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Regressive Effects of Various Chemopreventive Agents on Azoxymethane-induced Aberrant Crypt Foci in the Rat Colon

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Regressive effects of four chemopreventive agents [5-hydroxy-4-(2-phenyl-(*E*)-ethenyl)-2(5*H*)-furanone (KYN-54), *S*-methyl methanethiosulfonate (MMTS), chlorogenic acid (CA), and piroxicam] on azoxymethane (AOM)-induced aberrant crypt foci (ACF) in the colon of male F344 rats were examined by dietary exposure. At six weeks of age, 60 rats of groups 1 through 5 received subcutaneous injections of AOM (15 mg/kg body weight) once a week for three weeks. Twelve weeks after the first carcinogen injection, when the occurrence of ACF was maximal, the rats in groups 2 through 5 were started on diet containing the test chemicals as follows: group 2, KYN-54 (0.02%); group 3, MMTS (0.01%); group 4, CA (0.025%); and group 5, piroxicam (0.0125%). Group 1 (20 rats) was kept on the basal diet alone, and group 6 (12 rats) served as an untreated control. Rats in each group were killed at 6, 12, 18, or 24 weeks after the start of the experiment, and the yield of ACF in the colon of each group at 18 or 24 weeks was compared with that at 12 weeks. The number of ACF per rat colon of each group at 18 or 24 weeks was smaller than that at 12 weeks. The reduction rates at 18 weeks were 7% in group 1 (AOM alone), 11% in group 2 (AOM+KYN-54), 10% in group 3 (AOM+MMTS), 51% in group 4 (AOM+CA) ($P<0.01$), and 33% in group 5 (AOM+piroxicam) ($P<0.02$), while at 24 weeks they were 12%, 26%, 51% ($P<0.002$), 43% ($P<0.05$), and 70% ($P<0.001$), respectively. These results indicate that chemopreventive agents for large bowel carcinogenesis, i.e., KYN-54, MMTS, CA, and piroxicam, are not only able to prevent the development of ACF, but also can regress ACF, which are regarded as precursor lesions of colorectal cancer.

Key words: Regression — Chemoprevention — Azoxymethane — Aberrant crypt foci — Colon

Chemoprevention is the use of non-carcinogenic naturally occurring products or synthetic agents to inhibit the process of carcinogenesis in general.[1,2] The ideal situation would be that the initiating and promoting agents were known and efficacious countervailing agents with little or no toxicity and no significant side effects were available.[3] In several animal studies, a number of promising chemopreventive agents for colorectal cancers have been reported.[4,5]

Aberrant crypt foci (ACF) are putative preneoplastic lesions detected in the colon of carcinogen-treated rodents[6,7] and of humans with a high risk for cancer development.[8] ACF are easily observed in the whole specimen of colon stained with methylene blue,[9] and have been used as a marker to identify some chemopreventive agents.[7,10] In most studies examining inhibitory effects on ACF in the rat colon, however, the investigated agents have been administered during carcinogen exposure or immediately after the final treatment with carcinogen. However, Pereira *et al.*[11] reported that delayed treatment with piroxicam, a nonsteroidal antiinflammatory drug with chemopreventive properties, could regress azoxymethane (AOM)-induced ACF.

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We have demonstrated chemopreventive effects on large bowel carcinogenesis using a number of animal models.[12–14] The mechanisms of action of these chemopreventive agents, including the inhibition of carcinogen-induced cellular hyperproliferation, are becoming clearer,[15] but the details remain poorly understood. In this study, the regressive effects of several chemopreventive agents, which are considered to have different modes of action, on AOM-induced ACF were examined by dietary exposure to the chemicals, starting when the development of putative preneoplastic lesions was maximal.

MATERIALS AND METHODS

Animals Male F344 rats, four weeks of age, purchased from Shizuoka Laboratory Animal Center (Shizuoka), were quarantined for two weeks, randomized into experimental and control groups, and housed three to five to a wire cage. The holding room was maintained at 23±2°C and 50±10% humidity, on a 12 h light-dark cycle. Pellet CE-2 (Clea Japan, Inc., Tokyo) was used as a basal diet, and diet and tap water were freely available.

Chemicals AOM, *S*-methyl methanethiosulfonate (MMTS), and piroxicam were purchased from Sigma Chemical Co., St. Louis, MO, and chlorogenic acid (CA) was from Tokyo Chemical Industry Co., Ltd., Tokyo. 5-

Hydroxy-4-(2-phenyl-(*E*)-ethenyl)-2(5*H*)-furanone (KYN-54) was supplied by Kurarey Co., Ltd., Osaka.

Experimental procedure As shown in Fig. 1, 72 male rats were divided into six groups. At six weeks of age, the rats of groups 1 (20 rats), 2 (10 rats), 3 (10 rats), 4 (10 rats), and 5 (10 rats) were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for three weeks. Twelve weeks after the first carcinogen injection, the rats in groups 2 through 5 were started on diets containing the following chemicals, respectively: group 2, KYN-54 at a concentration of 0.02%; group 3, MMTS at 0.01%; group 4, CA at 0.025%; and group 5, piroxicam at 0.0125%. Group 1 was kept on the basal diet alone, and group 6 (12 rats) served as an untreated control. All rats were carefully observed and weighed at intervals of four weeks during the experiment.

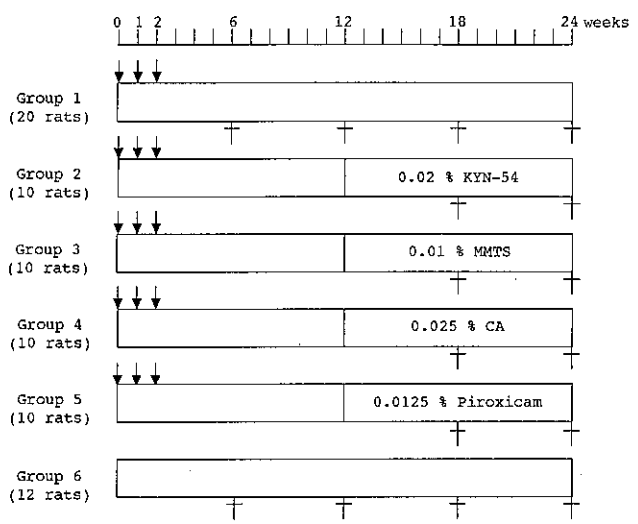

Fig. 1. Experimental protocol. Arrows (↓) show subcutaneous injection of azoxymethane (15 mg/kg body weight). KYN-54, MMTS, CA, and piroxicam were mixed with basal diet for administration at the indicated concentration. Open boxes (□) show the feeding of basal diet alone. Cruciform marks (+) indicate times at which rats were killed.

The rats were killed under ether anesthesia and autopsied at 6, 12, 18, and 24 weeks after the first exposure to AOM in groups 1 and 6, and at 18 and 24 weeks in groups 2 through 5. At autopsy, the intestine and liver were removed. The intestine was longitudinally cut open, flushed with saline to rinse out the contents, and observed carefully. For ACF analysis, the lower two-thirds of the large intestine was resected, placed on a piece of filter paper, and fixed in 10% neutral buffered formalin. The remaining organs were fixed in buffered formalin, then blocks were taken from suspected neoplastic lesions and routinely processed for histology: that is, embedded in paraffin, sliced at a thickness of 3 μm and stained with hematoxylin and eosin. All preparations were microscopically observed and histopathological diagnosis was made.

Evaluation for ACF After fixation, the resected colon was stained with 0.5% methylene blue in saline according to the procedure described by Bird,[9] and evaluated for ACF under a microscope. The numbers of ACF per colon and aberrant crypts in each focus were determined under a microscope at 40× magnification. The criteria used to identify an aberrant crypt focus topographically comprised: (i) increased size; (ii) thicker epithelial cell lining; and (iii) increased pericryptal zone relative to normal crypts.

Statistics For the statistical analysis of differences in body weights, liver weights, relative liver weights, and numbers of ACF, Student's *t* test was used.

RESULTS

When each rat was killed, body weight, liver weight, and relative liver weight were measured. At 12 and 18

Table I. Mean Body Weight of the Rats in Each Group

Group	Treatment	Body weight (g) of rats killed at			
		6 weeks	12 weeks	18 weeks	24 weeks
1	AOM alone	236±6[a)]	292±21[b)]	319±15[c)]	333±23
2	AOM→0.02% KYN-54	—	—	305±9	285±47
3	AOM→0.01% MMTS	—	—	307±24	330±17
4	AOM→0.025% CA	—	—	307±16	321±20
5	AOM→0.0125% piroxicam	—	—	303±19	288±14[d)]
6	Non-treatment	235±30	331±21	342±9	370±23

a) Mean±SD.
b) Significantly different from group 6 at 12 weeks by Student's *t* test ($P<0.05$).
c) Significantly different from group 6 at 18 weeks by Student's *t* test ($P<0.05$).
d) Significantly different from group 1 at 24 weeks by Student's *t* test ($P<0.01$).

weeks into the experiment, the body weight of rats in group 1 (12 weeks, 292±21 g; 18 weeks, 319±15 g) receiving AOM alone was significantly lower than those of non-treated rats in group 6 (12 weeks, 331±21 g, $P<0.05$; 18 weeks, 342±9 g, $P<0.05$) (Table I). The body weights of rats in groups 2 through 5 given the experimental diets tended to be lowered, when compared to group 1. At 24 weeks, the average body weight in group 5 given piroxicam was 288 g, while in group 1 it was 333 g ($P<0.01$). There were no specific changes of the liver weight and relative liver weight caused by the administration of carcinogen or experimental diets.

The results of sequential quantification of ACF per colon are shown in Fig. 2. There were no ACF in rats of group 6 (non-treated control). In rats of group 1 receiving AOM alone, ACF per colon at 6 weeks (69.2±14.7) increased 1.43 times at 12 weeks (98.8±19.1, $P<0.05$), and then decreased gradually (18 weeks, 91.6±27.6; 24 weeks, 86.6±59.2). Reduction rates were 7% at 18 weeks and 12% at 24 weeks. In groups 2, 3, 4, and 5 receiving the experimental diets, the reduction of ACF after 12 weeks was larger than that in group 1. The reduction rates at 18 weeks were 11% in group 2, 10% in group 3, 51% in group 4 ($P<0.01$), and 33% in group 5 ($P<0.02$), while at 24 weeks they were 26%, 51% ($P<0.002$), 43% ($P<0.05$), and 70% ($P<0.001$), respectively.

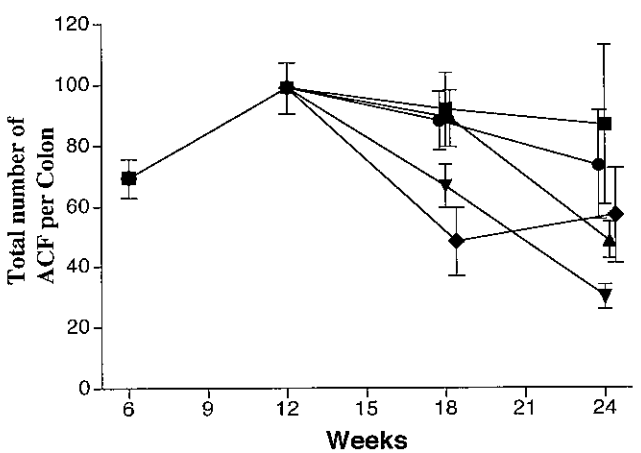

Fig. 2. Effects of delayed administration of candidate chemopreventive agents on AOM-induced total number of ACF per colon. Results are means of each group containing five rats at 6, 12, 18, and 24 weeks into the experimental period. Bars show standard errors. The symbols show the following treatments: ■ AOM alone; ● AOM+KYN-54; ▲ AOM+MMTS; ◆ AOM+CA; ▼ AOM+piroxicam.

In this study, ACF were categorized into small foci (one, two, or three crypts) and large foci (four or more crypts). The number of small ACF in group 1 was increased significantly between 6 weeks (50.6±12.1) and 12 weeks (68.0±10.4, $P<0.05$), and then decreased (18 weeks, 49.0±16.0; 24 weeks, 55.6±38.4) (Table II). In groups 2, 3, 4, and 5 receiving the experimental diets, the reduction of small ACF after 12 weeks was larger than that in group 1. The reduction rates at 18 weeks were 28% in group 1, 33% in group 2 ($P<0.02$), 21% in group 3, 66% in group 4 ($P<0.001$), and 46% in group 5 ($P<0.002$), while at 24 weeks they were 18%, 39% ($P<0.05$), 54% ($P<0.001$), 50% ($P<0.05$), and 71% ($P<0.001$), respectively.

Table III summarizes the numbers of large ACF composed of four or more crypts. In group 1, the number of large ACF at 18 weeks (42.6±18.0) was larger than at 6 weeks (18.6±4.6, $P<0.05$), and the number at 24 weeks decreased to 31.0±22.5. In groups 3 and 5, the yield of large ACF significantly regressed between 18 weeks (35.4±10.5 and 29.6±9.1, respectively) and 24 weeks (17.4±8.2, $P<0.02$ and 10.4±3.8, $P<0.005$, respectively). The rats in group 4 had a mild reduction of large ACF after 12 weeks. The changes in the number of large ACF in group 2 were similar to those in group 1.

Table II. Effects of Potential Chemopreventive Agents on Small Aberrant Crypt Foci

Group	Trearment	No. of aberrant crypt foci with 1–3 crypts/colon			
		6 weeks	12 weeks	18 weeks	24 weeks
1	AOM alone	50.6±12.1[a]	68.0±10.4[b]	49.0±16.0	55.6±38.4
2	AOM→0.02% KYN-54	—	—	45.4±13.0[c]	41.2±22.8[d]
3	AOM→0.01% MMTS	—	—	53.4±13.3	31.2±5.8[e]
4	AOM→0.025% CA	—	—	23.0±12.8[f]	34.0±24.8[g]
5	AOM→0.0125% piroxicam	—	—	36.8±10.8[h]	19.6±5.7[i]
6	Non-treatment	0	0	0	0

a) Mean±SD.
b) Significantly different from group 1 at 6 weeks by Student's *t* test ($P<0.05$).
c–i) Significantly different from group 1 at 12 weeks by Student's *t* test (*c*) $P<0.02$, *d, g*) $P<0.05$, *e, f, i*) $P<0.001$, *h*) $P<0.002$).

Table III. Effects of Potential Chemopreventive Agents on Large Aberrant Crypt Foci

Group	Treatment	No. of aberrant crypt foci with four or more crypts/colon			
		6 weeks	12 weeks	18 weeks	24 weeks
1	AOM alone	18.6±4.6[a]	30.8±10.9[b]	42.6±18.0[c]	31.0±22.5
2	AOM→0.02% KYN-54	—	—	42.6±7.8	32.2±17.5
3	AOM→0.01% MMTS	—	—	35.4±10.5	17.4±8.2[d]
4	AOM→0.025% CA	—	—	25.2±12.4	22.8±14.8
5	AOM→0.0125% piroxicam	—	—	29.6±9.1	10.4±3.8[e]
6	Non-treatment	0	0	0	0

a) Mean±SD.
b, c) Significantly different from group 1 at 6 weeks by Student's *t* test ($P<0.05$).
d) Significantly different from group 3 at 18 weeks by Student's *t* test ($P<0.02$).
e) Significantly different from group 5 at 18 weeks by Student's *t* test ($P<0.005$).

In this study, one rat in each of groups 1 and 4 developed a colon adenocarcinoma at 18 weeks, while one rat in each of groups 1, 2, and 3 developed a cancer at 24 weeks. Two or more tumors in the colon were not observed in any rat. Rats in groups 5 and 6 developed no large bowel tumors.

DISCUSSION

In the present study, the delayed administration of KYN-54, MMTS, CA, and piroxicam starting 12 weeks after the first AOM injection caused regression of ACF in the colon. These chemicals have already been reported to inhibit the development of colon tumor and ACF in animal models treated with chemical carcinogens.[5, 12–14, 16, 17] The examined chemicals are suggested to have different modes of chemopreventive action, such as inhibition of the formation of 8-hydroxyguanine by CA,[18] inhibition of cell proliferation due to a decrease of ornithine decarboxylase (ODC) activity[12, 13, 16] or inhibition of prostaglandin production.[5] In addition, KYN-54 has potential as a blocking agent.[19] Piroxicam was used as a positive control, based on the findings of Pereira *et al.*[11] In our study, delayed treatment with different types of chemopreventive agents, including piroxicam, exerted regressive effects on ACF. The effect of piroxicam was similar to that found by Pereira *et al.*[11]

As regards ACF of the colon, the larger foci with four or more crypts have been described to be more persistent or progressive than the smaller foci with one to three crypts.[20] In addition, although some chemicals can eliminate or remodel ACF, foci with larger crypt multiplicity, which might be more progressive among precancerous states, are resistant to these chemicals.[7, 21, 22] In the present study, small foci were significantly reduced by all tested agents. In contrast, KYN-54 and CA showed no reductive effects on large ACF, although MMTS and piroxicam caused significant regression. However, we think that there was no relationship between the strength of the suppression of colon cancer development and the ability to regress large ACF, because we had found no difference of chemopreventive effects between KYN-54 and MMTS, using the same protocol in our laboratory.[12, 13] These results, therefore, suggest that MMTS and piroxicam might have the ability to prevent the development of colon cancer after either delayed or early administration.

As mentioned above, KYN-54 and MMTS used in this study have been reported to suppress cell proliferation through inhibition of the activity of ODC, a key enzyme in mammalian polyamine synthesis.[12, 13, 16] Therefore, the regression of ACF might be caused by the inhibition of proliferative activity induced by potential chemopreventive agents. In addition, other possible mechanisms could be considered. Recently, there have been several reports describing the induction of apoptosis as one of the mechanisms of chemoprevention. Shiff *et al.*[23] reported that indomethacin, naproxen, and piroxicam induced apoptosis in HT-29 colon adenocarcinoma cells. Samaha *et al.*[24] also showed that sulindac, curcumin, and phenylethyl-3-methylcaffeate increased the apoptotic index in colon tumor cells of rats. We think that MMTS also has the potential to induce apoptosis of precancerous and cancer cells, since both MMTS and piroxicam regressed large ACF in this study. The regression of ACF in this experiment might be related to apoptotic induction in epithelial cells of ACF. It appears that some of the chemopreventive agents have not only inhibitory ability against tumorigenicity induced by chemical carcinogens, but also chemotherapeutic potential to induce apoptosis in preneoplastic lesions. Chemicals with a regressive effect on preneoplastic lesions such as ACF, that are expected to be associated with the development of colon cancer, may be useful in humans, since preventive substances are more realistically applicable after the development of precancerous lesions, such as adenomatous polyps, than at the initiation phase of carcinogenesis.[25]

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