finding suggests the inability of tumor promoters to induce apoptosis.

DISCUSSION

Although chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes of action of these compounds should provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy. This study shows in an in vivo model system that dietary administration of sulindac, curcumin, and PEMC, which were previously shown to inhibit colon carcinogenesis, induces apoptosis in colonic tumors. There are several reports referring to apoptosis as one of the mechanisms of caffeic acid- and sulindac-induced tumor inhibition. Su et al. (43) have demonstrated that caffeic acid phenyl ester induces its selective toxic effect toward oncogene-transformed rat embryo fibroblast cells as a consequence of apoptosis. Caffeic acid phenyl ester induces apoptosis in transformed rodent and human cells in culture; however, it has no apparent effect on nontumorigenic rat embryo fibroblast cells, which makes it a useful adjunct to chemotherapy (44). Schiff et al. (45) have shown that sulindac sulfide, in addition to its

![Fig. 2. Comparison of the morphology of colonic epithelial cells of rat tumors (paraffin-embedded section stained with H&E. A, adenocarcinoma of the colon of a rat fed the control diet, showing few apoptotic cells (×5750). B, adenocarcinoma of the colon of a rat fed the sulindac diet continuously, showing apoptotic cells; many cell are condensed and shrunk, and some cells were captured in the process of forming apoptotic bodies (×5000). C, adenocarcinoma of the colon of a rat fed PEMC; some cells are irregular, condensed, and shrunk, and some cells show apoptotic body formation (×5600). D, adenocarcinoma of the colon of a rat fed curcumin; many cells show shrinkage, nuclear condensation, and apoptotic bodies (×4800).](image)

![Fig. 3. Adenocarcinoma of the colon of a rat fed PHITC showing an absence of apoptotic cells (×4600).](image)
antiproliferative effects, induces apoptosis in HT-29 colon adenocarcinoma cells, supporting the findings of Pasricha et al. (25) and Boolbol et al. (26) and demonstrating the induction of apoptosis by sulindac in cases of familial adenomatous polyposis. Boolbol et al. (26) have found that sulindac induction of apoptosis is mediated through COX inhibition. Rao et al. (5, 6) have suggested that the chemopreventive action of curcumin and sulindac may be mediated through the inhibition of formation of COX metabolites, which could provide a mechanism for the induction of apoptosis. Sulindac-induced apoptosis in the promotion/progression stage holds clinical relevance in the prevention and treatment of colon cancer. Targeting the stage of promotion has the greatest potential for cancer prevention, whereas cancer therapy is generally administered during the stage of progression (46). Thus, understanding the mechanism of apoptosis and how it is regulated at postinitiation stage holds great promise in the development of new cancer-preventive strategies and therapies. That well-differentiated adenocarcinomas with well-differentiated goblet cells (which might be a treatment-related effect) did not show any induction of apoptosis, whereas mucinous carcinomas, which are large masses of mucus-containing complexes, did show induction of apoptosis suggests that mucin gene expression may play a role in the modulation of apoptosis in colon cancer. Neoplastic transformation is associated with altered mucin expression in the colon (47). The present study also demonstrates that PHITC, a tumor promoter in colon carcinogenesis, renders cells relatively resistant to induction of apoptosis. These results sum up the study of Wright et al. (48), who showed inhibition of cell death through apoptosis as a mechanism of tumor promotion. Rao et al. (22) suggested that the promoting effect of PHITC on AOM-induced colon carcinogenesis is likely mediated through an increase in COX activity. Tsujii and DuBois (49), who have implicated COX-2 activity in the regulation of apoptosis of rat intestinal epithelial cells, have demonstrated that overexpression of COX-2 can cause the cells to adhere more to the extracellular matrix and make them resistant to apoptosis. PHITC-induced COX activity may also play a role in the inhibition of apoptosis in colon tumors. Although further study of apoptosis as a biomarker is clearly warranted, an implication of these experiments seems to be that initiated cells are deleted by the induction of apoptosis by eicosanoid cascade inhibitors. The morphological evaluation described here is relatively inexpensive and could be used to evaluate several eicosanoid cascade inhibitors for potential chemopreventive properties.

REFERENCES

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