SHORT COMMUNICATION

Chemopreventive agents-induced regression of azoxymethane-induced aberrant crypt foci with the recovery of hexosaminidase activity

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Regression of precancerous lesions has been used in experimental and clinical studies to indicate chemoprevention in the colon, including the regression induced by piroxicam of the putative precancerous lesion, aberrant crypt foci (ACF). ACF lack hexosaminidase activity, while normal crypts possess the activity. We evaluated the ability of four potential chemopreventive agents to induce regression of ACF and to induce hexosaminidase activity in them. Male F-344 rats were i.p. administered 18 mg/kg body wt AOM at week 7 and again at week 8 of age. Five weeks after the first dose of AOM, curcumin (12 g/kg diet), ferulic acid (FA, 1 g/kg diet), or difluoromethylornithine (DFMO, 2 g/kg diet) were added to the diet. Also, at that time, other rats started to receive 0.3 g/kg body weight perillyl alcohol (PA) by gavage in corn oil or remained on the control diet. Rats were killed at 0, 10 and 28 days after the start of administering the chemopreventive agents. After 28 days of exposure, curcumin induced the regression of ACF, DFMO and PA prevented the occurrence of new ACF, and FA did not prevent ACF nor induce their regression. After 10 days of exposure, the order for the ability to induce hexosaminidase activity in ACF was curcumin > PA > DFMO > FA, which corresponded to the ability of these agents to induce regression and/or prevention of ACF. By 28 days of exposure, the percentage of foci that regained hexosaminidase activity was much less than at 10 days. The results demonstrated that another NSAID, curcumin, can induce the regression of ACF and that the reappearance of hexosaminidase activity might be a biomarker for the ability to induce the differentiation and regression of ACF.

Colorectal cancer accounts for ~20% of all cancer deaths in the US and is the second leading cause of cancer death in men and the third leading cause in women (1). One of the strategies to decrease its toll is the development of chemopreventive agents. Aspirin, piroxicam, sulindac and other non-steroidal anti-inflammatory drugs (NSAIDs*) are undergoing laboratory (2–7) and clinical investigation (8–10) for use as chemopreventive agents of colon cancer. One of the models used to evaluate agents for chemoprevention in the colon is the azoxymethane (AOM)-induced colon cancer model in rats (11). Using this model, NSAIDs including aspirin, curcumin, piroxicam, and sulindac have been shown to prevent colon cancer when administered prior to AOM and continuing until the death of the animals (2,3,6,7,12). Piroxicam (3,13) and, more recently, sulindac (12) have been shown to prevent AOM-induced colon cancer when administered 12–14 weeks after the last dose of AOM and continuing until death. In humans, sulindac has been shown to induce the regression of colon polyps in familial adenomatosis polyposis (FAP) patients (14–17). Hence, in both clinical and laboratory investigations, NSAIDs appear to prevent the progression of precancerous lesions in the colon.

In the AOM-induced colon cancer model, one of the earliest recognizable precancerous lesions is the appearance of aberrant crypt foci (ACF) (18–22). These foci are putative precancerous lesions that indicate at least the initiation of the carcinogenic process and predict an increased likelihood of cancer development (11). ACF are easily distinguished in methylene blue-stained whole mounts of rat colon by the procedure developed by Bird (18). In our previous studies, piroxicam was demonstrated not only to prevent AOM-induced ACF when administered prior to the carcinogen, but also to induce regression of ACF when administered after the carcinogen, both of which were associated with the prevention of colon tumors (13,22). Pretlow et al. have reported that AOM-induced ACF lacked hexosaminidase activity in contrast to normal crypts that contained activity (20). ACF do not differentiate normally; for example, they have a greatly reduced number of goblet cells. The association of a decrease in hexosaminidase activity with ACF, would suggest that it might be a biomarker for differentiation of colonic crypts. In this study, we evaluated the ability of four potential chemopreventive agents to induce regression of ACF in rat colons and determined whether recovery of hexosaminidase activity was associated with regression of ACF. The four agents were curcumin, ferulic acid (FA), difluoromethylornithine (DFMO), and perillyl alcohol (PA) which were chosen to represent a naturally occurring NSAID, an antioxidant, an ornithine decarboxylase inhibitor, and an inhibitor of farnesyl transferase, respectively.

Male F-344 rats (certified viral antibody-free) were obtained at 5 weeks of age from Charles River Laboratories (Raleigh, NC). The rats were maintained in an AAALAC accredited facility that was in accordance with the Animal Welfare Act (Public Law 89-544, 94-279) and NIH Publication no. 86-23 revised 1985 entitled Guide for the Care and Use of Laboratory Animals. Solid-bottom polycarbonate cages with stainless steel wire-bar lids and Bed-o-Cob bedding (Andersons, Toledo, OH) were used to house two rats per cage. The light cycle consisted of 12 h each of light and dark. The animal rooms were maintained at 64–76°F and 55 ± 15% relative humidity. The rats were fed AIN-76A diet (Bioserve, Frenchtown, NJ) consisting of 20% casein, 0.3% DL-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate. The diet and deionized water for drinking were provided ad libitum.

All the rats of the study were administered AOM (Sigma Chemical Co., St Louis, MO) at a dose of 18 mg/kg body wt by i.p. injection at 7 weeks and again at 8 weeks of age. The rats were randomly assigned to one of five treatment groups including the AOM + control diet group. The number of rats

*Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; DFMO, difluoromethylornithine; FA, ferulic acid; FAP, familial adenomatosus polyposis; NSAID, non-steroidal anti-inflammatory drug; PA, perillyl alcohol.
Table 1. Effect of chemopreventive agents upon body wt

<table>
<thead>
<tr>
<th>Agents</th>
<th>Body wt (g)</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>274.7 ± 2.6 (30)(^a)</td>
<td>292.6 ± 3.5 (20)</td>
<td>328.1 ± 4.7 (10)</td>
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</tr>
<tr>
<td>Curcumin</td>
<td>274.6 ± 3.0 (20)</td>
<td>290.4 ± 3.9 (20)</td>
<td>318.8 ± 9.7 (10)</td>
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</tr>
<tr>
<td>Ferulic acid</td>
<td>273.3 ± 3.6 (20)</td>
<td>309.0 ± 5.0 (20)</td>
<td>348.7 ± 5.3 (10)</td>
<td></td>
</tr>
<tr>
<td>DFMO</td>
<td>274.2 ± 3.7 (20)</td>
<td>306.1 ± 3.4 (20)</td>
<td>357.2 ± 3.7 (10)(^b)</td>
<td></td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>273.5 ± 3.4 (18)</td>
<td>287.6 ± 6.1 (18)</td>
<td>308.3 ± 3.6 (8)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SE for the number of animals indicated in the parentheses.\(^b\)Significantly different from control by one ANOVA followed by Tukey test (\(P < 0.05\)).

and their mean body weights in each treatment group prior to the start of administering the chemopreventive agents are given in Table 1. At 5 weeks after the first dose of AOM, 12 g/kg diet of curcumin (Sigma Chemical Co., St Louis, MO), 1 g/kg diet of FA (Sigma Chemical Co., St Louis, MO) or 2 g/kg diet of DFMO (Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH) were added to the diet. Also, at this time, other rats started to receive PA (Aldrich Chemical Co, Milwaukee, WI) at a dose of 0.3 g/kg body wt by gavage in corn oil for 6 days a week.

At 0, 10 and 28 days after the start of administering the chemopreventive agents, the animals were killed by carbon dioxide asphyxiation in order to determine the starting number of ACF (day 0) and regression/prevention of ACF (days 10 and 28). The colons were evaluated for ACF by the procedure described previously (18,22). Briefly, the colons were excised, cut open along the longitudinal axis, flushed with cold saline and fixed flat in 0.1 M phosphate buffered-10% formalin solution (pH 7.4, 4°C) for 24 h. The colons were then transferred to 70% ethanol and stored at 4°C until stained in 0.2% methylene blue in 70% ethanol solution for 5 min. The number of ACF/colon was determined with the use of a microscope at a magnification of \( \times 40 \). ACF were distinguished from surrounding non-involved crypts by their increased size, elongated luminal opening, increased distance from luminal to basal surface of cells, the easily discernible, thickened epithelial cell lining and enlarged pericryptal zone relative to surrounding normal crypts.

After evaluation for ACF, the colons from six animals in each treatment group were randomly selected and stained for hexosaminidase activity using the method described previously (20–22). Briefly, the colon was rinsed in phosphate buffered saline (PBS, pH 7.4) incubated in the same PBS at 4°C for 2 h, and then stained at 37°C for 3 h in a medium containing 0.1 M citrate buffer, 0.5% calcium chloride, 8% polyvinyl alcohol (mol. wt 13,000 ~ 23,000), 0.2% hexazonium salt of pararosaniline (Sigma Chemical Co., St Louis, MO), 0.04% naphthol AS-Bl-β-d-glucosaminide (Sigma Chemical Co., St Louis, MO), and 0.4% ethylene glycol monomethyl ether (Sigma Chemical Co., St Louis, MO), pH 4.7. The entire reddish brown appearing colon was rinsed briefly in PBS and counter-stained for 5 s in 0.2% methylene blue in 70% ethanol. ACF recognized by their aberrant morphology, were classified as either lacking, having less than normal or having normal levels of hexosaminidase activity relative to non-involved crypts of the colon. The appearance of ACF that lacked hexosaminidase activity were intensely blue-green, those with less than normal levels of activity were moderately brown with a tint of green and those with normal levels of activity were intensely reddish-brown.

The results were analysed for statistical significance by a one-way analysis of variance (ANOVA) followed by the Tukey test. A \( P \)-value of 0.05 was used to signify statistical significance.

Body weights of rats were monitored at 0, 10, and 28 days after the start of administering the test agents (Table 1). There was no significant difference (\( P > 0.05 \)) in body wt of the animals administered the chemopreventive agents and those fed the control diet, except at termination for the animals fed DFMO (2 g/kg diet) which had a statistically significant increase in the body wt.

The effect of the chemopreventive agents upon the number of ACF/colon induced by AOM is presented in Figure 1. After 10 and 28 days of exposure to curcumin, DFMO and PA, the yield of ACF was significantly reduced (\( P < 0.01 \)) compared to the control group. At both time points, the yield of ACF in animals fed FA was slightly lower, but not significantly different (\( P > 0.05 \)) from the control group. The yield of ACF in the animals exposed to curcumin decreased with time, from 108.4 ± 6.1 prior to curcumin to 104.1 ± 5.4 after 10 days and to 66.0 ± 7.8 (significantly different from prior to exposure, \( P < 0.01 \)) after 28 days of exposure. That is, the yield of ACF after 28 days of exposure to curcumin was ~40% less than the yield in the animals prior to its exposure. Hence, it appears that regression of ACF occurred after 28 days, but not after 10 days of exposure to curcumin. The number of ACF/colon in the animals exposed to DFMO or PA for 10 or 28 days were not significantly different (\( P > 0.05 \)) than the yield prior to their exposure. The increase in the yield of ACF in the control group at days 10 and 28, was reduced by both DFMO and PA, indicating their ability to prevent new foci. However, the results do not rule out the possibility that the decrease in ACF in animals administered either of the two agents is due to both the prevention of new ACF and the regression of others.

The level of hexosaminidase activity in ACF from animals on control diet was classified as being absent in 94%, less than normal in 5% and normal (same histochemical staining...
as surrounding non-involved crypts) in 1% of the foci (Figure 2A). After 10 days of exposure to curcumin, the percentage of ACF that lacked hexosaminidase activity decreased to 34%, while those with less than normal levels of the activity increased to 35% and with normal levels increased to 31% (P < 0.01 for the three comparisons to the control group) (Figure 2A). After 28 days of exposure to curcumin, the percentage of ACF with normal and less than normal levels of hexosaminidase activity decreased relative to 10 days of exposure from 31 to 6% and from 35 to 13%, respectively, and the percentage of ACF lacking activity increased from 34 to 81% (P < 0.05) (Figure 2A,B). After 10 days of exposure to FA, PA and DFMO, the percentage of ACF that lacked hexosaminidase activity also decreased with increases in the percentage with less than normal and normal levels (Figure 2A). However, the changes were less pronounced than with exposure to curcumin. Similar to animals exposed to curcumin for 28 days, the percentage of ACF with normal and less than normal levels of hexosaminidase activity decreased relative to 10 days of exposure (Figure 2B). The orders of the four chemopreventive agents for the ability to induce hexosaminidase activity in ACF corresponded to the ability of these four agents to induce regression and/or prevent ACF.

The recovery of hexosaminidase activity in ACF suggested that curcumin was inducing differentiation in them, since this enzymatic activity was found in normal crypts. After 28 days of exposure to curcumin both the yield of ACF and the percentage of remaining ACF with normal levels of hexosaminidase activity were reduced. This would suggest that the ACF in which curcumin induced hexosaminidase activity are the foci that differentiated and regressed. The other three agents did not demonstrate the ability to cause the regression of ACF and induced hexosaminidase activity in a smaller percentage of foci. However, it cannot be ruled out that DFMO and PA induced regression in a small number of foci. The correlation between the ability of these agents to induce hexosaminidase activity in foci and to induce the regression of ACF, indicates that the reappearance of hexosaminidase activity in ACF could be an early biomarker for the ability to induce regression of ACF.

Curcumin and DFMO have previously been reported to prevent AOM-induced ACF and cancer in the colon of rats (6,7,23–25). PA demonstrated in this study the ability to prevent the increase of ACF during the 28 days of its exposure. This would suggest that PA could prevent colon cancer. PA and other monoterpenes have demonstrated chemopreventive activity in rat mammary gland and liver (26–28). DFMO has been shown to inhibit rat tongue carcinogenesis when administered concurrently with the carcinogen, 4-nitroquinoline-1-oxide (29). PA did not demonstrate the ability to prevent ACF or to induce the regression of ACF, and was the weakest agent in inducing hexosaminidase activity in ACF. This indicates that its chemopreventive activity in the colon is likely to be weak.

The results of this study demonstrated that another NSAID, curcumin, similar to piroxicam (13) can induce the regression of ACF. It also demonstrated that the reappearance of hexosaminidase activity in ACF might be a biomarker for the ability to induce their differentiation and regression.

References


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