Effect of Age on Susceptibility to Azoxymethane-Induced Colonic Aberrant Crypt Foci Formation in C57BL/6JNIA Mice

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To determine the effect of age on susceptibility to azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) formation and its underlying mechanism, young and old mice were injected with AOM weekly for 4 or 5 weeks and euthanized 5 or 6 weeks later. Given the same (12 or 15) mg/kg body weight dose of AOM, old mice had significantly more ACF than young mice. However, given the same total dose of AOM (to avoid confounding effect of higher dose to heavier old mice), at a low total dose (1.5 mg) there was no age difference, but at higher total doses (1.8 and 2.2 mg) young mice had significantly more ACF than old mice. These results indicate that the age-related susceptibility to AOM differs depending on whether administration of the carcinogen is based on weight or total dose. These age differences are not due to variations in cyclooxygenase-2 expression, cell proliferation, or AOM hydroxylase activity.

AGING is associated with an increased incidence of many human malignancies, including colon cancer. However, it is not clear whether aging itself increases susceptibility to colon cancer or the higher incidence is due to the cumulative effect of long-term exposure to carcinogenic agents (1,2). Previous animal studies (3,4) on the effect of aging on colon cancer compared susceptibility in pups with adult or old animals. These comparisons are not appropriate to determine the effect of age on colon cancer because suckling or weanling animals, which were used in these studies, have a gut physiology that is quite distinct from that of young adult or old animals (5).

There are several characteristics that might increase the susceptibility to colon carcinogenesis in aged mice. For instance, cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) are contributing factors in the development of colonic carcinogenesis (6–15), and aged mice have higher levels of macrophage COX-2 expression, COX-2 activity, and PGE2 production than young mice (16,17). Thus, aged mice might be more susceptible to colon cancer due to their higher expression of COX-2 and PGE2 production.

Healthy aged rats and elder humans have been reported to have a significantly higher colonic crypt cell proliferation and an expanded proliferation zone, which are early biomarkers of colon cancer, compared with young rats and young humans (18–22). Therefore, an age-associated increase in colonic cell proliferation might predispose the aged (both rats and human subjects) to colon cancer. In addition, increases in oxidative stress with aging could contribute to a higher susceptibility to colon cancer in aged mice (23–25).

Azoxymethane (AOM), a chemical and metabolic derivative of 1,2-dimethylhydrazine, is a powerful and specific colon carcinogen in rodents (26). Azoxymethane-induced aberrant crypt foci (ACF) have been used as putative preneoplastic markers to evaluate colonic carcinogenesis (27–29). Thus, in this study we investigated the effect of age on susceptibility to colon cancer by comparing AOM-induced ACF formation between young and old mice. In addition, the underlying mechanism for the age-associated difference in AOM-induced ACF formation was examined.

METHODS

Animals and Diets

In experiment I and II (see experimental procedure), pathogen-free young (4–5 months) and old (21–22 months) male C57BL/6JNIA mice were watered and fed mouse chow (Harlan Teklad, Madison, WI) ad libitum. All mice were individually housed in cages (except in experiment I where 4 mice were housed per 1 cage) and maintained at a constant temperature (23°C) with a 12-hour light–dark cycle. Body weights were recorded weekly. All handling and care of the animal conditions were approved by the Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

Carcinogen

AOM was purchased from Sigma Chemical (St. Louis, MO), and dissolved in a fresh 0.9% sterile saline solution 1 hour before injection.
Experimental Procedure

As shown in Figure 1, 3 experiments were conducted to elucidate the effect of age on susceptibility to AOM-induced ACF formation. Mice were randomly assigned to groups in all experiments. In experiment I, young and old mice were administered, subcutaneously, saline or 15 mg AOM/kg body weight weekly for 4 weeks. All mice were euthanized via CO2 asphyxiation, and colons were collected at 9 weeks for determination of ACF. Our preliminary experiment in which 2 different doses of AOM (10 and 15 mg AOM/kg body wt) in young mice showed that the injection of 15 mg AOM/kg body weight weekly for 4 weeks induced detectable numbers of ACF (data not shown). Thus, this dose was used in experiment I. In experiment II, young and old mice were administered, subcutaneously, saline or 12 mg AOM/kg body weight, and a group of old mice were administered the same total amount of AOM as that received by young mice injected with 12 mg AOM/kg body weight (8.9 mg AOM/kg body wt) weekly for 4 weeks. Colons were collected at 11 weeks for determination of ACF. In experiment III, young mice were administered, subcutaneously, saline or 12 (the first injection)/10 (injections 2–5), or 15 (the first injection)/12 (injections 2–5) mg AOM/kg body weight and old mice were administered, subcutaneously, saline or the same total amount of AOM as that received by young mice injected with 12/10 or 15/12 mg AOM/kg body weight weekly for 5 weeks (1.8 or 2.2 mg total dose of AOM). We had few dead mice after the first injection, therefore we reduced the AOM doses for injections performed in week 2–5 as described above. At 11 weeks, colons were collected for determination of ACF. Multi-injections of high-dose of AOM were used in this study to induce reasonable number of ACF formation in C57BL/6 mice to be able to detect age difference because C57BL/6 mice are less susceptible to ACF formation compared to rats and certain mice species.

Quantification of ACF

After fixation in 70% ethanol, the colon was dyed in a solution of 0.2% methylene blue for 1 minute. It was then placed with mucosal side up on a microscope slide and viewed with a light microscope. ACF were distinguished from the surrounding normal crypts by their increased size of crypts, darker staining, and enlarged pericryptal zone as previously described (30). The number of ACF per colon, and the shape and location of each focus, were recorded blindly. After quantification of ACF, the dye was removed and the colon was embedded in paraffin. Serial longitudinal sections were made onto microscope slides for COX-1, COX-2, and proliferating cellular nuclear antigen (PCNA) immunohistochemistry.

Immunohistochemical Analysis of COX-1 and COX-2 Expression in the Colon

Immunostaining for COX-1 and COX-2 were performed with the avidin-biotin-peroxidase complex (ABC) kit (Vectastain, Burlingame, CA). Deparaffinized tissue sections were incubated with 3% hydrogen peroxide (H2O2) for 30 minutes to block endogenous peroxidase activity. Nonspecific binding sites were blocked with normal goat serum for 30 minutes at room temperature (RT). Rabbit polyclonal antimurine COX-1 and COX-2 antibodies (Abs) (Cayman Chemical, Ann Arbor, MI) were incubated overnight at 4°C. Biotinylated antirabbit IgG (Vectastain) was used for 45 minutes at RT. Tissues were then incubated with ABC reagent for 30 minutes. Color was developed by treatment with liquid diaminobenzidine tetrahydrochloride (DAB, Sigma) for 4 minutes. The section was counterstained with Mayer’s hematoxylin (Sigma). For each tissue specimen, the extent and intensity of staining with COX-1 and –2 Abs were graded on a scale of 0–3 by a blinded observer on two separate occasions, and the average scores were calculated.

Measurement of Colonic Cell Proliferation

Similar to COX-1 and COX-2 staining, PCNA staining was performed by using the ABC system. Anti-PCNA mouse monoclonal Ab (PC-10, Dako, Carpinteria, CA) and biotinylated antimouse IgG (Vectastain) were used. To determine PCNA labeling index (LI), 8–20 open U-shaped crypts were identified, and the number of PCNA positive and negative epithelial cells per crypt were counted by a blinded observer on 2 separate occasions; the average of the 2 determinations was calculated. The PCNA LI was calculated by the following formula: The PCNA LI = (number of positive epithelial cells per crypt/total number of epithelial cells per crypt) × 100.
Determination of AOM Hydroxylase Enzyme Activity

Hepatic microsomes were prepared from C57BL/6JNIA mice as previously described (31). The AOM hydroxylase activity was determined by measuring hydroxylation of AOM to methylazoxymethanol (MAM) as described by McMahon and colleagues (32). High performance liquid chromatography (HPLC) was used to quantify MAM as described before (32).

Statistical Analysis

Data were analyzed by student’s t test or analysis of variance (ANOVA) for overall effect of age and AOM followed by Tukey’s honestly significant difference (HSD) post hoc test for individual comparisons using Systat 9 statistical software (Systat, Evanston, IL). Student’s paired t test was used to determine the difference in body weights before and after AOM injection. Data are reported as mean ± SEM, and the significance was set at p < .05.

RESULTS

Effect of Age on Susceptibility to AOM-Induced ACF Formation in Mouse Colon

In experiment I, when mice were given the same per kilogram body weight of AOM (method used in previous reports), old mice had significantly more ACF than young mice (17.0 ± 1.16 versus 10.0 ± 0.82, n = 11, p < .001). In addition, we observed a higher mortality in old mice compared with young mice (data not shown). Since old mice are heavier than young mice, they received a higher total dose of AOM than young mice (1.5 mg and 1.8 mg in young and old mice, respectively). Thus, the higher ACF formation and mortality observed in old mice might not be due to an age-related phenomenon; rather, it might reflect the higher total dose received by the old mice. To distinguish the effect of aging from that of total dose and reduce mortality, experiment II was conducted, in which young and old mice were administered a lower per kilogram body weight AOM dose (12 mg AOM/kg body wt). In addition, a group of old mice were administered the same total amount (1.5 mg) of AOM as that received by young mice injected with 12 mg AOM/kg body weight (see Figure 1). There was no significant effect of age or AOM injection on mortality. As shown in Figure 2, given the same [12] milligram AOM/kg body weight, which resulted in a higher total dose (2.0 mg) in old mice, old mice had significantly higher ACF formation than young mice (p < .03). However, there was no age difference in ACF formation when young and old mice were administered the same total dose of AOM (1.5 mg).

To further investigate if old mice are more susceptible to ACF formation at a higher (than 1.5 mg) dose of AOM than young mice, experiment III was conducted, in which young and old mice were administered a “medium” or “high” total dose of AOM (1.8 or 2.2 mg). Table 1 shows ACF formation in young and old mice. Young mice given the same medium and high total dose of AOM had significantly higher ACF formation compared with old mice (p < .001). Thus, different patterns of ACF formation are observed in response to a low, medium, and high total dose of AOM in young and old mice. Figure 3 demonstrates that smaller young mice are significantly more sensitive to increasing doses of AOM than old mice (p < .001).

Effect of AOM and Age on COX-1 and COX-2 Expression

COX-1 expression was predominantly localized in interstitial cells, and levels of COX-1 expression in normal cells and ACF were consistent between saline and AOM-injected mice, as well as between young and old mice (data not shown).

AOM injection produced a significant increase in COX-2 expression (p < .01), but no effect of age alone or age–AOM interaction was observed on COX-2 expression (Table 2). These observations were consistent between normal colonic tissue and ACF. In normal colonic tissue of saline or AOM-injected mice, COX-2 was expressed in epithelial, interstitial, and lamina propria cells; in ACF, however, COX-2 was predominantly expressed in epithelial cells. The level of COX-2 expression in ACF was not significantly higher compared to that in normal epithelial cells in mice injected with AOM, but COX-2 expression

Table 1. Effect of Age on Colonic Aberrant Crypt Foci Formation of Mice Injected With a Medium or High Total Dose of Azoxymethane in Experiment III

<table>
<thead>
<tr>
<th>Total Dose of AOM</th>
<th>ACF/colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Old</td>
</tr>
<tr>
<td>1.8 mg</td>
<td>21.3 ± 1.1*</td>
</tr>
<tr>
<td>2.2 mg</td>
<td>19.9 ± 1.8*</td>
</tr>
</tbody>
</table>

Notes: Values are expressed as mean ± SEM, n = 13–17. No ACF were found in mice injected with saline.

*Significantly higher than old mice by analysis of variance (p < .001).

ACF = aberrant crypt foci; AOM = azoxymethane.
was significantly higher compared to that in normal epithelial cells of saline-injected mice \( (p < .05) \). There was no significant difference in COX-2 expression by normal cells or ACF between medium and high total doses of AOM-injected young or old mice. Control sections incubated without primary Abs did not show any staining (data not shown).

Effect of AOM and Age on Cell Proliferation

As shown in Figure 4, AOM injection caused a higher colonic epithelial cell proliferation \( (p < .01) \), the magnitude of which was similar when either medium or high total dose of AOM was used. There was no effect of age or age–AOM interaction on PCNA LI.

Effect of Age on ACF Formation

As shown in Figure 3, the effect of age on AOM-induced ACF formation in 3 experiments. Values are expressed as mean \( \pm \) SEM, \( n = 16–32 \). Low, medium, and high total dose of AOM is 1.5, 1.8, and 2.0–2.2 mg, respectively. \*Significantly higher compared with old mice, as determined by Tukey’s honestly significant difference post hoc test at \( p < .03 \). \#Significantly higher compared with mice treated with low total dose of AOM, as determined by Tukey’s HSD post hoc test at \( p < .001 \). \( p = .06 \) compared with mice treated with low total dose of AOM, as determined by Tukey’s HSD post hoc test.

Effect of AOM Metabolism

McMahon and colleagues (32) reported that the livers from old animals are less able to convert AOM to MAM by the enzyme AOM hydroxylase. Thus we hypothesized that the lower AOM-induced ACF formation in old mice might be due to their reduced activity of hepatic AOM hydroxylase. MAM is the intermediate in the conversion of AOM to carbonium ion, the active carcinogen in the colon. There was no significant difference in specific activity of hepatic AOM hydroxylase between young and old mice \( (0.29 \pm 0.04 \) and \( 0.34 \pm 0.05 \) nmol/min/mg protein in young and old mice, \( n = 5 \), respectively).

Effect of Age on AOM-Induced Weight Loss

Since the young mice were in a growth phase of their life cycle, there was significant weight gain in the young mice injected with saline \( (p < .05) \), but, as expected, there was no significant weight gain in old mice injected with saline during the experimental period (data not shown). Both young and old mice injected with AOM had significantly lower body weight following 4 or 5 weeks of AOM injection compared with their baseline weights \( (p < .01, \) data not shown). As seen in Figure 5, old mice had significantly greater weight loss following 4 or 5 weeks of AOM injection compared with young mice at all different total doses of AOM \( (p < .001) \).

### Table 2. Effect of Age on Colon Cyclooxygenase-2 Expression of Mice Injected With a Medium or High Total Dose of Azoxymethane (AOM) in Experiment III

<table>
<thead>
<tr>
<th>Age</th>
<th>Normal cells</th>
<th>ACF</th>
<th>Score</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td></td>
<td>NP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0.3 ± 0.2</td>
<td>1.1 ± 0.3*</td>
<td>1.3 ± 0.4*</td>
<td>NP</td>
<td>1.6 ± 0.4*</td>
</tr>
<tr>
<td>Old</td>
<td>0.2 ± 0.1</td>
<td>1.1 ± 0.4*</td>
<td>1.1 ± 0.5*</td>
<td>NP</td>
<td>1.3 ± 0.4*</td>
</tr>
</tbody>
</table>

Notes: Values are expressed as mean \( \pm \) SEM, \( n = 5–8 \), 0 = no staining: 1 = weak staining; 2 = moderate staining; 3 = strong staining. COX-2 immunostaining of normal cells was observed evenly in epithelial cells and lamina propria.

\*Significantly higher than their saline-injected counterparts by analysis of variance (ANOVA) \( (p < .05) \).

\#Significantly higher than that of normal cells of their saline-injected counterparts by ANOVA \( (p < .05) \).

COX-2 = cyclooxygenase-2; AOM = azoxymethane; ACF = aberrant crypt foci; NP = not present.

DISCUSSION

This is the first study to investigate the effect of age on the susceptibility to AOM-induced ACF formation and weight loss in adult mice. The results of this study indicate that, (a) old mice are more susceptible to AOM-induced ACF formation when a higher dose is administered to correct for body weight, and (b) young mice are more susceptible to AOM-induced ACF formation than old mice at medium and high total dose of AOM.

To determine the possible underlying mechanisms for this age-associated difference in ACF formation given the same
total dose of AOM, we investigated several factors that are thought to be mechanistically related to colon cancer, including cell proliferation, COX-2 expression, and AOM metabolism.

As shown by others (21,33), mice treated with AOM had a significantly higher colonic cell proliferation compared with mice injected with saline. However, unlike previous reports in rats (18,20), there was no age difference in colonic cell proliferation in saline-treated mice. This might be due to species differences. In addition, there was no age-related difference in colonic cell proliferation in AOM-treated mice.

Young and old mice injected with AOM had significantly higher COX-2 expression in normal cells as well as ACF compared with that in normal cells of saline-injected mice. However, levels of COX-2 expression were not significantly different between ACF and normal cells of AOM-injected mice. Takahashi and colleagues (34) reported that there was no or low COX-2 expression in colonic ACF, while high COX-2 expression was observed in colonic tumors. COX-2 appears to be strongly correlated to the late stages of colon cancer rather than the early stages. There was no significant difference in COX-2 expression between young and old mice in normal cells or ACF. In addition, we found no difference in specific activity of hepatic AOM hydroxylase between young and old mice. Taken together, these results suggest that the higher susceptibility of young mice to AOM-induced ACF formation at certain doses is not due to the difference in cell proliferation, COX-2 expression, or AOM hydroxylase activity.

Despite the lower ACF formation at a certain total AOM dose in old mice compared with young mice, the old mice lost significantly more weight than the young mice following AOM injection. Further studies are needed to determine other underlying mechanisms that contribute to the higher susceptibility of young mice to AOM-induced ACF formation and to determine underlying mechanisms of the age-related difference in AOM-induced weight loss.

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