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Piroxicam and Acarbose as Chemopreventive Agents for Spontaneous Intestinal Adenomas in APC Gene 1309 Knockout Mice

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The use of nonsteroidal anti-inflammatory drugs has been suggested to have a chemopreventive effect against colon carcinoma, through the inhibition of cyclooxygenases 1 and 2, in patients with familial adenomatous polyposis and in animal models. Acarbose, an alpha-glycosidase inhibitor, may also be chemopreventive. In order to examine the effects of these drugs we employed APC gene knockout mice randomized into 3 groups, one for treatment with piroxicam (0.05% concentration in drinking water), one for acarbose (0.04% concentration in food) and another for the control. After 14 weeks of treatment, mice were killed for quantitation of gastric and intestinal adenomas. Tumor multiplicity in the whole gastrointestinal tract decreased from 33.89±13.07 tumors/mouse in the control group to 17.05±7 tumors/mouse in the piroxicam-treated group ($P<0.001$). The decrease in the acarbose-treated group (29.68±12.86 tumors/mouse) was not significant ($P>0.05$). The number of tumors ≥3 mm in diameter was also quantified in all gastrointestinal segments. The number of such tumors in the piroxicam group was decreased to 0.56±1.2 tumors/mouse from the control value of 3.78±1.17 tumors/mouse ($P<0.001$), while in the acarbose-treated group the number decreased to 2.36±1.7 tumors/mouse ($P<0.01$). Thus, piroxicam decreases the size and number of gastrointestinal adenomas in APC 1309 knockout mice, while acarbose decreases only the size.

Key words: APC 1309 knockout mice — Chemoprevention — Piroxicam — Acarbose — Intestinal adenoma

Epidemiological studies indicate a marked decrease in the relative risk (40–50%) of colorectal cancer among continuous users of nonsteroidal anti-inflammatory drugs (NSAIDs) compared to those who do not take these agents.[1–3] The exact molecular mechanism by which NSAIDs reduce colorectal neoplasms is still unknown, but a possible explanation is that NSAIDs alter arachidonic acid metabolism by inhibiting cyclooxygenase (COX) enzymes. This, in turn, would inhibit prostaglandin synthesis, which plays an important role in immune response, cell proliferation and neoplasia. Two isoforms of COX enzymes have been identified: constitutive COX-1 and inducible COX-2. Induction of COX-2 is considered to be a very early event (perhaps at the stage of polyp formation) in colon carcinogenesis.[1, 4–13] The administration of NSAIDs at high concentrations also induces programmed cell death (apoptosis) directly in cultured cells.[14]

Piroxicam (4-hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide) is an inhibitor of COX-1 and COX-2, and is the first chemically available member of the new class of NSAIDs known as oxicams. The inhibitory effect of this agent on the induction of chemically induced colon tumors was studied in rats and mice.[15, 16]

Acarbose, an alpha-glycosidase inhibitor, acts to delay the intestinal absorption of carbohydrates, and hence, allows some passage of carbohydrates into the colon. It is effective for the treatment of type I and type II diabetes mellitus. Moreover, some investigators found that this drug also decreases fecal pH and increases total fecal output of short-chain fatty acids (SCFA), butyrate and acetate. These changes have been proposed to be protective factors against colonic cancer.[17, 18]

To investigate further the chemopreventive effect of NSAIDs and other drugs, we have begun a series of experiments using the APC 1309 gene knockout mouse model. This mutation is an autosomal dominant, heterozygous nonsense mutation of the mouse APC gene codon 1309. It is highly homologous to the APC mutations in humans with familial adenomatous polyposis (FAP).[19, 20] APC 1309 knockout mice develop multiple intestinal adenomas throughout the duodenal-to-colonic axis, and have an average life-span of 120 days. This mouse is a good model system for analyzing the mechanisms of balance

between proliferation and differentiation in the renewing gut epithelium and for assessment of the adenoma-to-carcinoma sequence. We consider this animal suitable for testing chemopreventive agents targeted against polypoid lesions.

Our purpose in this preliminary investigation was to examine the effects of piroxicam and acarbose drugs on the size and number of gastrointestinal adenomas in this animal model.

MATERIALS AND METHODS

APC 1309 knockout mouse Male and female APC 1309 knockout mice were obtained from the Japanese Foundation for Cancer Research (Cell Biology Department, Cancer Institute, Tokyo). Progeny were genotyped in order to maintain the pedigree before breeding.

Genotyping The genotyping for selection of the mice was done by allele-specific polymerase chain reaction (AS-PCR) and agarose gel electrophoresis. Three PCR primers were used. Primer M was designed as complementary to the 1309 mutant-type allele. Primer W was designed to be complementary to the wild-type allele. This primer is not complementary to the mutant-type sequence. Another common primer, C, was designed for the common sequence that exists in both alleles.[19, 20] Genomic DNA was typically isolated from tail tissue of each mouse.

Drug treatments Four weeks after birth, mice were randomized into three groups, maintaining a sexual balance in each group. They were treated until 14 weeks after birth. The piroxicam group, consisting of 20 mice, received the drug in tap water at 0.05% concentration every day; the piroxicam-containing water was replaced twice a week. This group received a total amount of 116 mg per mouse (1.18 mg per day). The acarbose group, consisting of 22 mice, received the drug at 0.04% concentration mixed with Certified Diet-MF (Oriental Yeast Co., Ltd., Tokyo) every day (fresh diet was provided once a week), making a total amount of 102.8 mg of drug per mouse (1.05 mg per day). The Control group (20 mice), received only Certified Diet-MF and water every day. Fresh diet was provided once a week.

Tumor scoring Animals were killed by diethyl ether inhalation. The entire gastrointestinal (G-I) tract of each mouse was quickly removed and fixed in buffered formaldehyde solution for approximately one week. Then the G-I tracts were opened from the stomach to the anus longitudinally with fine scissors, rinsed with water, spread and fixed on individual cork boards. Stomach and intestinal segments were examined after gentian-violet staining. Tumors which were larger than or equal to 3 mm (≥3 mm) in diameter were designated as "large" tumors. We used this size (3 mm in diameter) as a reference to determine the effect of treatment on tumor size. Total number of tumors and tumors ≥3 mm in diameter were quantified using a Video scope CF 200Z Magnifying Endoscope (Olympus Corp., Tokyo) (Fig. 1).

Statistical analysis The significance of differences between the control and treatment groups in relation to number and size of tumors was determined by use of the Fisher (PLSD) test.

RESULTS

Since four mice, two from the piroxicam group and two from the control group, died during the experiment for unknown reasons, tumor data for them were not available. In general all the animals seemed healthy in appearance

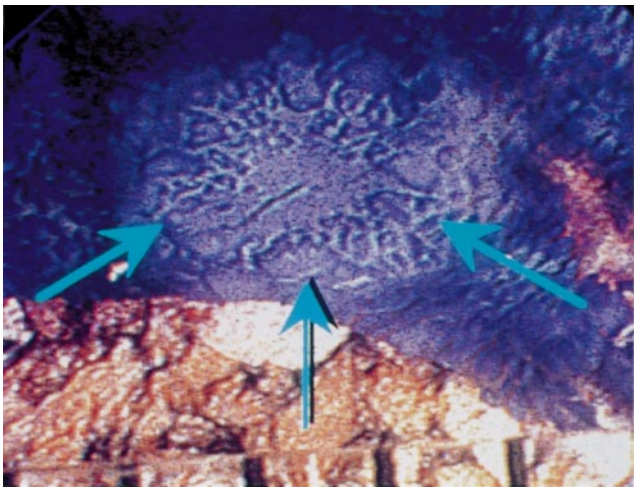

Fig. 1. This picture shows the features of one tumor in the small intestine of an APC gene 1309 knockout mouse, after Gentian-violet staining. A Video scope CF 200Z Magnifying Endoscope (Olympus Corp.) was used.

Table I. Effects of Piroxicam and Acarbose Treatment on Size of Gastrointestinal Tumors in APC 1309 Knockout Mice

Treatment[a)]	No. of mice	Number of tumors[b)]	Significance[c)]
Control	18	3.78±1.17	
Piroxicam[d)]	18	0.56±1.2	$P<0.001$
Acarbose[d)]	22	2.36±1.7	$P<0.01$

a) See text for details of treatment.
b) Mean number of tumors≥3mm in size in the gastrointestinal tract.
c) *P* values obtained with the Fisher test.
d) Mice were maintained on piroxicam and acarbose from 4 weeks after birth for 14 weeks.
Data are presented as mean±SD.

Table II. Tumors in Each Gastrointestinal Segment in Control and Treated APC 1309 Knockout Mice

Gastrointestinal segment[a)]	Number of tumors per mouse		
	Control diet[b)]	Piroxicam group[b)]	Acarbose group[b)]
Stomach	2.72±1.6	1.44±1.92 (P<0.05)*	0.82±1.05 (P<0.001)*
Small intestine	28.6±12.14	14.7±6.13 (P<0.001)*	26.73±12.38
Colon	2.56±1.82	1.44±1.1 (P<0.05)*	2.14±1.7
Total	33.89±13.07	17.05±7 (P<0.001)*	29.68±12.86

a) Tumors were counted with a Video scope CF 200Z Magnifying Endoscope (manufactured by Olympus Corp.).
b) See text for details of treatment.
∗ P values <0.05 are indicated in the table. The Fisher test was used for statistical analysis to compare control and treated groups. P values over 0.05 are not shown.
Data are presented as mean±SD.

and there were no statistically significant differences in body weights among treated and control groups.

Tumor multiplicity in the whole gastrointestinal tract was decreased from 33.89±13.07 tumors/mouse in the control group to 17.05±7 tumors/mouse in the piroxicam-treated group (P<0.001), but there was no significant difference in the acarbose-treated group (29.68±12.86 tumors/mouse with a P value >0.05).

The number of tumors ≥3mm in diameter was quantified in all gastrointestinal segments. Mice treated with piroxicam carried 0.56±1.2 tumors compared with 3.78±1.17 among untreated controls (P<0.001). In the acarbose-treated group, too, the number of these tumors decreased to an average of 2.36±1.7 tumors/mouse (P<0.01). The results are summarized in Table I and Table II.

DISCUSSION

Germline mutation in the APC gene leads to FAP, an autosomal dominant inherited predisposition to colorectal cancer.[21, 22)] Although FAP patients with germline mutations of APC account for less than 1% of colorectal cancer in the United States, somatic mutations of the APC gene occur in the majority of sporadic colorectal cancers.[23)]

Mice with germline mutations of the murine homologue APC gene develop intestinal neoplasias, as in human FAP patients. The Min mouse was the first such mouse to be described,[17, 24–26)] but here we employed the APC 1309 knockout mouse, because this animal model is a good autochthonous tumor system for analyzing the effects of chemopreventive agents on natural adenoma development. Patients with mutation in the APC gene codon 1309 have a tendency to develop colon carcinoma 10 years earlier than patients with mutations in other sites of the APC gene.

Investigations on the mechanism of action of piroxicam and other NSAIDs, such as aspirin, showed that COX enzymes are responsible for prostanoid production.[9, 27)] We think that NSAIDs reduce colorectal neoplasms by inhibiting COX enzymes. There is evidence that levels of COX-2 in colorectal carcinoma tissues from humans and rodent models are high compared to those in accompanying normal mucosa.[10, 28)] Oshima *et al*. demonstrated that COX-2 is induced in small polyps of the intestine and colon of APC 716 knockout mice, suggesting that COX-2 plays an important role in the polyp development.[9)]

Giardello *et al*. concluded that sulindac, another NSAID, reduces the number and size of colorectal adenomas in patients with FAP.[24)] Shiff *et al*. demonstrated that sulindac and sulindac sulfide inhibit cell cycle progression and induce apoptotic cell death of HT-29 cells.[14)]

Jacoby *et al*. and some other groups obtained good results using piroxicam as a chemopreventive drug for FAP disease.[6, 29–31)] In agreement with their results, our analysis also indicates that piroxicam has profound effects on the size and number of polyps in the G-I tract of the APC 1309 knockout mouse. From a clinical perspective, piroxicam seems to be a suitable drug for Japanese patients since it is less damaging to the intestinal tract and suppositories are available. Further investigations should be performed using piroxicam in patients with FAP disease.

The production of SCFAs is associated with a reduction of colonic pH. This pH reduction will affect the intraluminal concentration of secondary bile acids by precipitation and by inhibition of their enzymatic formation from primary bile acids. Since secondary bile acids have a cytotoxic effect on colonic mucosa, an increase in their level will cause compensatory cell proliferation. Special attention has focused on butyric acid, which has antineoplastic properties *in vitro* and *in vivo*. Butyrate is considered to be the primary energy source for colonocytes. *In vitro* studies have shown that butyrate favors differentiation and decrease in cell proliferation, whereas *in vivo* studies have shown the opposite effects.[32, 33)] Lupton and Kurtz showed that butyrate has a stimulatory effect in the distal colon *in vivo*, because butyrate concentration was positively correlated with number of cells per crypt column and the total number of cells per crypt at that site. However, butyrate failed to stimulate cell proliferation in the cecum and proximal colon.[34)] There is good evidence that the effects of SCFA on colonocytes in normal cells and in transformed cells are different. Butyrate seems to inhibit growth in transformed cells, while enhancing proliferation in normal human mucosa.[35)] Bradburn *et al*. found that

FAP gene carriers with polyps produced less butyrate than normal controls or gene carriers without polyps.[36)]

Considering the above findings and also taking into account the effects of acarbose in increasing SCFAs and decreasing the pH of feces, we also chose acarbose for our study. We found that acarbose decreases the size of polyps. However, it did not significantly decrease the number of polyps. To obtain a better understanding of the effects of acarbose on polyps, further investigations are needed.

Our results indicate that piroxicam, which has both preventive and regressive effects on the polyps, can be used as a chemopreventive agent for FAP disease. To confirm these promising initial results, a second study has been initiated to explore the efficacy of this drug at different doses and to examine the efficacy of other chemopreventive agents.

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