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Food and Chemical Toxicology 42 (2004) 1339–1347
Food and Chemical Toxicology
www.elsevier.com/locate/foodchemtox

Effects of adlay on azoxymethane-induced colon carcinogenesis in rats

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Received 20 November 2003; accepted 22 March 2004

Abstract

Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop used in traditional Chinese medicine and as a nutritious food. It has been reported that adlay has anti-inflammatory and anti-tumor activity. Cyclooxygenase-2 (COX-2) is an inducible enzyme functionally related to both inflammation and colon carcinogenesis and is the target of many chemopreventive agents. This study investigated the effect of adlay on colon carcinogenesis and COX-2 expression. In a short-term experiment, male F344 rats were fed diets containing different doses of dehulled adlay and received the colon-specific carcinogen, azoxymethane (AOM), by intraperitoneal injection. All rats were killed after 5 weeks of feeding, and the colons were examined for the preneoplastic lesion, aberrant crypt foci (ACF). Dietary dehulled adlay at levels of 10%, 20%, or 40% significantly reduced the numbers of ACF and aberrant crypts. Dehulled adlay reduced the number of ACF of different sizes but did not affect the crypt multiplicity. Most ACF were found in the middle and distal colons; dehulled adlay significantly suppressed the formation of ACF in the middle colon. In a long-term experiment, male F344 rats were fed diets containing different doses of dehulled adlay and injected with AOM. All rats were killed after 52 weeks of feeding, and colons were examined for tumors and COX-2 protein expression. The results indicated that dehulled adlay did not inhibit colon tumors in spite of a slight suppressing effect in the proximal colon. Rats fed diets containing 20% dehulled adlay had less COX-2 protein expression in both proximal and distal colon tumors. The inconsistent effects between COX-2 protein expression and tumor outcome may be due to regional differences in the colon and the malignancy of the tumors. These findings suggest that dehulled adlay suppresses early events in colon carcinogenesis but not the formation of tumors.

Keywords: Adlay; Colon cancer; Aberrant crypt foci; Cyclooxygenase-2; Azoxymethane

1. Introduction

Colorectal cancer is the fourth most common cancer worldwide (Talbot and Neugut, 2002) and the third most common cause of cancer death in Taiwan. Despite the development of new screening strategies, aggressive surgical and adjuvant therapy, and intensive research effects, little progress has been made in the successful management of this disease (Williams et al., 1999). The cure rate for this cancer has remained at 50% for several decades (Burnstein, 1993). The etiology of colorectal cancer is complex and may be attributable to combined actions of inherited and environmental factors. Epidemiological studies have indicated that colorectal cancer is strongly associated with diet (Slattery et al., 1999), and thus it may be possible to prevent the occurrence of this cancer by dietary modification. Chemoprevention refers to the use of natural or synthetic compounds to prevent, reverse, or delay the development of cancer (Swan and Ford, 1997). Because food-derived products exist universally and are expected to be safe, they are highly interesting for development as chemopreventive agents (Kelloff et al., 2000).

Adlay (soft-shelled job's tears, *Coix lachryma-jobi* L. var. *ma-yuen* Stapf), widely planted in some Asian

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; COX-2, cyclooxygenase-2; PBS, phosphate-buffered saline

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doi:10.1016/j.fct.2004.03.011

countries including Taiwan, is a grass crop that has long been used in traditional Chinese medicine and as a nutritious food. The seed of adlay was used in China to treat warts, chapped skin, rheumatism, and neuralgia, and as an antihelminth and anti-inflammatory agent. In addition, adlay has been widely used as a diuretic, stomachic, analgesic, and antispasmodic agent from ancient times. Adlay is believed to be beneficial to the gastrointestinal tract and may be used as a prebiotic due to its modifying effect on some intestinal bacteria (Chiang et al., 2000a). A number of recent studies have shown that adlay may have an anti-tumor effect. Ukita and Tanimura (1961) reported that an acetone extract of adlay showed a growth-inhibitory activity on the Ehrlich ascites sarcoma of mice and identified the active component as coixenolide (Tanimura, 1961). Tokuda et al. (1990) found that a methanolic extract of adlay exhibited an anti-tumor promoting activity and that α-monolinolein is one of the active constituents. Numata et al. (1994) indicated that an acetone extract of adlay inhibited transplantable mouse tumors and that the active components contain four free fatty acids (palmitic, stearic, oleic, and linoleic acids). Chiang et al. (2000b) evaluated the anti-tumor effect of adlay in the form of a processed food and found that this product inhibited sarcoma-180 tumors in mice. Kuo et al. (2001) reported that a methanolic extract from adlay hull exhibited antiproliferative activity against human histolytic lymphoma U937 monocytic cells; this effect appeared to be attributable to apoptosis. Chang et al. (2003) showed that a methanolic extract of adlay exerted an antiproliferative effect on A549 lung cancer cells by inducing cell cycle arrest and apoptosis and that feeding a diet containing adlay reduced the number of surface lung tumors in mice.

These studies suggest that adlay and its components may have an anti-tumor effect and have the potential to be developed as chemopreventive agents. However, most of these studies used cancer models not similar to human cancer, and none of them investigated the effect of adlay on diet-related colorectal cancer. In addition, most of these studies administered adlay extracts by injection, not feeding. In order to evaluate the chemopreventive effect of adlay on colorectal cancer, this study examined the effect of dietary adlay on colonic aberrant crypt foci (ACF), a preneoplastic lesion of the colon, and on colon tumors. Recent studies have demonstrated that cyclooxygenase-2 (COX-2), an inducible enzyme that catalyses the conversion of arachidonic acid into prostaglandins, is up-regulated during inflammation and colorectal carcinogenesis and that COX-2 inhibitors offer real hope for safe anti-inflammatory drugs and chemopreventive agents for colorectal cancer (Kam and See, 2000; Church et al., 2003). Since the COX-2-mediated inflammation is critical for colorectal carcinogenesis, we explored whether adlay, a natural anti-inflammatory agent, could affect colorectal carcinogenesis through modulation of COX-2 expression.

2. Materials and methods

2.1. Adlay

Adlay was purchased from a local farmer who planted Taichung Shuenyu No. 4 (TCS4) of *Coix lachryma-jobi* L. var. *ma-yuen* Stapf in Taichung, Taiwan, in March 2000 and harvested it in July of the same year. The air-dried adlay seeds were dehulled, blended into powder, and screened through a 40-mesh sieve. The composition of dehulled adlay was analyzed according to the AOAC method (Association of Official Analytical Chemists, 1980) and the method of Prosky et al. (1988) and determined to be (on a wet basis): moisture, 8.5%; ash, 1.7%; crude protein, 13.4%; crude fat, 7.4%; dietary fiber, 3.9%; nitrogen-free extract, 65.1%; and energy, 15,985 kJ/kg dehulled adlay.

2.2. Animals and diets

Male F344 rats were obtained at age 5 weeks from the Laboratory Animal Center of the College of Medicine at National Taiwan University (Taipei, Taiwan). Animals were housed in stainless steel, wire-bottomed cages (three or four rats per cage) in a room under controlled conditions of 23 ± 1 °C and $50 \pm 10\%$ relative humidity, with a 12-h light/dark cycle. They were allowed free access to feed and water. The experimental diets were formulated on the basis of a modified AIN-76 diet (American Institute of Nutrition, 1977; American Institute of Nutrition, 1980) and substituted with 0%, 10%, 20%, or 40% dehulled adlay. The composition of the experimental diets is shown in Table 1.

2.3. Experimental procedures

After 1 week of acclimation, animals were grouped and fed the experimental diets. One week later, animals scheduled to receive carcinogen treatment were injected intraperitoneally with azoxymethane (AOM) purchased from Sigma Chemical Co. (St. Louis, MO) once weekly for 2 weeks at a dose level of 20 mg/kg body weight. Animals intended for vehicle treatment were intraperitoneally injected with equal volumes of phosphate-buffered saline (PBS). The body weight of animals and the consumption of experimental diets were recorded weekly. At the end of the experiment, all rats were killed by CO_2 asphyxiation.

2.3.1. Experiment 1

Thirty-eight rats were randomly divided into five groups. Rats in groups 1.1 and 1.2 were fed the AIN-76

Table 1
Composition of experimental diets (g/kg diet)[a,b]

	C	10A	20A	40A
Dehulled adlay	–	100	200	400
Corn starch	650	574	500	350
Casein	200	187	173	146
Soybean oil	50	43	35	20
Cellulose	50	46	42	34
AIN-76 mineral mixture	35	35	35	35
AIN-76 vitamin mixture	10	10	10	10
DL-Methionine	3	3	3	3
Choline bitartrate	2	2	2	2

[a] C, control diet; 10A, 10% adlay diet; 20A, 20% adlay diet; 40A, 40% adlay diet.

[b] Corn starch, Samyang Genex Co. (Seoul, Korea); casein, methionine, and choline bitartrate, Sigma Chemical Co. (St. Louis, MO); soybean oil, President Co. (Tainan, Taiwan); cellulose, AIN-76 mineral mixture, and AIN-76 vitamin mixture, ICN Biochemicals Co. (Costa Mesa, CA).

diet and injected with PBS and AOM, respectively. Rats in groups 1.3, 1.4, and 1.5 were fed diets containing 10%, 20%, and 40% dehulled adlay, respectively, and they were injected with AOM. After 5 weeks of feeding, all rats were killed and the colons removed for evaluation of ACF.

2.3.2. Experiment 2

Sixty rats were randomly divided into six groups. Rats in groups 2.1, 2.2, and 2.3 were fed the AIN-76 diet or diets containing 10% and 20% dehulled adlay, respectively, and they were injected with PBS. Rats in groups 2.4, 2.5, and 2.6 were fed the AIN-76 diet or diets containing 10% and 20% dehulled adlay, respectively, and they were injected with AOM. After 52 weeks of feeding, all rats were killed and the colons removed for evaluation of tumors and COX-2 expression.

2.4. ACF assay

The method of Bird (1987, 1998) was used to assess ACF. Colons (from the cecum–colon connection to the anus) were removed, cut along the longitudinal axis, and flushed with PBS. Each colon was cut into three (the proximal, middle, and distal) sections of equal lengths and fixed flat between filter papers in 10% buffered formalin (Mallinckrodt Specialty Chemicals Co., Paris, KY) for at least 24 h. The fixed colon sections were stained with a 0.2% solution of methylene blue (Sigma, St. Louis, MO) and placed on microscopic slides with the mucosal side up. ACF were examined under 40× magnification using a light microscope (Nikon, Japan) and identified by their increased size, irregular and dilated luminal opening, and thicker epithelial lining and pericryptal zone. The number of ACF per colon, the number of aberrant crypts observed in each focus, and the location of each focus were recorded.

2.5. Tumor assay

The method of Rao et al. (1995, 2001) was used to assess tumors. Colons (from the cecum–colon connection to the anus) were resected and opened longitudinally, and the contents were flushed with PBS. Each colon was divided into two (the proximal and distal) sections of equal lengths, and tumors were examined grossly for their location, number, and size. For each tumor, the length (L), width (W), and depth (D) were measured with calipers. Estimates of tumor volume (V) were made using the formula $V = L \times W \times D \times \pi/6$. For the analysis of COX-2, colon tumors with diameters of more than 0.5 cm were cut, and the tumor-free colonic mucosa of all animals was scraped with a microscope slide. These samples were frozen quickly in liquid nitrogen and stored at −80 °C until analysis.

2.6. Measurement of COX-2 protein expression

The method for measurement of COX-2 protein expression was modified from Singh et al. (1997) and Tanaka et al. (2001). Frozen tissues (colon tumors or colonic mucosa) were thawed in ice-cold homogenization buffer (RIPA buffer containing 1 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethylsufonylfluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin; pH 7.4) and homogenized on ice. The homogenates were centrifuged at 19,300×g at 4 °C for 20 min. The protein contents of the supernatants were determined using BCA protein assay reagents (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard. Supernatants corresponding to 75 μg of protein were mixed with sample buffer [10% sodium dodecylsulfate (SDS), 600 mM Tris–HCl, pH 6.8, 50% glycerol] containing 2-mercaptoethanol and 50 μg/ml bromophenol blue. Samples were boiled for 5 min and electrophoretically resolved on 10% SDS–polyacrylamide gels. Electrophoretically resolved proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk, membranes were incubated with a goat anti-rat COX-2 antibody (1:1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then a secondary antibody (donkey anti-goat IgG-HRP, 1:5000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were developed using enhanced chemiluminescence reagents (Perkin Elmer Life Sciences Inc., Boston, MA) and exposed to medical X-ray film (Kodak Company, Rochester, NY). Alpha-tubulin was used as an internal standard.

2.7. Statistical analysis

Tumor incidence among groups was compared by chi-squared test. The other results among groups were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test. Differences were considered statistically significant at $P < 0.05$.

3. Results

In experiment 1, there were no significant differences in body weight gain and daily feed intake in all groups. During the 5-week experiment, no clinical signs of toxicity were observed in any animals (data not shown).

As shown in Table 2, there were no ACF in the colons of rats injected with PBS (group 1.1); however, colonic ACF appeared in all rats injected with AOM (groups 1.2–1.5). Dehulled adlay at 10%, 20%, or 40% levels significantly reduced the number of ACF by 26–32% ($P < 0.05$) as compared to the control group (group 1.2). The total numbers of aberrant crypts were also significantly lower in groups fed dehulled adlay ($P < 0.05$). The crypt multiplicity (number of aberrant crypts/focus) was not affected by dehulled adlay, but the numbers of ACF consisting of 1 crypt as well as 4 or more crypts were significantly decreased in groups fed dehulled adlay. ACF were mainly observed in the middle and distal colons. Dehulled adlay significantly suppressed the formation of ACF in the middle colon ($P < 0.05$).

In experiment 2, the body weight gain of PBS-treated rats fed the 20% dehulled adlay diet (group 2.3) was significantly lower than that of rats fed the control diet (group 2.1) after 52 weeks of feeding. However, the body weight gains of AOM-treated rats (groups 2.4–2.6) were comparable (Table 3). Feed intake was not affected by dehulled adlay in PBS- or AOM-treated rats but was significantly decreased in rats treated with AOM and fed the control diet (group 2.4) as compared to group 2.1. The feed efficiency did not differ among groups. Dehulled adlay did not produce any gross changes attributable to toxicity in the liver, kidney, or lung.

As shown in Table 4, none of the PBS-treated rats on control or experimental diets developed colon tumors (groups 2.1–2.3). In AOM-treated rats, the tumor incidences were 64–79%. Dehulled adlay at the 20% level (group 2.6) reduced tumor incidence by 19%, but this was not statistically significant. Differences between the proximal and distal tumor incidence in dehulled adlay-fed rats were found. The tumor incidence was significantly lower in the proximal than in the distal colon in rats fed 20% dehulled adlay ($P < 0.05$). Dehulled adlay did not affect the tumor development (the multiplicity and volume of tumors) in total or in each section of the colon.

Regardless of diet, very low to undetectable levels of COX-2 protein were observed in PBS-treated rats (data not shown). A representative immunoblot analysis of COX-2 protein expression in the colonic mucosa and tumors of AOM-treated rats on different diets is shown in Fig. 1. The levels of COX-2 protein expression were increased in colon tumors as compared to those in their paired mucosa. The 20% dehulled adlay diet resulted in decreased expression of AOM-induced COX-2 both in proximal (Fig. 1A) and distal (Fig. 1B) colon tumors.

Table 2
Effect of dehulled adlay on AOM-induced ACF in the colon of male F344 rats[a,b]

	Experimental group[c]				
	1.1 C/PBS	1.2 C/AOM	1.3 10A/AOM	1.4 20A/AOM	1.5 40A/AOM
ACF incidence	0/6	8/8	8/8	8/8	8/8
Number of ACF	0	66 ± 18^{e}	40 ± 15^{f}	45 ± 15^{f}	45 ± 19^{f}
Number of aberrant crypts	0	117 ± 29^{e}	69 ± 29^{f}	77 ± 27^{f}	75 ± 38^{f}
Crypt multiplicity[d]	0	1.8 ± 0.1	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.1
Number of foci containing					
1 crypt	0	33 ± 12^{e}	18 ± 6^{f}	21 ± 9^{f}	22 ± 6^{f}
2 crypts	0	22 ± 6	15 ± 7	18 ± 6	16 ± 10
3 crypts	0	6 ± 3	5 ± 3	4 ± 3	5 ± 5
$\geqslant 4$ crypts	0	5 ± 1^{e}	2 ± 2^{f}	2 ± 2^{f}	1 ± 1^{f}
ACF distribution					
Proximal colon	0	5 ± 10	1 ± 1	1 ± 1	0 ± 1
Middle colon	0	38 ± 9^{e}	24 ± 13^{f}	24 ± 16^{f}	20 ± 12^{f}
Distal colon	0	$23 \pm 8^{e,f}$	16 ± 7^{f}	$21 \pm 7^{e,f}$	25 ± 10^{e}

[a] Values except for ACF incidence (number of rats with ACF/total rats) are means ± SD; $n = 6$ for group 1.1 and $n = 8$ for the other groups.
[b] Values in a row without a common letter are significantly different, $P < 0.05$.
[c] C/PBS, control diet and PBS injection; C/AOM, control diet and AOM injection; 10A/AOM, 10% adlay diet and AOM injection; 20A/AOM, 20% adlay diet and AOM injection; 40A/AOM, 40% adlay diet and AOM injection.
[d] Number of aberrant crypts/focus.

Table 3
Effect of dehulled adlay on body weight, food intake, and food efficiency in male F344 rats[a,b]

	Experimental group[c]					
	2.1 C/PBS	2.2 10A/PBS	2.3 20A/PBS	2.4 C/AOM	2.5 10A/AOM	2.6 20A/AOM
Body weight						
Initial (g)	115 ± 10	115 ± 10	115 ± 10	113 ± 10	114 ± 10	116 ± 7
Final (g)	465 ± 31	445 ± 29	424 ± 37	429 ± 51	438 ± 35	424 ± 48
Gain (g/d)	0.97 ± 0.08[e]	0.92 ± 0.06[e,f]	0.86 ± 0.10[f]	0.88 ± 0.13[e,f]	0.90 ± 0.09[e,f]	0.86 ± 0.13[f]
Feed intake (g/d)	19.8 ± 0.5[e]	18.4 ± 0.1[e,f]	19.0 ± 0.8[e,f]	17.8 ± 0.4[f]	18.7 ± 1.4[e,f]	18.7 ± 0.4[e,f]
Feed efficiency[d] (%)	4.9 ± 0.4	5.0 ± 0.3	4.5 ± 0.5	5.0 ± 0.7	4.8 ± 0.4	4.6 ± 0.7

[a] Values are means ± SD; $n = 6$ for group 2.1–2.3 and $n = 14$ for group 2.4–2.6.
[b] Values in a row without a common letter are significantly different, $P < 0.05$.
[c] C/PBS, control diet and PBS injection; 10A/PBS, 10% adlay diet and PBS injection; 20A/PBS, 20% adlay diet and PBS injection; C/AOM, control diet and AOM injection; 10A/AOM, 10% adlay diet and AOM injection; 20A/AOM, 20% adlay diet and AOM injection.
[d] Weight gain/feed intake × 100%.

Table 4
Effect of dehulled adlay on AOM-induced colon tumors in male F344 rats[a]

	Experimental group[b]					
	2.1 C/PBS	2.2 10A/PBS	2.3 20A/PBS	2.4 C/AOM	2.5 10A/AOM	2.6 20A/AOM
Tumor incidence						
Total colon	0	0	0	79%	79%	64%
Proximal colon	0	0	0	36%	29%	14%[d]
Distal colon	0	0	0	57%	64%	57%
Tumor multiplicity (n)[c]						
Total colon	0	0	0	1.4 ± 0.9	1.4 ± 0.5	1.2 ± 0.4
Proximal colon	0	0	0	1.4 ± 0.9	1.0 ± 0	1.0 ± 0
Distal colon	0	0	0	1.0 ± 0	1.2 ± 0.4	1.1 ± 0.4
Tumor volume (mm^3)						
Total colon	0	0	0	152 ± 229	144 ± 141	166 ± 130
Proximal colon	0	0	0	139 ± 157	105 ± 111	240 ± 11
Distal colon	0	0	0	164 ± 289	158 ± 153	149 ± 140

[a] Values except for tumor incidence (percentage of rats with tumors) are means ± SD; $n = 6$ for group 2.1–2.3 and $n = 14$ for group 2.4–2.6.
[b] C/PBS, control diet and PBS injection; 10A/PBS, 10% adlay diet and PBS injection; 20A/PBS, 20% adlay diet and PBS injection; C/AOM, control diet and AOM injection; 10A/AOM, 10% adlay diet and AOM injection; 20A/AOM, 20% adlay diet and AOM injection.
[c] Tumors/tumor-bearing rat.
[d] Values are significantly different from that in the distal colon, $P < 0.05$.

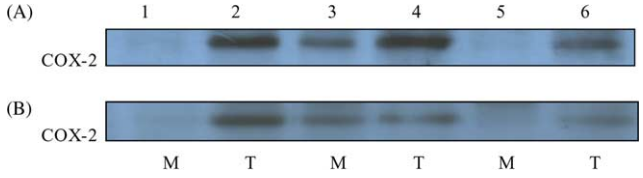

Fig. 1. Effect of dehulled adlay on COX-2 protein expression of mucosa and tumors in the proximal (A) and distal (B) colons of rats treated with AOM. Male F344 rats were grouped and fed different diets throughout the experimental period. After 1 week of feeding, rats were injected intraperitoneally with AOM once weekly for 2 weeks at a dose level of 20 mg/kg body weight, and rats for vehicle treatment were injected with PBS. Colons were obtained 51 weeks after the first injection, and the COX-2 protein expression of colonic mucosa (M) and tumors (T) was examined by immunoblotting as described in Section 2. Lanes 1 and 2, control diet; Lanes 3 and 4, 10% dehulled adlay; Lanes 5 and 6, 20% dehulled adlay.

4. Discussion

To our understanding, this is the first study to examine the short- and long-term effects of adlay on colon carcinogenesis. The results demonstrated that administration of dehulled adlay suppressed AOM-induced ACF development and, to some extent, colon tumor formation in rats. We have also shown for the first time that dehulled adlay reduced AOM-induced COX-2 protein expression in rat colon tumors. ACF have been accepted as preneoplastic markers in a number of investigators and are used extensively to identify modulators of colon carcinogenesis (Bird and Good, 2000). Many different ACF parameters have been measured, including the number of ACF, the number of aberrant crypts (AC), ACF size, ACF multiplicity, the

number of ACF per length or per square centimeter of the colon, and the degree of luminal alterations of ACF (Jenab et al., 2001). Although it is still unclear which of these ACF parameters offers the best estimate of future colon cancer risk, most studies use the number of total ACF per colon, the number of large ACF per colon, and the number of crypts per ACF (ACF multiplicity) to evaluate potential colon cancer chemopreventive agents (Corpet and Tache, 2002). Dehulled adlay significantly reduced both the numbers of ACF and AC, but no dose effect was observed in groups fed 10, 20, and 40% dehulled adlay (Table 2). This suggests that dehulled adlay is able to exert a chemopreventive effect on preneoplastic ACF even at a low dose. Tsukamoto et al. (1999) illustrated the development of ACF by a fission mechanism. It is evident that the more crypts a focus has, the more advanced it is. The decrease in numbers of ACF consisting of various numbers of crypts suggests that dehulled adlay may inhibit the growth of ACF.

In the large intestine of F344 rats, there is no distinct demarcation of the colon into ascending, transverse, and descending segments (Elsayed and Shamsuddin, 1990), so we divided the colon into three equal parts (the proximal, middle, and distal colons) to examine the effect of dehulled adlay on the distribution of ACF. It is interesting to note that dehulled adlay not only reduced the number of ACF in the middle colon but also altered the distribution of ACF in the entire colon (Table 2). In rats fed the control diet (group 1.2), ACF appeared mainly in the middle colon (59% of total ACF), with a few ACF (35%) in the distal and very few (6%) in the proximal colon. The percentage of ACF in the middle colon decreased with an increase in dosage of dehulled adlay. In contrast, the percentage of ACF in the distal colon increased with the increase in dosage of dehulled adlay. This does not mean that dehulled adlay enhanced the formation of ACF in the distal colon because the numbers of ACF in the distal colon were comparable in control and dehulled adlay-fed groups. ACF develop as early as 2–4 weeks after carcinogen administration (Caderni et al., 1995) and appear predominantly in the distal colon during early time points; as time progresses, ACF appear in the proximal colon, and a proportion of ACF start to exhibit focal expansion and may contain one to several crypts (Bird, 1998). In the present study, colons were examined for ACF 4 weeks after the first injection of AOM. Data from ACF incidence indicated that all rats treated with AOM developed ACF; data from ACF growth features indicated that there were many large ACF in the colon. In addition, dehulled adlay did not affect the number of ACF in the distal colon but actually suppressed the formation of ACF in the middle colon. Taken together, our results suggest that dehulled adlay may intervene in the development of ACF at later time points, i.e., the post-initiation or promotion stage.

The short-term experiment demonstrated that dehulled adlay at low doses was able to inhibit the growth of ACF, so we further investigated the long-term effect of dehulled adlay at 10% and 20% levels on colon carcinogenesis. Dehulled adlay at the 20% level reduced body weight gain but did not produce any clinical signs of toxicity after 52 weeks of feeding. AOM injection reduced the feed intake of rats fed the control diet while the feed intake was not affected in rats fed dehulled adlay (Table 3). Cameron et al. (1997) also observed that the daily caloric intake was significantly lower in carcinogen-treated rats. It is possible that the appetite of rats decreases due to AOM carcinogenicity and the consequent tumor burden, and dehulled adlay may improve this phenomenon. The relative rarity of spontaneous epithelial tumors of the colon in experimental animals provides a rationale to use chemically induced experimental models of colon carcinogenesis (Reddy, 1998). Using this model, the present study showed that the tumor incidences were 64–79%, consistent with those in other studies (Corpet and Tache, 2002). Dehulled adlay at the 20% level reduced the tumor incidence in the whole colon by 19% and in the proximal colon by 61%. Although striking, this effect was not found to be significant at the $P < 0.05$ level, in part due to the small number and/or the low incidence of tumors in experimental animals, as reported by Beaty et al. (1993). The multiplicity and volume of colon tumors were not different in all groups mainly due to the small number of tumors and the large individual difference. Similar to the ACF data, dehulled adlay caused different effects in different sections of the colon. In dehulled adlay-fed rats, both the tumor incidence and multiplicity in the proximal colon tended to be lower. Exon and South (2003) indicated that the distribution of ACF in the colons of older rats was shifted more to the proximal region as opposed to that in younger animals, and this appears to mimic the shift in distribution of cancer in the aging human colon. In addition, a slow change in the distribution of colon cancer, with a shift to the proximal, has been reported in some high-incidence countries during the past two decades (Hermanek, 2002). Proximal colon tumors in humans are more silent and clinically inapparent and are diagnosed later (Holt et al., 1996). The predominantly proximal effect of dehulled adlay suggests that it may be potentially protective of colon carcinogenesis in spite of the necessity of further investigation with a larger sample size.

The role of COX-2 as an enhancer of carcinogenesis in many organs including the colon is receiving increasing attention. Therefore, assays of COX-2 expression may be used to monitor the process of carcinogenesis, and the suppression of COX-2 expression has become a target for cancer chemoprevention (Singh et al., 1997; Tanaka et al., 2001). In the present study, the expression of COX-2 protein in colon tumors was

higher than that in colonic mucosa, suggesting that this differential expression of COX-2 protein is closely related to events leading to the development of colon tumors. The effect of dehulled adlay on COX-2 protein expression was dependent on the dose of dehulled adlay and the subsite of the tumors. In the proximal colon, the expression of COX-2 protein in tumors was slightly suppressed by 20% dehulled adlay but not by 10% dehulled adlay. This trend in COX-2 protein expression is consistent with that in tumor incidence, suggesting that the suppression of COX-2 protein expression is related to the inhibition of tumor formation. In contrast, no relationship was observed between COX-2 protein expression and either the incidence or the multiplicity of tumors in the distal colon. The regional distribution of COX-2 expression in the colorectal tumors is controversial. Dimberg et al. (1999) reported a significant prevalence of up-regulated COX-2 in cancerous tissue originating from the rectum in relation to tumors of colonic origin. In contrast, Wiese et al. (2003) found that the rectum shows lower in COX-2 expression, as opposed to the colon, and rectal tumors may be less dependent on COX-2 expression than tumors of the colon are. Roy et al. (2001) detected considerably greater COX-2 in the distal as opposed to the proximal colon. These studies suggest that subsites of origin within the large intestine may be subject to different initiating events, leading to various phenotypes in tumors originating in different sites. Therefore, regional disparity within the large intestine may be one cause of the discrepancy between COX-2 expression and tumor outcome.

Several lines of evidence suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) are protective against colon cancer and that COX-2 is a major molecular target for chemoprevention (Dannenberg and Zakim, 1999; Fournier and Gordon, 2000). However, there is no evidence showing that NSAIDs can cause the regression or cure of colon tumors (Ota et al., 2002). Results from this study show that adlay fails to inhibit the formation of malignant colon tumors. This observation is in agreement with studies on the action of NSAIDs. Induction of COX-2 is an early event during colon carcinogenesis especially at the adenoma stage (Singh et al., 1997; Ota et al., 2002). In the present study, rats were sacrificed up to 51 weeks after the first AOM injection, and colon tumors were examined by the histopathological method. All tumors examined were carcinomas, which represent the advanced phase in the adenoma–carcinoma sequence. It appears that dehulled adlay, like NSAIDs, interrupts the development of colon tumors at a rather early stage but becomes ineffective at later stage, in particular at the malignant stage.

The expression of COX-2 protein in colonic mucosa seemed to be increased in rats fed 10% dehulled adlay (Fig. 1). In fact, this phenomenon existed only in a few animals. It is well accepted that COX-2 protein levels are elevated in carcinogen-induced rodent colon tumors (DuBois et al., 1996; Singh et al., 1997) and in most human colorectal tumors (Bamba et al., 1999; Chen et al., 2001). However, the expression of COX-2 protein in normal mucosa varies from study to study. Singh et al. (1997) reported increasingly high levels of AOM-induced COX-2 in the colonic mucosa of rats. In contrast, Shao et al. (1999) indicted that COX-2 protein was not detectable in normal mucosa in the colon of AOM-treated rats. The conflicting results on the detection of COX-2 in mucosa were also observed in human colorectal tissues (Bamba et al., 1999; Dimberg et al., 1999; Chen et al., 2001; Elder et al., 2002; Zhang and Sun, 2002; Wiese et al., 2003). Some studies suggest that COX-2 is overexpressed in endocrine cells of normal colorectal mucosa and that COX-2 is expressed in interstitial tissue during the early period of carcinogenesis, and as cancer spreads, it is expressed in cancer cells and epithelial cells (Sakuma et al., 1999; Fujita et al., 2000). Based on these studies, we suggest that the altered COX-2 level is possibly due to the existence of preneoplastic or inflammatory cells in the neighboring normal mucosa.

Adlay is a natural food containing many biologically active components. Some studies indicated that the components with anti-tumor potential are in lipid-associated fraction of adlay (Tanimura, 1961; Tokuda et al., 1990; Numata et al., 1994). The action of lipid on colon cancer is mediated by changing prostaglandin as well as bile acid/cholesterol metabolism (Davis and Iwahashi, 2001). We have previously reported that inclusion of adlay in the diet increased the number of fecal lactic acid bacteria (generally considered beneficial to health) while decreasing the number of enterics (opportunistic pathogens) in rats (Chiang et al., 2000a). We also found that rats fed with adlay had higher concentrations of short-chain fatty acids in the gastrointestinal tract. These effects may be due to fermentation of dietary fiber. Butyrate, a fermentation product of dietary fiber, may be protective of tumor development because it appears to have contrasting effects on proliferation, differentiation, and apoptosis in normal and neoplastic tissues (Zoran et al., 1997). Nevertheless, the role of dietary fiber in prevention of colon cancer is still controversial. Poorly fermented fiber, such as wheat bran, is protective against colon cancer, whereas the readily fermented fiber, such as oat bran, is not protective and may possibly promotive of tumor development (Cameron et al., 1997; Zoran et al., 1997). Whether these components of adlay would regulate the ACF development and COX-2 expression and thus suppress the colon carcinogenesis remains to be investigated.

In conclusion, the present study demonstrated that dietary dehulled adlay had a modulating effect on chemically induced colon carcinogenesis. The

preneoplastic ACF were significantly suppressed by dehulled adlay, especially in the middle colon. COX-2 protein expression in colon tumors was also suppressed by dehulled adlay; nevertheless, tumors were not inhibited. The inconsistent effects between COX-2 protein expression and tumor outcome may be due to regional differences within the colon and the malignancy of tumors. These findings suggest that dehulled adlay suppresses early events in colon carcinogenesis but not the formation of tumors. Further investigation is needed to elucidate the active component of dehulled adlay and the mechanism of action.

Acknowledgements

This study was supported by grants "NSC 89-2316-B-002-031" and "NSC 90-2316-B-002-008" from the National Science Council, Taipei, Taiwan.

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