[15] J.F. Ruiz-Rabelo, R. Vázquez, M.D. Perea, A. Cruz, R. González, A. Romero, M.C. Muñoz-Villanueva, I. Túnez, P. Montilla, J. Muntané, F.J. Padillo, Beneficial properties of melatonin in an experimental model of pancreatic cancer, J. Pineal Res. 43 (2007) 270–275.
[16] M. Karbownik, A. Lewinski, R.J. Reiter, Anticarcinogenic actions of melatonin which involve antioxidative processes: comparison with other antioxidants, Int. J. Biochem. Cell. Biol. 33 (2001) 735–753.
[17] S.A. Musatov, V.N. Anisimov, V. André, C. Vigreux, T. Godard, F. Sichel, Effects of melatonin on N-nitroso-N-methylurea-induced carcinogenesis in rats and mutagenesis in vitro (Ames test and COMET assay), Cancer Lett. 138 (1999) 37–44.
[18] D.E. Blask, R.T. Dauchy, L.A. Sauer, J.A. Krause, Melatonin uptake and growth prevention in rat hepatoma 7288CTC in response to dietary melatonin: melatonin receptor-mediated inhibition of tumor linoleic acid metabolism to the growth signaling molecule 13-hydroxyoctadecadienoic acid and the potential role of phytomelatonin, Carcinogenesis 25 (2004) 951–960.
[19] D.E. Blask, R.T. Dauchy, L.A. Sauer, Putting cancer to sleep at night: the neuroendocrine/circadian melatonin signal, Endocrine 27 (2005) 179–188.
[20] W.G. Dong, Q. Mei, J.P. Yu, J.M. Xu, L. Xiang, Y. Xu, Effects of melatonin on the expression of iNOS and COX-2 in rat models of colitis, World J. Gastroenterol. 9 (2003) 1307–1311.
[21] J.H. Li, J.P. Yu, H.G. Yu, X.M. Xu, L.L. Yu, J. Liu, H.S. Luo, Melatonin reduces inflammatory injury through inhibiting NF-kappaB activation in rats with colitis, Mediators Inflamm. 2005 (2005) 185–193.
[22] J.C. Mayo, R.M. Sainz, D.X. Tan, R. Hardeland, J. Leon, C. Rodriguez, R.J. Reiter, Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages, J. Neuroimmunol. 165 (2005) 139–149.
[23] WHO Media Centre, Cancer, Fact sheet No. 297, WHO, 2006.
[24] F. Balkwill, A. Mantovani, Inflammation and cancer: back to Virchow? Lancet 357 (2001) 539–545.
[25] D.T. Rubin, N. Parekh, Colorectal cancer in inflammatory bowel disease: molecular and clinical considerations, Curr. Treat. Opt. Gastroenterol. 9 (2006) 211–220.
[26] T. Tanaka, H. Kohno, R. Suzuki, Y. Yamada, S. Sugie, H. Mori, A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate, Cancer Sci. 94 (2003) 965–973.
[27] R. Suzuki, H. Kohno, S. Sugie, H. Nakagama, T. Tanaka, Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice, Carcinogenesis 27 (2006) 162–169.
[28] R. Suzuki, H. Kohno, S. Sugie, T. Tanaka, Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate, Cancer Sci. 95 (2004) 721–727.
[29] R. Suzuki, S. Miyamoto, Y. Yasui, S. Sugie, T. Tanaka, Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate, BMC Cancer 7 (2007) 84.
[30] H. Kohno, R. Suzuki, M. Curini, F. Epifano, F. Maltese, S.P. Gonzales, T. Tanaka, Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice, Int. J. Cancer 118 (2006) 2936–2942.
[31] H. Kohno, R. Suzuki, Y. Yasui, S. Miyamoto, K. Wakabayashi, T. Tanaka, Ursodeoxycholic acid versus sulfasalazine in colitis-related colon carcinogenesis in mice, Clin. Cancer Res. 13 (2007) 2519–2525.
[32] H. Kohno, R. Suzuki, S. Sugie, T. Tanaka, Beta-Catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate, Cancer Sci. 2005 (2005) 69–76.
[33] T. Tanaka, R. Suzuki, H. Kohno, S. Sugie, M. Takahashi, K. Wakabayashi, Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase, Carcinogenesis 26 (2005) 229–238.
[34] T. Tanaka, H. Kohno, R. Suzuki, K. Hata, S. Sugie, N. Niho, K. Sakano, M. Takahashi, K. Wakabayashi, Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms, Int. J. Cancer 118 (2006) 25–34.
[35] H. Ohshima, H. Tazawa, B.S. Sylla, T. Sawa, Prevention of human cancer by modulation of chronic inflammatory processes, Mutat. Res. 591 (2005) 110–122.
[36] R. D'Inca, R. Cardin, L. Benazzato, I. Angriman, D. Martines, G.C. Sturniolo, Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia, Inflamm. Bowel Dis. 10 (2004) 23–27.
[37] E.P. Chan, G.R. Lichtenstein, Chemoprevention: risk reduction with medical therapy of inflammatory bowel disease, Gastroenterol. Clin. N. Am. 35 (2006) 675–712.
[38] Y.M. Cho, T. Imai, Y. Ota, M. Hasumura, S. Takami, M. Hirose, A. Nishikawa, A new medium-term rat colorectal bioassay applying neoplastic lesions as end points for detection of carcinogenesis modifiers effects with weak or controversial modifiers, Toxicol. Pathol. 36 (2008) 459–464.
[39] T. Tanaka, H. Kohno, S. Yoshitani, S. Takashima, A. Okumura, A. Murakami, M. Hosokawa, Ligands for peroxisome proliferator-activated receptors alpha and gamma inhibit chemically induced colitis and formation of aberrant crypt foci in rats, Cancer Res. 61 (2001), 2424–2428.
[40] N.B. Janakiram, C.V. Rao, Molecular markers and targets for colorectal cancer prevention, Acta Pharmacol. Sin. 29 (2008) 1–20.
[41] Y. Yasui, M. Kim, T. Tanaka, Colorectal carcinogenesis and suppression of tumor development by inhibition of enzymes and molecular targets, Curr. Enzyme Inhib., (2009) in press.
[42] J.M. Ward, Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats, Lab. Invest. 30 (1974) 505–513.
[43] Y. Yasui, R. Suzuki, S. Miyamoto, T. Tsukamoto, S. Sugie, H. Kohno, T. Tanaka, A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis, Int. J. Cancer 121 (2007) 2331–2339.
[44] V.N. Anisimov, I.G. Popovich, M.A. Zabezhinski, S.V. Anisimov, G.M. Vesnushkin, I.A. Vinogradova, Melatonin as antioxidant, geroprotector and anticarcinogen, Biochim. Biophys. Acta 1757 (2006) 573–589.
[45] G. Sener, J. Balkan, U. Cevikbaş, M. Keyer-Uysal, M. Uysal, Melatonin reduces cholesterol accumulation and prooxidant state induced by high cholesterol diet in the plasma, the liver and probably in the aorta of C57BL/6J mice, J. Pineal Res. 36 (2004) 212–216.
[46] B.B. Aggarwal, S. Shishodia, S.K. Sandur, M.K. Pandey, G. Sethi, Inflammation and cancer: how hot is the link? Biochem. Pharmacol. 72 (2006) 1605–1621.
[47] W.G. Deng, S.T. Tang, H.P. Tseng, K.K. Wu, Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding, Blood 108 (2006) 518–524.
[48] H. Kohno, R. Suzuki, S. Sugie, T. Tanaka, Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands, BMC Cancer 5 (2005) 46.
[49] B.B. Aggarwal, S. Shishodia, Molecular targets of dietary agents for prevention and therapy of cancer, Biochem. Pharmacol. 71 (2006) 1397–1421.
[50] M. Dong, K. Guda, P.R. Nambiar, A. Rezaie, G.S. Belinsky, G. Lambeau, C. Giardina, D.W. Rosenberg, Inverse association between phospholipase A2 and COX-2 expression during mouse colon tumorigenesis, Carcinogenesis 24 (2003) 307–315.
[51] M.S. Inan, R. Place, V. Tolmacheva, Q.S. Wang, A.K. Hubbard, D.W. Rosenberg, C. Giardina, IkappaBbeta-related proteins in normal and transformed colonic epithelial cells, Mol. Carcinog. 29 (2000) 25–36.
[52] M.S. Inan, V. Tolmacheva, Q.S. Wang, D.W. Rosenberg, C. Giardina, Transcription factor NF-kappaB participates in regulation of epithelial cell turnover in the colon, Am. J. Physiol. Gastrointest. Liver Physiol. 279 (2000) G1282–G1291.
[53] X. Tong, L. Yin, R. Washington, D.W. Rosenberg, C. Giardina, The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation, Mol. Cell. Biochem. 265 (2004) 171–183.
[54] T. Kusaba, T. Nakayama, K. Yamazumi, Y. Yakata, A. Yoshizaki, T. Nagayasu, I. Sekine, Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma, correlation with clinicopathological factors, J. Clin. Pathol. 58 (2005) 833–838.
[55] J.I. Fenton, S.D. Hursting, S.N. Perkins, N.G. Hord, Interleukin-6 production by leptin treatment promotes cell proliferation in an Apc(Min/+) colon epithelial cell line, Carcinogenesis 27 (2006) 1507–1515.
[56] J.H. Li, W. Zhou, K. Liu, H.X. Li, L. Wang, Melatonin reduces the expression of chemokines in rat with trinitrobenzene sulfonic acid-induced colitis, Saudi Med. J. 29 (2008) 1088–1094.
[57] Y. Ohta, M. Kongo-Nishimura, Y. Imai, T. Matsura, A. Kitagawa, K. Yamada, alpha-Tocopherol protects against alpha-naphthylisothiocyanate-induced hepatotoxicity in rats less effectively than melatonin, Chem. Biol. Interact. 131 (2006) 115–124.
[58] Y. Ohta, M. Kongo-Nishimura, Y. Imai, A. Kitagawa, Melatonin attenuates disruption of serum cholesterol status in rats with a single alpha-naphthylisothiocyanate treatment, J. Pineal Res. 42 (2007) 159–165.
[59] S. Nishida, T. Segawa, I. Murai, S. Nakagawa, Long-term melatonin administration reduces hyperinsulinemia and improves the altered fatty-acid compositions in type 2 diabetic rats via the restoration of Delta-5 desaturase activity, J. Pineal Res. 32 (2002) 26–33.
[60] S. Sanchez-Mateos, C. Alonso-Gonzalez, A. Gonzalez, C.M. Martinez-Campa, M.D. Mediavilla, S. Cos, E.J. Sanchez-Barcelo, Melatonin and estradiol effects on food intake, body weight, and leptin in ovariectomized rats, Maturitas 58 (2007) 91–101.
[61] M.R. Hussein, O.G. Ahmed, A.F. Hassan, M.A. Ahmed, Intake of melatonin is associated with amelioration of physiological changes, both metabolic and morphological pathologies associated with obesity: an animal model, Int. J. Exp. Pathol. 88 (2007) 19–29.

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Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice

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Zerumbone (ZER), present in subtropical ginger *Zingiber zerumbet* Smith, possesses anti-growth and anti-inflammatory properties in several human cancer cell lines. ZER also down-regulates the cyclooxygenase-2 and inducible nitric oxide synthase expression *via* modulation of nuclear factor (NF)-κB activation in cell culture systems. These findings led us to investigate whether ZER is able to inhibit carcinogenesis in the colon and lung, using 2 different preclinical mouse models. In Exp. 1, a total of 85 male ICR mice were initiated using a single intraperitoneal (i.p.) injection with azoxymethane (AOM, 10 mg/kg bw) and promoted by 1.5% dextran sulfate sodium (DSS) in drinking water for 7 days for rapid induction of colonic neoplasms. Animals were then fed the diet containing 100, 250 or 500 ppm ZER for 17 weeks. In Exp. 2, a total of 50 female A/J mice were given a single i.p. injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (10 μmol/mouse) to induce lung proliferative lesions. They were then fed the diet mixed with 100, 250 or 500 ppm ZER for 21 weeks. At the termination of the experiments (wk 20 of Exp. 1 and wk 22 of Exp. 2), all animals were subjected to complete necropsy examination to determine the pathological lesions in both tissues. Oral administration of ZER at 100, 250 and 500 ppm significantly inhibited the multiplicity of colonic adenocarcinomas. The treatment also suppressed colonic inflammation. In the lung carcinogenesis, ZER feeding at 250 and 500 ppm significantly inhibited the multiplicity of lung adenomas in a dose-dependent manner. Feeding with ZER resulted in inhibition of proliferation, induction of apoptosis, and suppression of NFκB and heme oxygenase (HO)-1 expression in tumors developed in both tissues. Our findings suggest that dietary administration of ZER effectively suppresses mouse colon and lung carcinogenesis through multiple modulatory mechanisms of growth, apoptosis, inflammation and expression of NFκB and HO-1 that are involved in carcinogenesis in the colon and lung.

Key words: zerumbone; colon; lung; carcinogenesis; chemoprevention

A sesquiterpenoid, zerumbone (ZER), is a major constituent of the subtropical ginger plant *Zingiber zerumbet* Smith. The essential oil of the rhizomes contains large amount of ZER and is used as an anti-inflammatory medicine.[1] Recent studies revealed several biological properties of ZER that may be responsible for inhibition of carcinognesis. They include suppression of skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation in Raji cells,[1] inhibition of free radical generation, inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression, inhibition of tumor necrosis factor (TNF)-α-release in activated leukocytes and induction of apoptosis in human colonic adenocarcinoma (ADC) cell lines.[2] *In vivo* studies demonstrated that dietary feeding with ZER markedly suppressed dextran sulfate sodium (DSS)-induced acute colitis in mice[3] and a putative precursor lesions for colonic ADC, aberrant crypt foci (ACF), produced by azoxymethane (AOM) in rat colon.[4] The findings were accompanied by reductions of prostaglandin E_2 and COX-2 protein expression in colonic mucosa.[3,4] Additionally, ZER suppresses the combined lipopolysaccharide- and interferon-γ-induced IκB protein degradation in macrophages.[5] More recently, Takada *et al.*[6] reported that ZER suppresses nuclear factor (NF)-κB activation induced by TNF, okadaic acid, cigarette smoke condensate, TPA and H_2O_2.

Colorectal cancer (CRC) and lung cancer are major epithelial malignancies and both are increasing in developed countries. An association between inflammation and cancer has long been suspected[7] and inflammatory condition is a risk for cancer development in colon and lung.[8–12] A representative example is that inflamed colon has a high risk for CRC development. In patients with inflammatory bowel disease, including ulcerative colitis and Crohn's disease, the risk of CRC development is greater than in the general population.[13] Smoking is a risk factor of development of different types of cancer in different tissues, including lung[14] and colon.[15] Also, inflammation caused by tobacco enhances lung carcinogenesis.[11] Despite well-developed diagnostic and therapeutic techniques, and novel anti-cancer drugs against both malignancies have been introduced, mortality rates of CRC and lung cancer have not remarkably been improved. Therefore, we need some new weapons and strategies for fighting against these malignancies. Cancer chemoprevention is one of such strategies. For clinical use of candidate cancer chemopreventive agents, they need to be determined preclinical efficacy using appropriate animal carcinogenesis models. As to inflammation-associated colon carcinogenesis, we have developed a mouse model utilizing a colon carcinogen AOM and a colitis-inducing agent DSS.[16] 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced mouse lung tumorigenesis model is frequently used for carcinogenesis and chemoprevention studies, as NNK is a tobacco-specific carcinogenic nitrosamine, which derived from nicotine.[14,17] Hecht *et al.*[18] developed a relatively rapid single-dose model for induction of lung adenomas (ADs) in female A/J mice initiated with NNK. The fact that nonsteroidal anti-inflammatory drugs can inhibit NNK-induced lung tumors[19] suggests that inflammation is involved in NNK-induced lung tumorigenesis.

In the current study, we investigated the chemopreventive ability of ZER in colon and lung carcinogenesis using a mouse colitis-related CRC model[16] and a NNK-induced mouse lung carcinogenesis model.[18] Also, the effects of ZER on the immunohistochemical

Abbreviations: ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; ARE, antioxidant response element; CRC, colorectal cancer; DAB, 3,3'-diaminobenzidine; DSS, dextran sulfate sodium; GPx, γ-glutamylcystein glutathione peroxidase; HO, heme oxygenase; H&E, hematoxylin and eosin; HP, hyperplasia; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NFκB, nuclear factor-kappaB; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NrF, nuclear factor-erythroid 2-related factor; PCNA, proliferating cell nuclear antigen (PCNA); TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TUNEL, TdT-mediated dUTP nick-end labeling; ZER, zerumbone.

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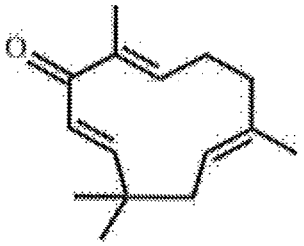

FIGURE 1 – Chemical structure of zerumbone (2,6,9,9-tetramethyl-[2*E*,6*E*,10*E*]-cycloundeca-2,6-10-trien-1-one, MW 218, purity>95%).

expression of heme oxygenase (HO)-1 and NFκB in colonic and lung neoplasms were examined, since the expression of these molecules are involved in carcinogenesis and inflammation[20–22] and certain suppressors of these molecules' expression are candidates for cancer chemopreventive agents.[23,24]

Material and methods

Chemicals

ZER (2,6,9,9-tetramethyl-[2*E*,6*E*,10*E*]-cycloundeca-2,6-10-trien-1-one, MW 218, purity > 95%, Fig. 1) was isolated and purified from a chloroform extract of the rhizomes of *Z. zerumbet* Smith, as previously reported.[1]

AOM was purchased from SIGMA-ALDRICH (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 (Cat. no. 160110) was obtained from MP Biomedicals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 1.5% (*w/v*) just before it was used. NNK was obtained from Toronto Research Chemical Inc. (Ontario, Canada).

Animals and diets

The animal experiments were approved by the Committee of the Institutional Animal Experiments and were conducted at the Kanazawa Medical University Animal Facility under the Institutional Animal Care Guideline. Five-week-old male ICR mice for Exp. 1 (CRC chemoprevention study) and 5-week-old female A/J mice for Exp. 2 (lung cancer chemoprevention study) were purchased from Charles River Laboratories, Inc. (Tokyo, Japan). All animals were housed in plastic cages (5 mice/cages) and had free access to drinking water and a basal diet (CRF-1, Oriental Yeast, Co., Ltd., Tokyo, Japan) *ad libitum*, under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2 C). After arrival, they were quarantined for the first 7 days, and then randomized by their body weights into experimental and control groups. Experimental diets were prepared by mixing zerumone in powdered basal diet CRF-1 at dose levels (w/w) of 100, 250 and 500 ppm, based on our previous study.[4] We confirmed that ZER is quite stable in the diets (data not shown). Animals had access to food and water at all times. Cups were replenished with fresh food everyday. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

Exp. 1: Effect of ZER on AOM/DSS-induced colon carcinogenesis in mice

A total of 85 male ICR mice were divided into 7 experimental and control groups. They were given a single intraperitoneal (i.p.) injection with AOM (10 mg/kg, bw). Starting 7 days after the AOM injection, they received 1.5% (*w/v*) DSS in the drinking water for 7 days. Seven days later, ZER-containing diets were started and the mice continued on these experimental diets for 17 weeks. Experimental groups included group 1 (n = 20) that received AOM and DSS; groups 2–4 (n = 15 for each group) were treated with AOM, DSS and ZER (100 ppm for group 2, 250 ppm for group 3, and 500 ppm for group 4) in diet; group 5 (n = 5) was treated with DSS and ZER (500 ppm); group 6 (n = 5) was treated with DSS alone; group 7 (n = 5) was given the diet containing 500 ppm ZER alone; group 8 was an untreated group. All animals were subjected to a complete gross necropsy examination at the time of euthanasia (wk 20) to determine the incidence and multiplicity of tumors in the large bowel. At sacrifice, the large bowel was removed and macroscopically inspected for the presence of tumors. After measuring the colon length (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis and gently washed with saline. Fifteen mice in group 1, 10 each from groups 2–4, and 2 each from groups 5–8 were randomly selected for histopathology. They were cut along the vertical axis and then fixed in 10% buffered formalin for at least 24 hr. The colons of remaining animals were saved for future molecular analysis. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections made from paraffin-embedded blocks. Colitis was recorded and scored according to the following morphological criteria described by Cooper *et al.*[25]: Grade 0, normal colonic mucosa; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa: Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of all crypts with severe inflammation in the mucosa, but with the surface epithelium still remaining; and Grade 4, loss of all crypts and the surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. Colonic tumors were diagnosed according to the criteria described by Ward.[26]

Exp. 2: Effect of ZER on NNK-induced lung carcinogenesis

To determine the effect of ZER on lung carcinogenesis, a total of 50 female A/J mice were divided into 5 experimental and control groups. The mice were given a single i.p. injection with NNK (10 μmol/mouse). Seven days after the injection, they were fed the diets mixed with ZER at 3 different dose levels (100, 250 and 500 ppm) for 21 weeks. Experimental groups were: group 1 (n = 10) given NNK alone; Groups 2 (n = 10) given NNK and 100 ppm ZER; group 3 received NNK and 250 ppm ZER; group 4 (n = 10) treated with NNK and 500 ppm ZER; group 5 received 500 ppm ZER alone; and group 6 was an untreated control. All mice were subjected to a complete gross necropsy examination at the time of euthanasia (wk 22) to histopathologically investigate proliferative lesions, alveolar cell hyperplasia (HP) and neoplasms in the lung. At sacrifice, all lobes of the lung were removed, weighed and macroscopically inspected for the presence of tumors and/or nodules. They were fixed in 10% buffered formalin for at least 24 hr for histopathological examination on H&E-stained sections made from paraffin-embedded blocks. Lung proliferative lesions including HP and neoplasms were diagnosed according to the criteria Nikitin *et al.*[27]

Immunohistochemistry of proliferating cell nuclear antigen (PCNA), apoptotic nuclei, NF-κB and HO-1

Immunohistochemical analysis for the PCNA, NF-κB and HO-1-positive neoplastic cells in the colon and lung was performed on 4-μm-thick paraffin-embedded sections by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65 C, de-paraffinized in xylene and rehydrated through grade ethanol at room temperature. Tris HCl buffer (0.05 M, pH 7.6) was used to prepare the solutions and was used for washes between the various steps. Incubations were performed in a humidified chamber.

The sections were treated for 40 min at room temperature with 2% bovine serum albumin, and incubated overnight at 4 C with primary antibodies. The primary antibodies included anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1000 dilution; DAKO Japan), anti-HO-1 rabbit polyclonal antibody (OSA-150C, diluted 1:200, StressGen Biotechnologies, Ann Arbor, MI)

TABLE I – EFFECTS OF DIETARY ZEROMBONE ON COLONIC INFLAMMATION AND DEVELOPMENT OF MUCOSAL ULCER AND HIGH GRADE DYSPLASIA (EXP. 1)

Group no.	Treatment	No. of mice examined	Inflammatory score	Number of mucosal ulcer	No. of dysplasia (high grade)
1	AOM[1]/DSS	15	1.80 ± 0.77[2]	2.00 ± 1.00	1.80 ± 1.01
2	AOM/DSS + 100 ppm ZER	10	0.80 ± 0.63[3]	1.20 ± 0.63	1.70 ± 1.49
3	AOM/DSS + 250 ppm ZER	10	0.50 ± 0.71[4]	0.60 ± 0.84[3]	1.00 ± 1.63
4	AOM/DSS + 500 ppm ZER	10	0.30 ± 0.48[4]	0.50 ± 0.71[4]	1.10 ± 1.66
5	DSS + 500 ppm ZER	5	2.50 ± 0.71	1.50 ± 0.71	0
6	DSS	5	4.00 ± 0.00	4.50 ± 0.71	0
7	500 ppm ZER	5	0	0	0
8	Untreated	5	0	0	0

[1]AOM, azoxymethane; DSS, dextran sulfate sodium; and ZER, zerumbone.–[2]Mean ± SD.–[3,4]Significantly different from the AOM/DSS group (group 1) by Tukey-Kramer multiple comparison posttest ([3]$p < 0.01$ and [4]$p < 0.001$).

and anti-NF-κB p50 (H-119) rabbit polyclonal antibody (sc-7178, diluted 1: 500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). These antibodies were applied to the sections according to the manufacturer's protocol. To reduce the nonspecific staining of mouse tissue by a mouse antibodies, a Mouse On Mouse IgG blocking reagent (MKB-2213, Vector Laboratories, Burlingame, CA) was applied for 1 hr. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine (DAB) for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections.

Levels of apoptosis in tumor tissues were determined by the TdT-mediated dUTP nick-end labeling (TUNEL) method. Four-micrometer formalin-fixed, paraffin-embedded tissue sections of colonic ADCs and lung ADs from groups 1 through 4 of Exp. 1 and 2 were processed according to manufacturer's instructions using Apoptosis in situ Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The kit is based on TUNEL procedure. Appropriate positive and negative controls for determining the specificity of staining were generated. Negative controls were processed in the absence of the terminal deoxynucleotidyl transferase (TdT) enzyme in the reaction buffer. Sections of tissue digested with nuclease enzyme and colon lymphoid nodules, which are known to exhibit high rates of apoptosis, were used as positive controls. Color was developed with the peroxidase substrate DAB, and sections were counterstained with hematoxylin.

The numbers of nuclei with positive reactivity for PCNA- and TUNEL-immunohistochemistry were counted in a total of 3 × 100 cells in 3 different areas of the tumors, and expressed as percentage (mean ± SD). Intensity and localization of immunoreactivities against the primary antibodies, NF-κB and HO-1, were assessed using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). Each slide for the immunohistochemical expression of NF-κB p50 and HO-1 was observed with the grading intensity of the immunoreactivity in neoplasms of the large bowel and lung. Two observers (T.T. and T.O.) were unaware of the treatment groups to which the slides belonged and evaluated the immunoreactivity with grading between 0 and 5: 0 (15% of the colonic mucosa examined shows positive reactivity), 1 (16 30% of the colonic mucosa examined shows positive reactivity), 2 (31 45% of the colonic mucosa examined shows positive reactivity), 3 (46–60% of the colonic mucosa examined shows positive reactivity), 4 (61 75% of the colonic mucosa examined shows positive reactivity) and 5 (75 % of the colonic mucosa examined shows positive reactivity). Care was taken to exclude the possibility of any inflammatory cells that were mistakenly identified as positive epithelial cells.

Statistical analysis

The incidences among the groups were compared using Chi-square test or Fisher's exact probability test with the GraphPad Instat Software (version 3.05; GraphPad software Inc., San Diego, CA). Other measurements expressing mean ± SD were statistically analyzed using Tukey-Kramer multiple comparison post test (GraphPad Instat version 3.05). Differences were considered statistically significant at $p < 0.05$.

Results

Exp. 1: Effect of ZER on AOM/DSS-induced colon carcinogenesis in mice

General observation. All animals remained healthy throughout the experimental period. At sacrifice, the mean body weight (51.5 ± 6.4 g, $p < 0.01$) and colon length (14.9 ± 0.7 cm, $p < 0.05$) of the DSS alone group (group 6) were significantly higher than those of the AOM/DSS (group 1, 44.6 ± 2.1 g and 13.8 ± 0.3 cm). Other measures (liver, kidney and spleen weights) did not significantly differ among the groups.

The inflammation scores and numbers of mucosal ulcer and high grade dysplasia in the colon. As summarized in Table I, colonic inflammation with or without mucosal ulcer was observed in the mice of groups 1 through 6. The mean score of inflammation and mean number of mucosal ulcer of group 6 (DSS alone) were the highest among the groups. Oral administration of ZER (groups 2–4) significantly and dose-dependently decreased the inflammatory score (group 2: 0.80 ± 0.63, $p < 0.01$; group 3: 0.50 ± 0.71, $p < 0.001$; and group 4 0.30 ± 0.48, $p < 0.001$) as compared to that of the AOM/DSS group (1.80 ± 0.77). Likewise, 250 ppm (0.60 ± 0.84; $p < 0.01$) and 500 ppm ZER (0.50 ± 0.71; $p < 0.001$) significantly lowered the number of mucosal ulcer. ZER feeding lowered the multiplicity of high grade dysplasia, but the differences were insignificant among the groups.

Colonic neoplasms. Macroscopically, colonic neoplasms developed in the mice of groups 1 through 4 with different incidence and multiplicity. The incidences and multiplicities of histopatholgically confirmed colonic tubular AD, ADC (Fig. 2*a*), and total tumors are given in Table II. Group 1 (AOM/DSS group) had a 93% incidence of colon ADC with a multiplicity of 3.87 ± 2.90. The incidences of ADC in groups 2–4 were smaller than that of group 1 and the value (50%, $p = 0.0225$) of group 4 (AOM/DSS + 500 ppm ZER) was significantly lower than group 1. The multiplicities of colonic ADC in group 2 ($p < 0.05$), 3 ($p < 0.01$) and 4 ($p < 0.01$) were also significantly smaller than group 1.

Effects of ZER on proliferation and apoptosis of colonic ADCs. As given in Figure 3, ZER feeding significantly decreased the PCNA-labeling index at 3 doses ($p < 0.001$ for each dose) of cancer cells (Fig. 3*a*) and significantly increased TUNEL-positive apoptotic nuclei ($p < 0.05$ at 100 ppm, and $p < 0.001$ at 250 and 500 ppm) of cancer cells (Fig. 3*b*).

Immunohistochemical scores of NFκB and HO-1 in colonic ADCs. Immunohistochemistry of NFκB revealed that strong reactivity of inflammatory cells in the inflamed colon (Fig. 4*a*) and ADC cells (Fig. 4*b*). Relative weak reactivity against HO-1 antibody was observed inflammatory cells (Fig. 4*c*) and ADC cells (Fig. 4*d*). As illustrated in Figure 3, ZER feeding dose-dependently lowered the immunohistochemical scores of NFκB (Fig. 3*c*) and HO-1 (Fig. 3*d*).

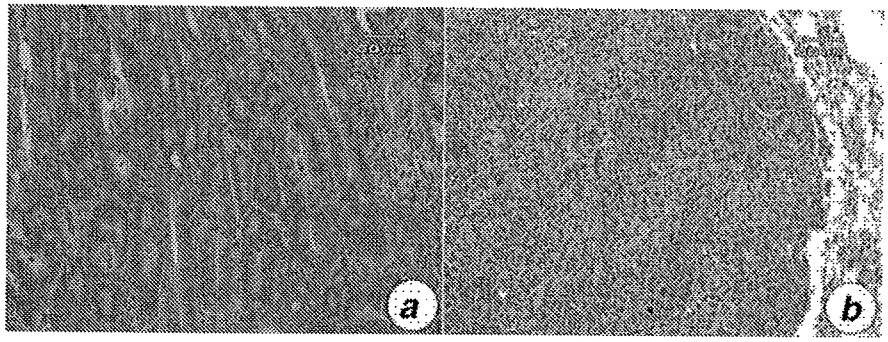

FIGURE 2 – Histopathology of (*a*) colonic tubular ADC induced by AOM/DSS and (b) lung tubular adenoma induced by NNK. Bars in the photos indicate magnification (μm).

TABLE II – EFFECTS OF DIETARY ZERUMBONE ON THE DEVELOPMENT OF COLONIC ADENOMA AND ADENOCARCINOMA (EXP. 1)

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of tumors/colon)		
			AD	ADC	Total	AD	ADC	Total tumors
1	AOM[1]/DSS	15	60	93	93	1.60 ± 1.55[2]	3.87 ± 2.90	5.47 ± 4.02
2	AOM/DSS + 100 ppm ZER	10	70	70	80	1.60 ± 1.58	1.50 ± 1.51[3]	3.10 ± 2.64
3	AOM/DSS + 250 ppm ZER	10	90	60	90	2.00 ± 1.33	1.20 ± 1.23[4]	3.20 ± 2.39
4	AOM/DSS + 500 ppm ZER	10	50	50[5]	60	0.90 ± 1.10	0.60 ± 0.70[4]	1.50 ± 1.72[3]
5	DSS + 500 ppm ZER	5	0	0	0	0	0	0
6	DSS	5	0	0	0	0	0	0
7	500 ppm ZER	5	0	0	0	0	0	0
8	Untreated	5	0	0	0	0	0	0

[1]AOM, azoxymethane; DSS, dextran sulfate sodium; ZER, zerumbobe; AD, adenoma; and ADC, adenocarcinoma.–[2]Mean ± SD.–[3,4]Significantly different from the AOM/DSS group (group 1) by Tukey-Kramer multiple comparison posttest ([3]$p < 0.05$ and [4]$p < 0.01$).–[5]Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0225$).

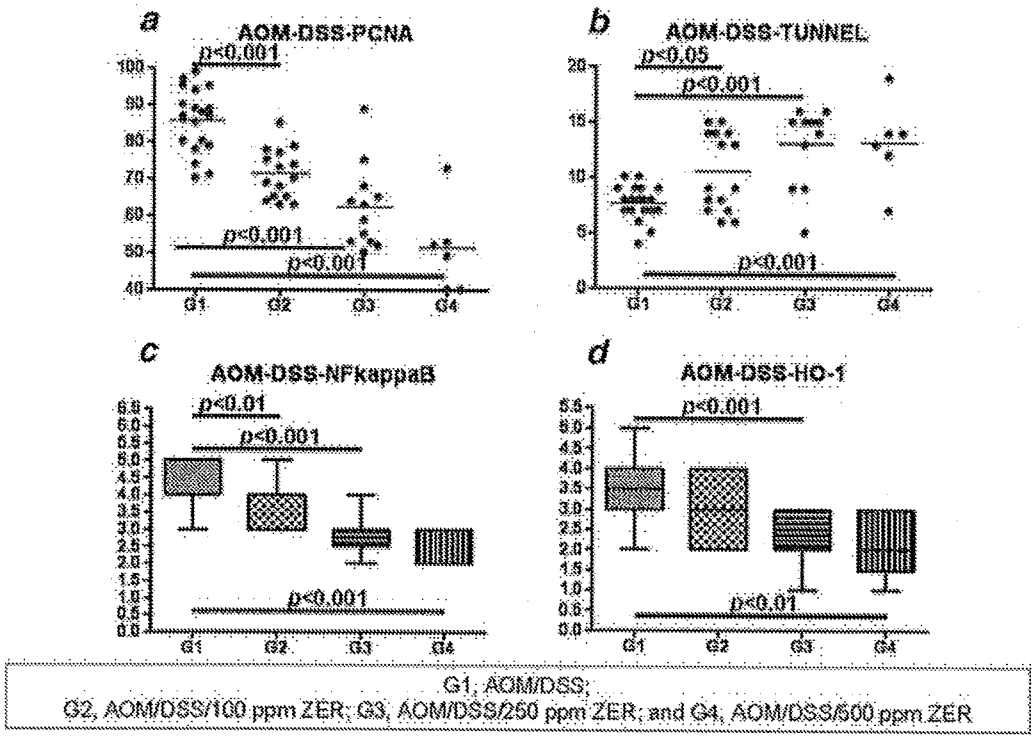

FIGURE 3 – Immunohistochemical scores of (*a*) PCNA-labeling index, (*b*) apoptotic index, (*c*) NF-κB and (*d*) HO-1, which were determined in colonic ADCs developed in mice of groups 1 through 4.

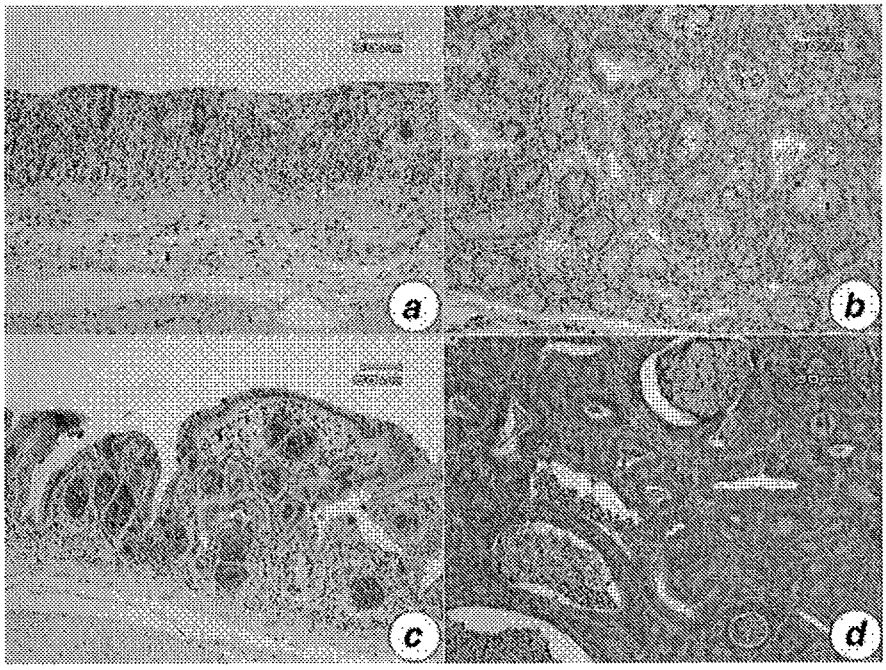

FIGURE 4 – Immunohistochemistry of NF-κB (*a, b*) and HO-1 (*c, d*). Strong NF-kB expression is observed in (*a*) mononuclear inflammatory cells in the colonic mucosa and (*b*) colonic ADC cells. HO-1 expression is relatively weak in (*c*) mononuclear inflammatory cells in the colonic mucosa and strong in (*d*) some of ADC cells. Bars in the photos indicate magnification (μm).

TABLE III – EFFECTS OF DIETARY ZERUMBONE ON THE DEVELOPMENT OF LUNG PROLIFERATIVE LESIONS INDUCED BY NNK (EXP. 2)

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of prolierative lesions/lung)		
			HP	AD	Total	HP	AD	Total
1	NNK[1]	12	100[2]	100[2]	100	2.75 ± 0.97[3,4]	8.25 ± 2.83[4]	11.00 ± 2.92[4]
2	NNK + 100 ppm ZER	10	70	100	100	1.50 ± 1.18[5]	6.40 ± 2.63	7.90 ± 2.64
3	NNK + 250 ppm ZER	10	40[6]	90	90	0.50 ± 0.71[7]	4.70 ± 3.33[5]	5.20 ± 3.74[7]
4	NNK + 500 ppm ZER	10	90	80	90	0.90 ± 0.32[7]	2.60 ± 2.17[7]	3.50 ± 2.32[7]
5	500 ppm ZER	5	40	20	40	0.40 ± 0.55	0.20 ± 0.45	0.60 ± 0.89
6	Untreated	5	40	40	60	0.40 ± 0.55	0.60 ± 0.89	1.00 ± 1.22

[1]NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HP, hyperplasia; AD, adenoma; and ZER, zerumbone.–[2]Significantly different from the untreated group (group 6) by Fisher's exact probability test ($p = 0.0147$).–[3]Mean ± SD.–[4]Significantly different from the untreated group (group 6) by Tukey-Kramer multiple comparison posttest ($p < 0.001$).–[5]Significantly different from the NNK group (group 1) by Tukey-Kramer multiple comparison posttest ($p < 0.05$).–[6]Significantly different from the NNK group (group 1) by Fisher's exact probability test ($p = 0.0028$).–[7]Significantly different from the NNK group (group 1) by Tukey-Kramer multiple comparison posttest ($p < 0.001$).

Exp. 2: Effect of ZER on NNK-induced lung carcinogenesis

General observation. Any clinical signs of toxicity of dietary ZER were not noted during the experiment. At sacrifice, the mean weight of lungs of the NNK-treated mice (group 1, 0.45 ± 0.05 g, $p < 0.05$) was significantly greater than that of the untreated mice (group 6, 0.35 ± 0.02 g). The mean weight of lungs of the mice in groups 2 (0.39 ± 0.05 g, $p < 0.05$), 3 (0.35 ± 0.05 g, $p < 0.05$), and 4 (0.36 ± 0.03 g, $p < 0.05$) were significantly lower as compared with that of group 1. Other measures (body, liver, kidney and spleen weights) did not significantly differ among the groups.

Effects of dietary ZER on the development of lung proliferative lesions. Table III summarizes the data on the incidence and multiplicity of lung proliferative lesions (HP and AD) induced by NNK and/or ZER. All mice belonging to group 1 developed alveolar cell HP and AD (Fig. 2*b*) with 100% incidences ($p = 0.0147$ for each) with high multiplicities of HP (2.75 ± 0.97, $p < 0.001$) and AD (8.25 ± 2.83, $p < 0.001$), as compared with an untreated group (group 6). Dietary ZER slightly affected the incidences of lung HP and AD, but significantly lowered the multiplicity of HP (100 ppm ZER: 1.50 ± 1.18, $p < 0.05$; 250 ppm ZER: 0.50 ± 0.71, $p < 0.001$; and 500 ppm ZER: 0.90 ± 0.32, $p < 0.001$) as compared group 1. Likewise, the supplementation of ZER at 250 (4.70 ± 3.33, $p < 0.05$) and 500 ppm (2.60 ± 2.17, $p < 0.001$) to the diet significantly reduced the multiplicity of AD when compared to group 1. The inhibition by 100 ppm ZER feeding was insignificant. Suppression effects of ZER at 3 dose levels demonstrated an inverse relationship of inhibition in the multiplicity of lung AD (Pearson $r = -0.9855$, $p < 0.00145$).

Effects of ZER on proliferation and apoptosis of lung ADs. As shown in Figure 5, ZER feeding at 250 ppm ($p < 0.01$) and 500 pmm ($p < 0.001$) significantly decreased the PCNA-labeling index of adenoma cells (Fig. 5*a*) and significantly increased TUNEL-positive apoptotic nuclei ($p < 0.01$ at 100 and 250 ppm, and $p < 0.001$ at 500 ppm) of lung adenoma cells (Fig. 5*b*).

Immunohistochemical scores of NFκB and HO-1 in lung ADs. Positive immunohistochemical reactions of NFκB (Fig. 6*a*) and HO-1 (Fig. 6*c*) were observed in lung adenomas that developed in NNK-treated mice. The positive reactions were reduced in

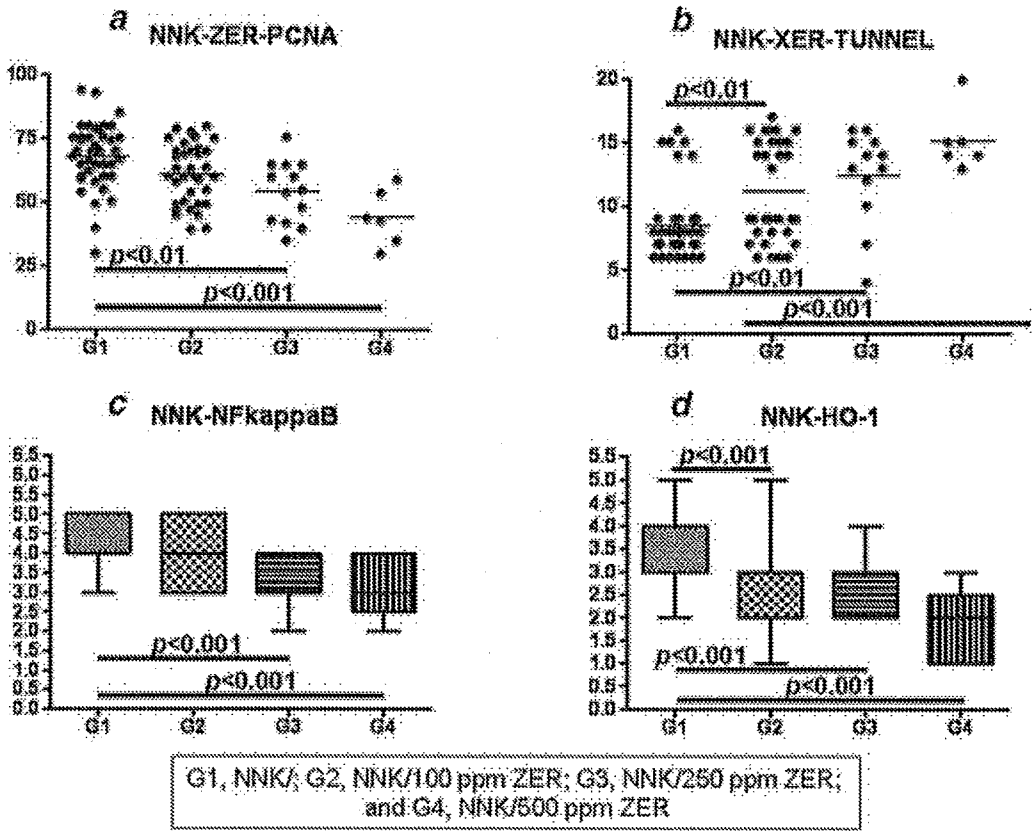

FIGURE 5 – Immunohistochemical scores of (*a*) PCNA-labeling index, (*b*) apoptotic index, (*c*) NF-κB and (*d*) HO-1, which were determined in lung adenoma developed in mice of groups 1 through 4.

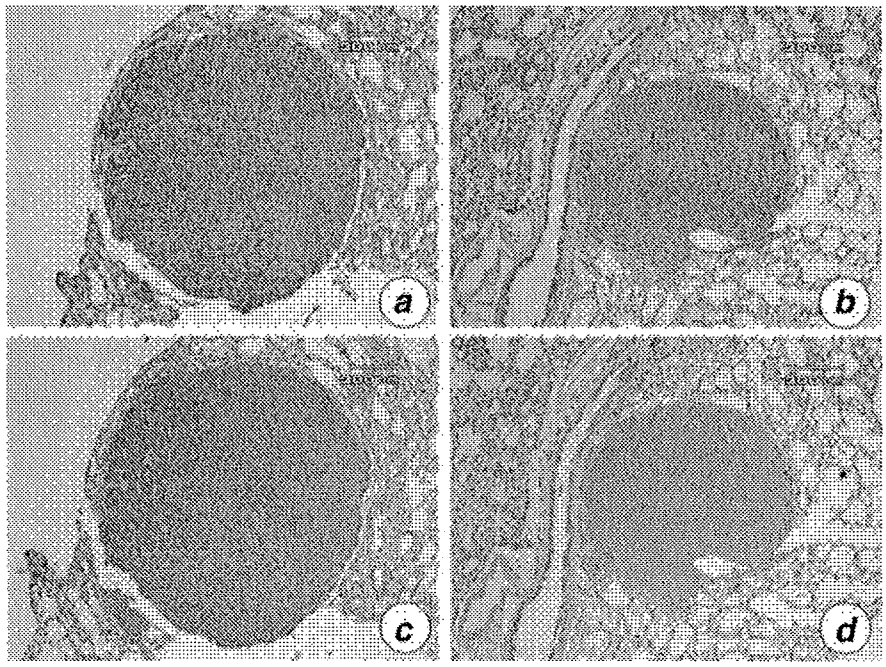

FIGURE 6 – Representative immunohistochemical reactions of NF-κB (*a*, *b*) and HO-1 (*c*, *d*) of lung adenomas. While strong reactivities of (*a*) NF-κB and (*c*) HO-1 are observed in the lung adenoma from group 1 (NNK alone), those of (*c*) NF-κB and (*c*) HO-1 from group 4 (NNK + 500 ppm ZER) are weak.

the lung AD (Figs. 6*b* and 6*d*) of mice that received NNK and ZER. As illustrated in Figure 5, feeding with ZER dose-dependently reduced the immunohistochemical scores and the inhibition of the NF-kB score (Fig. 5*c*) by ZER at 250 ($p < 0.001$) and 500 ppm ($p < 0.001$) and that of the HO-1 score (Fig. 5*d*) by ZER at 100 ($p < 0.001$), 250 ($p < 0.001$) and 500 ppm ($p < 0.001$) were statistically significant.

Discussion

The findings described here clearly indicated the chemopreventive effects of ZER derived from wild ginger in the mouse colon and lung carcinogenesis models. The protective ability of ZER is considered to be mediated by its anti-proliferative, apoptosis-inducing, anti-inflammatory and suppression of NF-kB and HO-1 expression. Together with our previous findings,[4,28] ZER is one of the possible chemopreventive agents against carcinogenesis in different tissues, including colon, lung and skin.[28] As large amount of ZER is readily available from the rhizomes of *Z. zerumbet*, further investigations for clarifying for detailed mechanisms of inhibition can be conducted in multiple organs of preclinical and clinical chemopreventive studies.

We assessed in this study chemopreventive ability of ZER at 3 dose levels (100, 250 and 500 ppm in diet) using 2 different mouse carcinogenesis models. All doses of ZER suppressed colonic inflammation and reduced the multiplicity of colonic ADC formation induced by AOM/DSS in a dose-dependent manner. In the NNK-induced lung tumorigenesis, ZER at all doses reduced the multiplicity of proliferative lesions, HP and ADs. The suppressing effects of ZER on the multiplicity of lung ADs showed clear dose-dependency. Importantly, we did not observe any toxicity of ZER in 2 different experiments.

As expected from our previous study[4] showing the inhibitory effects of ZER on the development of AOM-induced ACF, which is putative precursor lesion for colonic ADCs,[29,30] dietary ZER inhibited the occurrence of colonic ADC induced by the AOM/DSS treatment. Although the model system of colitis-related colon carcinogenesis[16] used in this study to induce colonic preneoplastic and neoplastic lesions was different from that for sporadic CRC,[4,29,30] protective effects of ZER on colon tumorigenesis are more likely in the inflamed colon, where the risk for ADC development is quite high.[31] Previous *in vitro* and *in vivo* investigations revealed strong anti-inflammatory properties of ZER.[1–3,5,28,32,33] Moreover, ZER can induce detoxifying enzymes.[34] ZER, thus, might be a cancer chemopreventive agent for the high-risk groups for CRC, such as ulcerative colitis and patients who receive polypectomy or surgical resection of CRC.

NF-κB is the key transcriptional factor for synthesis of proinflammatory mediators, including iNOS, COX-2 and TNF-α. NF-κB also plays central roles in carcinogenesis and inflammation,[21] and thus it is one of the molecular targets of cancer chemoprevention and therapy.[24,35] In fact, NF-κB activation is reported to be involved in colon[36] and lung carcinogenesis[37] and certain NF-κB inhibitors are able to suppress cancer development in these tissues.[38,39] In this study, dietary administration of ZER reduced the immunohistochemical expression of NFκB in colonic and lung tumors. Also, we observed that ZER causes suppression of cell proliferation, and induction of apoptosis. ZER is reported to suppress proinflammatory protein production and oxidative/nitrosative stress, and to induce apoptosis in human colon cancer cell lines *via* suppressing the expression of COX-2 and iNOS.[2] ZER induces nuclear localization of nuclear factor-erythroid 2-related factor (Nrf)-2 that binds to antioxidant response element (ARE) of the phase II enzyme genes, suggesting that ZER is a potential activator of the Nrf-2/ARE-dependent phase II enzyme genes, including manganese superoxide dismutase (MnSOD), γ-glutamylcysteine glutathione peroxidase (GPx) 1[28] and HO-1.[34] In the current study, dietary feeding with ZER inhibited the immunohistochemical expression of HO-1 in the tumors developed in the colon and lung. Both *MnSOD* and *GPx1* are regulated, at least in part, by Nrf-2 transcription factor.[40] Therefore, ZER may induce these genes' expression *via* possibly Nrf-2 activation An anti-oxidative enzyme, HO-1, is protective against oxidative stress in the damaged tissues without neoplastic alterations.[41] However, overexpression of HO-1 is observed in preneoplastic[42] and neoplastic tissues[43–45] to growth and to survive against cancer therapy.[44] In this context, the effects of ZER on the expression of NF–κB and HO-1 in tumors developed in the colon and lung are of interest. We observed that dietary ZER effectively inhibits immunohistochemical expression of colonic ADCs and lung AD, suggesting that ZER has cancer chemopreventive as well as cancer chemotherapeutic potentials.

In the current experiments, we noted that ZER treatment induces apoptosis in the neoplasms of colon and lung. ZER is previously reported to induce apoptosis in a variety of colon cancer cell lines (LS174, LS180, COLO205 and COLO320DM) with different degree.[2] The α,β-unsaturated carbonyl group of ZER is suspected to be responsible for the effects.[2] A recent report by Sakinah *et al.*[46] showing that ZER treatment results in decreased expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax in human hepatocellular cancer cells, HepG2, confirmed apoptosis-inducing effects of ZER in malignant epithelial cells.

In conclusion, our findings indicate that a sesquiterpenoid, zerumbone, being a major constituent of the subtropical ginger plant *Zingiber zerumbet* Smith is one of the good candidates with multiple targets for cancer chemopreventive agent in colon and lung carcinogenesis related with inflammation.

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References

1. Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, Ohigashi H. Identification of zerumbone in Zingiber zerumbet Smith as a potent inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation. Biosci Biotechnol Biochem 1999;63:1811–2.
2. Murakami A, Takahashi D, Kinoshita T, Koshimizu K, Kim HW, Yoshihiro A, Nakamura Y, Jiwajinda S, Terao J, Ohigashi H. Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production, and cancer cell proliferation accompanied by apoptosis: the alpha,beta-unsaturated carbonyl group is a prerequisite. Carcinogenesis 2002;23:795–802.
3. Murakami A, Hayashi R, Tanaka T, Kwon KH, Ohigashi H, Safitri R. Suppression of dextran sodium sulfate-induced colitis in mice by zerumbone, a subtropical ginger sesquiterpene, and nimesulide: separately and in combination. Biochem Pharmacol 2003;66:1253–61.
4. Tanaka T, Shimizu M, Kohno H, Yoshitani S, Tsukio Y, Murakami A, Safitri R, Takahashi D, Yamamoto K, Koshimizu K, Ohigashi H, Mori H. Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary zerumbone isolated from Zingiber zerumbet. Life Sci 2001;69:1935–45.
5. Murakami A, Matsumoto K, Koshimizu K, Ohigashi H. Effects of selected food factors with chemopreventive properties on combined lipopolysaccharide- and interferon-gamma-induced IkappaB degradation in RAW264.7 macrophages. Cancer Lett 2003;195:17–25.
6. Takada Y, Murakami A, Aggarwal BB. Zerumbone abolishes NF-kappaB and IkappaBalpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion. Oncogene 2005;24:6957–69.

7. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.
8. Azad N, Rojanasakul Y, Vallyathan V. Inflammation and lung cancer: roles of reactive oxygen/nitrogen species. J Toxicol Environ Health B Crit Rev 2008;11:1–15.
9. Engels EA. Inflammation in the development of lung cancer: epidemiological evidence. Expert Rev Anticancer Ther 2008;8:605–15.
10. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol 2001;2:149–56.
11. Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA. Environmental and chemical carcinogenesis. Semin Cancer Biol 2004;14:473–86.
12. Xie J, Itzkowitz SH. Cancer in inflammatory bowel disease. World J Gastroenterol 2008;14:378–89.
13. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol 2004;287:G7–17.
14. Hecht SS, Hoffmann D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 1988;9:875–84.
15. Kim M, Miyamoto S, Sugie S, Yasui Y, Ishigamori-Suzuki R, Murakami A, Nakagama H, Tanaka T. A tobacco-specific carcinogen, NNK, enhances AOM/DSS-induced colon carcinogenesis in male A/J mice. In Vivo, in press.
16. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci 2003;94:965–73.
17. Hecht SS, Hochalter JB, Villalta PW, Murphy SE. 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. Proc Natl Acad Sci U S A 2000;97:12493–7.
18. Hecht SS, Morse MA, Amin S, Stoner GD, Jordan KG, Choi CI, Chung FL. Rapid single-dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. Carcinogenesis 1989;10:1901–4.
19. Yao R, Rioux N, Castonguay A, You M. Inhibition of COX-2 and induction of apoptosis: two determinants of nonsteroidal anti-inflammatory drugs' chemopreventive efficacies in mouse lung tumorigenesis. Exp Lung Res 2000;26:731–42.
20. Prawan A, Kundu JK, Surh YJ. Molecular basis of heme oxygenase-1 induction: implications for chemoprevention and chemoprotection. Antioxid Redox Signal 2005;7:1688–703.
21. Maeda S, Omata M. Inflammation and cancer: role of nuclear factor-kappaB activation. Cancer Sci 2008;99:836–42.
22. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. Curr Opin Genet Dev 2008;18:19–26.
23. Miyamoto S, Epifano F, Curini M, Genovese S, Kim M, Ishigamori-Suzuki R, Yasui Y, Sugie S, Tanaka T. A novel prodrug of 4'-geranyloxy-ferulic acid suppresses colitis-related colon carcinogenesis in mice. Nutr Cancer, in press.
24. Surh YJ. NF-kappa B and Nrf2 as potential chemopreventive targets of some anti-inflammatory and antioxidative phytonutrients with anti-inflammatory and antioxidative activities. Asia Pac J Clin Nutr 2008;17 (Suppl. 1):269–72.
25. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 1993;69:238–49.
26. Ward JM. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. Lab Invest 1974;30:505–13.
27. Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, Fraire AE, Gabrielson EW, Gunning WT, Haines DC, Kaufman MH, Linnoila RI, et al. Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. Cancer Res 2004;64:2307–16.
28. Murakami A, Tanaka T, Lee JY, Surh YJ, Kim HW, Kawabata K, Nakamura Y, Jiwajinda S, Ohigashi H. Zerumbone, a sesquiterpene in subtropical ginger, suppresses skin tumor initiation and promotion stages in ICR mice. Int J Cancer 2004;110:481–90.
29. Tanaka T, Miyamoto S, Suzuki R, Yasui Y. Chemoprevention of colon carcinogenesis in dietary non-nutritive compounds. Curr Topics Neutraceut Res 2006;4:127–52.
30. Tanaka T, Sugie S. Inhibition of colon carcinogenesis by dietary non-nutritive compounds. J. Toxicol Pathol 2007;20:215–35.
31. Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S. Colitis-related rat colon carcinogenesis induced by 1-hydroxy-anthraquinone and methylazoxymethanol acetate (Review). Oncol Rep 2000;7:501–8.
32. Murakami A, Miyamoto M, Ohigashi H. Zerumbone, an anti-inflammatory phytochemical, induces expression of proinflammatory cytokine genes in human colon adenocarcinoma cell lines. Biofactors 2004;21:95–101.
33. Murakami A, Shigemori T, Ohigashi H. Zingiberaceous and citrus constituents, 1'-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action. J Nutr 2005;135:2987S–92S.
34. Nakamura Y, Yoshida C, Murakami A, Ohigashi H, Osawa T, Uchida K. Zerumbone, a tropical ginger sesquiterpene, activates phase II drug metabolizing enzymes. FEBS Lett 2004;572:245–50.
35. Gopalakrishnan A, Kong AN. Anticarcinogenesis by dietary phytochemicals: cytoprotection by Nrf2 in normal cells and cytotoxicity by modulation of transcription factors NF-kappa B and AP-1 in abnormal cancer cells. Food Chem Toxicol 2008;46:1257–70.
36. Clemo NK, Collard TJ, Southern SL, Edwards KD, Moorghen M, Packham G, Hague A, Paraskeva C, Williams AC. BAG-1 is up-regulated in colorectal tumour progression and promotes colorectal tumour cell survival through increased NF-kappaB activity. Carcinogenesis 2008;29:849–57.
37. Stathopoulos GT, Sherrill TP, Cheng DS, Scoggins RM, Han W, Polosukhin VV, Connelly L, Yull FE, Fingleton B, Blackwell TS. Epithelial NF-kappaB activation promotes urethane-induced lung carcinogenesis. Proc Natl Acad Sci USA 2007;104:18514–9.
38. Rajakangas J, Misikangas M, Paivarinta E, Mutanen M. Chemoprevention by white currant is mediated by the reduction of nuclear beta-catenin and NF-kappaB levels in Min mice adenomas. Eur J Nutr 2008;47:115–22.
39. Anto RJ, Mukhopadhyay A, Shishodia S, Gairola CG, Aggarwal BB. Cigarette smoke condensate activates nuclear transcription factor-kappaB through phosphorylation and degradation of IkappaB(alpha): correlation with induction of cyclooxygenase-2. Carcinogenesis 2002;23:1511–8.
40. Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J Biol Chem 2003;278:12029–38.
41. Abraham NG, Tsenovoy PL, McClung J, Drummond GS. Heme oxygenase: a target gene for anti-diabetic and obesity. Curr Pharm Des 2008;14:412–21.
42. Lee J, Lee SK, Lee BU, Lee HJ, Cho NP, Yoon JH, Choi HR, Lee SK, Kim EC. Upregulation of heme oxygenase-1 in oral epithelial dysplasias. Int J Oral Maxillofac Surg 2008;37:287–92.
43. Kim HR, Kim S, Kim EJ, Park JH, Yang SH, Jeong ET, Park C, Youn MJ, So HS, Park R. Suppression of Nrf2-driven heme oxygenase-1 enhances the chemosensitivity of lung cancer A549 cells toward cisplatin. Lung Cancer 2008;60:47–56.
44. Loboda A, Was H, Jozkowicz A, Dulak J. Janus face of Nrf2-HO-1 axis in cancer—friend in chemoprevention, foe in anticancer therapy. Lung Cancer 2008;60:1–3.
45. Maines MD, Abrahamsson PA. Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. Urology 1996;47:717–33.
46. Sakinah SA, Handayani ST, Hawariah LP. Zerumbone induced apoptosis in liver cancer cells via modulation of Bax/Bcl-2 ratio. Cancer Cell Int 2007;7:4.

Supplementation with Branched-chain Amino Acids Inhibits Azoxymethane-induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db*/*db* Mice

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Abstract

Purpose: Obesity and related metabolic abnormalities, including insulin resistance and activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, are risk factors for colon cancer. Supplementation with branched-chain amino acids (BCAA) reduces the risk of liver cancer in cirrhotic patients who are obese, and this has been associated with an improvement of insulin resistance. The present study examined the effects of BCAA on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db*/*db* (*db*/*db*) mice that were obese and had hyperinsulinemia.

Experimental Design: Male *db*/*db* mice were given 4 weekly s.c. injections of AOM (15 mg/kg of body weight) and then they were fed a diet containing 3.0% BCAA or casein, a nitrogenc content–matched control diet, for 7 weeks.

Results: Feeding with BCAA caused a significant reduction in the number of total aberrant crypt foci and β-catenin accumulated crypts, both of which are premalignant lesions of the colon, compared with the control diet–fed groups. BCAA supplementation caused a marked decrease in the expression of IGF-IR, the phosphorylated form of IGF-IR, phosphorylated glycogen synthase kinase 3β, phosphorylated Akt, and cyclooxygenase-2 proteins on the colonic mucosa of AOM-treated mice. The serum levels of insulin, IGF-I, IGF-II, triglyceride, total cholesterol, and leptin were also decreased by supplementation with BCAA.

Conclusion: BCAA supplementation in diet improves insulin resistance and inhibits the activation of the IGF/IGF-IR axis, thereby preventing the development of colonic premalignancies in an obesity-related colon cancer model that was also associated with hyperlipidemia and hyperinsulinemia. BCAA, therefore, may be a useful chemoprevention modality for colon cancer in obese people.

Colorectal cancer (CRC) is a major health problem worldwide. Recent evidence indicates that the risk of CRC is elevated in patients with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and related metabolic abnormalities (1, 2). Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is also a possible risk factor for CRC (3). CRC occurs more frequently in patients with diabetes mellitus, a condition associated with hyperinsulinemia (4, 5). Insulin has growth-promoting properties in CRC cells, and exogenous insulin injection stimulates the growth of CRC precursors in rodent models (6–8). In addition, elevated circulating levels of insulin causes alterations in the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, which is involved in the development and progression of CRC (9, 10). Therefore, increased insulin resistance and abnormalities in the IGF/IGF-IR axis might be a critical target to prevent the development of obesity-related malignancies, including CRC. For instance, (-)-epigallocatechin gallate, the major biologically active component of green tea, inhibited the development of colonic premalignant lesions in an obesity-related colon cancer that was associated with improvement in insulin resistance and inhibition of the IGF/IGF-IR axis (11).

Diet supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine) has been suggested to improve protein malnutrition in patients with liver cirrhosis (12). Recent studies have revealed that BCAA is useful for both preventing progressive hepatic failure and improving event-free survival in patients with chronic liver diseases, such as liver cirrhosis, and these beneficial effects are associated with the improvement of insulin resistance by BCAA (13–15). In addition, oral supplemental treatment with BCAA can reduce the risk of hepatocellular carcinoma in cirrhotic patients who

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Translational Relevance

Obesity and related metabolic abnormalities, including insulin resistance and the activation of the insulin-like growth factor (IGF)/IGF-I receptor axis, are associated with colorectal cancer (CRC) development. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis might be a promising strategy for obese people who are at increased risks of CRC. We believe that this study is novel and clinically relevant because this article is the first report indicating that supplementation with branched-chain amino acids (BCAA) effectively suppressed the development of azoxymethane-induced putative precursor lesions of colonic adenocarcinoma in C57BL/KsJ-*db/db* mice that are obese and developed diabetes mellitus. Our studies indicate that this suppressing effect of BCAA was associated with improvement of hyperlipidemia and hyperleptinemia. BCAA supplementation could also improve insulin resistance and exert a depressant effect on the IGF/IGF-IR axis. The current findings suggest the possibility of using BCAA as a chemopreventive agent for obesity-related malignancies.

are obese (with a body mass index ≥25; ref. 16). Obesity, hyperinsulinemia, and diabetes mellitus are possible risk factors for hepatocellular carcinoma, which commonly develops in cirrhotic livers (16–18). Based on these findings, BCAA supplementation in diet may also reduce the risk of other obesity-related human malignancies, including CRC, by improving insulin resistance. However, no detailed studies on whether BCAA can prevent the development of obesity-related CRC have yet been conducted.

In previous studies, we have established a useful preclinical animal model to determine the possible underlying mechanisms of how specific agents prevent the development of obesity-related CRC with the use of C57BL/KsJ-*db/db* (*db/db*) mice with obesity, hyperinsulinemia, and hyperleptinemia (19–21). The mice are susceptible to the colonic carcinogen azoxymethane (AOM) because the development of AOM-induced aberrant crypt foci (ACF) and β-catenin–accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma (22, 23), is enhanced in *db/db* mice compared with *db*/+ or +/+ mice (19, 20). In the present study, we investigated in detail the effects of BCAA on the development of colonic premalignant lesions, ACF and BCAC, in *db/db* mice initiated with AOM, focusing on the improvement of hyperinsulinemia, hyperlipidemia, and hyperleptinemia. In addition, we also determined whether BCAA supplementation in the diet inhibits the activation of the IGF/IGF-IR axis in this animal model.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male homozygous *db/db* mice were obtained from Japan SLC, Inc.. All mice were maintained at the Gifu University Life Science Research Center according to the Institutional Animal Care Guidelines. AOM was purchased from Sigma Chemical Co.. BCAA and casein were obtained from Ajinomoto Co., Ltd.. The BCAA composition (2:1:1.2, leucine/isoleucine/valine) was set at the clinical dosage that is used for the treatment of hypoalbuminemia in patients with decompensated liver cirrhosis in Japan.

Experimental procedure. The animal experiment was approved by the Institutional Committee of Animal Experiments of Gifu University. A total of 54 male *db/db* mice were divided into 6 groups. At 5 wk of age, the mice in groups 1 to 3 were s.c. injected with AOM (15 mg/kg of body weight) weekly for 4 wk. As controls, the mice in groups 4 to 6 were given s.c. injections of saline. Groups 1 (12 mice) and 4 (6 mice) were fed a basal diet, corticotropin-releasing factor (CRF)-1 (Oriental Yeast Co., Ltd.), throughout the experiment. Groups 3 (12 mice) and 6 (6 mice) were given a basal diet containing 3.0% BCAA (weight for weight) for 7 wk, starting 1 wk after the last injection of AOM. The BCAA concentration (3.0%) was determined by the previous study, which indicated the same intake to improve insulin resistance in C57BL/6J mice (24). The mice in groups 2 (12 mice) and 5 (6 mice) were given a basal diet containing 3.0% casein (weight for weight). The casein-fed groups were served as nitrogen content–matched controls for the BCAA-treated groups to eliminate the possibility that the nitrogen content itself affects the promotion or the prevention of colonic premalignant lesions. At the termination of the study (16 wk of age), the mice were sacrificed by CO_2 asphyxiation to analyze the number of colonic ACF and BCAC.

Counting the number of ACF and BCAC. The ACF and BCAC were determined according to the standard procedures described previously (20, 21, 25). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts (22). BCAC, which have high frequency mutations in the *β-catenin* gene, show histologic dysplasia with a disruption of the cellular morphology and an accumulation of this protein (Fig. 1A; ref. 23). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histologic sections of en face preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive properties (26). After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water), and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from the anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area, 0.7 cm^2 per colon) was embedded in paraffin, and then a total of 20 serial sections (4-μm thick each) per colon were made by an en face preparation (20, 21, 25). For each case, 2 serial sections were used to analyze BCAC.

Histopathology and immunohistochemical analyses for β-catenin and PCNA. Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. The other section was used for the proliferating cell nuclear antigen (PCNA), a G_1-to-S phase marker, immunohistochemistry to estimate the cell proliferative activity in the colonic mucosa. Immunohistochemical analyses for β-catenin and PCNA were done with the labeled streptavidin-biotin method (LSAB kit; DAKO) as previously described (20, 21). Anti–β-catenin antibody (1:1,000 final dilution) was obtained from Transduction Laboratories (catalogue no. 610154). Anti-PCNA antibody (1:100 final dilution) was from Santa Cruz Biotechnology, Inc. (sc-7907). Negative control sections were immunostained without the primary antibody. PCNA-positive cells in the colonic mucosa, which seemed normal by H&E staining, were counted and expressed as a percentage of the total number of normal crypt cells. The PCNA labeling index (%) was determined by counting at least 200 crypt cells in each mouse (a total of 1,000 crypt cells per group). Two experienced pathologists (Y. Hirose and T. Tanaka) immunohistologically determined the BCAC and PCNA-positive cells.

Protein extraction and western blot analysis. Total proteins were extracted from the scraped mucosa from the remaining colon of the AOM-treated mice (groups 1 to 3), and equivalent amounts of proteins

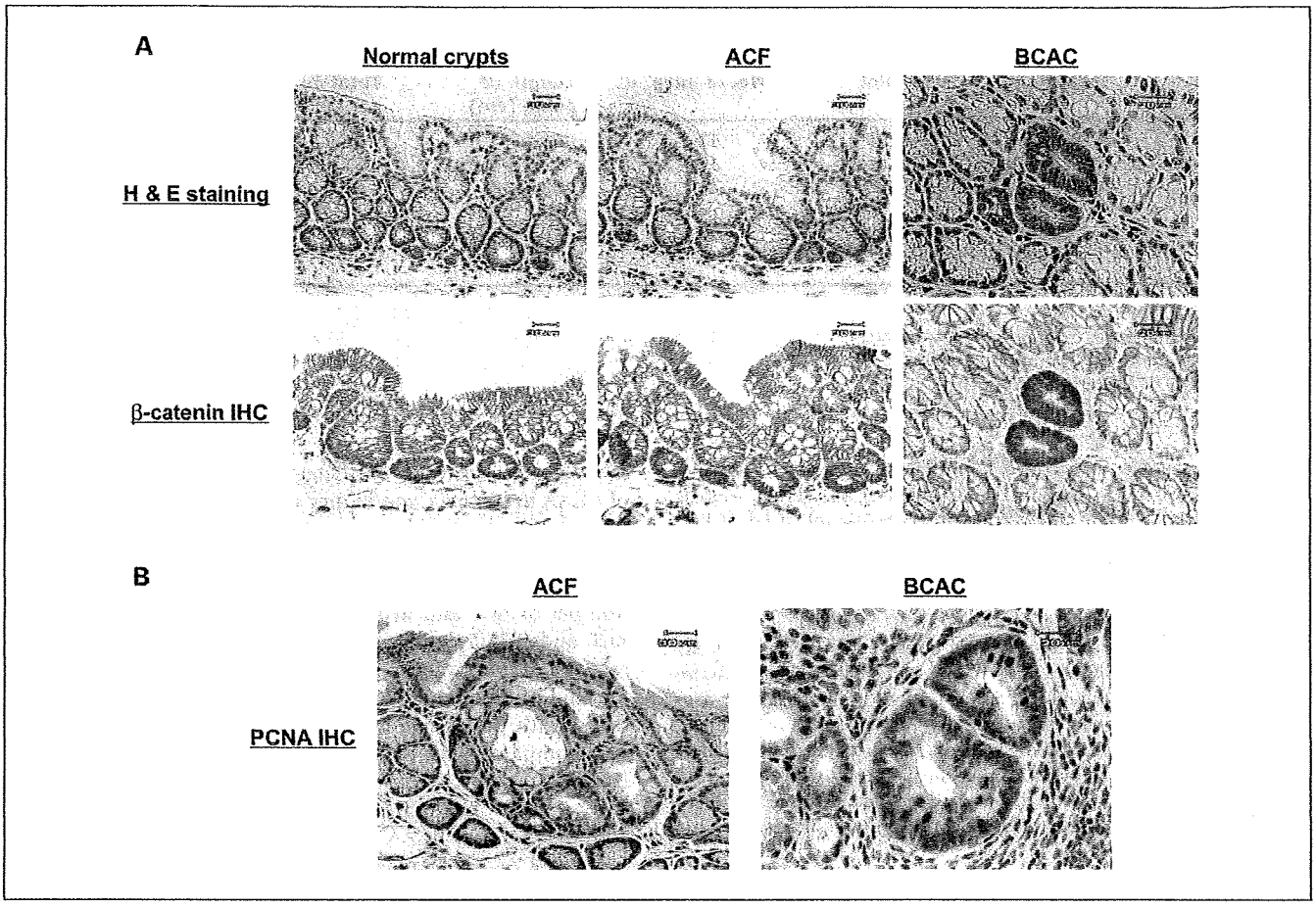

Fig. 1. Histopathology and immunohistochemical expression of β-catenin and PCNA proteins in ACF and BCAC. *A*, representative photographs of ACF and BCAC induced by AOM in *db/db* mice. Top, H&E staining; bottom, β-catenin immunohistochemistry. Left, normal crypts; middle, ACF; right, BCAC. The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells in BCAC. *B*, immunohistochemical pattern of PCNA protein in ACF and BCAC. The nuclear expression of the PCNA protein significantly increased in BCAC compared with ACF and surrounding normal crypts. Bar, 20 or 30 μm, respectively.

(40 μg per lane) were examined by a western blot analysis with the use of the primary antibodies for IGF-IR, phosphorylated IGF-IR (p-IGF-IR), phosphorylated glycogen synthase kinase 3β (p-GSK-3β), Akt, phosphorylated Akt (p-Akt), cyclooxygenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase as described previously (11, 27, 28). An antibody to glyceraldehyde-3-phosphate dehydrogenase served as a loading control. The intensities of the blots were quantified with the NIH Image software version 1.62. The intensities of the blots found at the CRF-fed mice in each antibody was set at 1, and the changes in expression were shown as the fold difference.

Table 1. Body, liver, kidney, and white adipose tissue weights of the experimental mice

Group no.	Treatment	Diet	No. of mice	Final body weight (g)	Body length (cm)	BMI	Absolute organ weight		
							Liver	Kidney	White adipose tissue
1	AOM 15 mg/kg	CRF-1	12	49.7 ± 8.3*,†	9.25 ± 0.77	0.58 ± 0.05	2.64 ± 0.76	0.38 ± 0.04†	2.67 ± 0.64
2	AOM 15 mg/kg	Casein	12	51.7 ± 4.8	9.43 ± 0.37	0.58 ± 0.02	2.75 ± 0.48‡	0.41 ± 0.04	2.74 ± 0.37
3	AOM 15 mg/kg	BCAA	12	50.3 ± 5.0§	9.47 ± 0.25	0.56 ± 0.04	2.58 ± 0.64§	0.40 ± 0.05	2.51 ± 0.42
4	Saline	CRF-1	6	58.1 ± 2.5	9.63 ± 0.22	0.63 ± 0.02	3.35 ± 0.72	0.45 ± 0.06	3.02 ± 0.32
5	Saline	Casein	6	58.0 ± 2.1	9.70 ± 0.18	0.62 ± 0.01	3.87 ± 1.04	0.44 ± 0.04	2.70 ± 0.38
6	Saline	BCAA	6	58.5 ± 2.5	9.63 ± 0.17	0.63 ± 0.01	3.83 ± 0.86	0.44 ± 0.01	2.60 ± 0.35

*Mean ± SD.
†Significantly different from group 4 ($P < 0.05$).
‡Significantly different from group 5 ($P < 0.05$).
§Significantly different from group 6 ($P < 0.05$).

Table 2. Effects of BCAA on AOM-induced ACF and BCAC formation in the experimental mice

Group no.	Treatment	Diet	No. of mice	Length of colon (cm)	Total no. of ACFs per colon	Total no. of BCACs/cm^2
1	AOM 15 mg/kg	CRF-1	12	12.4 ± 1.4*	85.9 ± 8.1	11.7 ± 8.4
2	AOM 15 mg/kg	Casein	12	12.5 ± 0.5	83.4 ± 11.2	8.3 ± 3.9
3	AOM 15 mg/kg	BCAA	12	12.0 ± 0.7	54.5 ± 8.6†,‡	4.2 ± 6.7§
4	Saline	CRF-1	6	12.5 ± 1.0	0	0
5	Saline	Casein	6	11.5 ± 0.7	0	0
6	Saline	BCAA	6	11.3 ± 0.5	0	0

*Mean ± SD.
†Significantly different from group 1 ($P < 0.001$).
‡Significantly different from group 2 ($P < 0.001$).
§Significantly different from group 1 ($P < 0.05$).

Clinical chemistry. At sacrifice, blood samples were collected from the AOM-treated mice (groups 1-3) to measure the serum concentrations of insulin, leptin, triglyceride, total cholesterol, IGF-I, IGF-II, and BCAA. The serum triglyceride, total cholesterol, and BCAA levels were assayed as described previously (20, 29). The serum insulin, leptin, IGF-I, and IGF-II were determined by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems).

Statistical analysis. The results were presented as the mean ± SD and were analyzed with the use of the GraphPad InStat software program version 3.05 (GraphPad Software) for Macintosh. Differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ($P < 0.05$), comparisons of each experimental group with the control group were then made with the use of the Tukey-Kramer multiple comparisons test. The differences were considered significant when the two-tailed P was <0.05.

Results

General observations. As shown in Table 1, the average body weights of groups 1 (CRF-1) and 3 (BCAA) in the AOM-injected mice at the termination of this experiment were smaller than those of the saline-injected groups 4 (CRF-1; $P < 0.05$) and 6 (BCAA; $P < 0.05$). The mean liver weights in the AOM-treated groups 2 (casein) and 3 (BCAA) were significantly lower than those in the saline-treated groups 5 (casein; $P < 0.05$) and 6 (BCAA; $P < 0.05$). Among CRF-1–fed mice, the mean kidney weight in the AOM-treated group 1 was also significantly lower than that of the saline-treated group 4 ($P < 0.05$). No significant difference was observed in the body length, body mass index, and mean white adipose tissue weight among the experimental mice. A histopathologic examination also revealed no alteration, thus suggesting the absence of toxicity of BCAA in the liver and kidney of the mice in groups 3 and 6 (data not shown).

Effects of BCAA supplementation on AOM-induced ACF and BCAC formations in db/db *mice.* Table 2 summarizes the total number of ACF and BCAC (Fig. 1) in the mice of all groups. ACF and BCAC developed in the colons of all the mice that received AOM (groups 1 to 3) but not in the colons of the mice that did not receive AOM (groups 4 to 6). Dietary supplementation with BCAA significantly decreased the number of total ACF compared with those of the CRF-1–fed (37% reduction; $P < 0.001$) and casein-supplemented groups (35% reduction; $P < 0.001$). Compared with the CRF-1–fed group, the administration of BCAA also significantly reduced the number of total BCAC (64% reduction; $P < 0.05$).

Effects of BCAA supplementation on the serum levels of BCAA in AOM-treated db/db *mice.* Because the colonic premalignant lesions developed only in the AOM-injected mice (Table 2), the following experiments were done among the mice thatz received AOM (groups 1 to 3). BCAA supplementation caused a significant increase in the serum concentrations of total BCAA (valine, isoleucine, and leucine; 1736 ± 179 nmol/mL) compared with the CRF-1–fed (882 ± 160 nmol/mL; $P < 0.001$) and casein-supplemented groups (853 ± 51 nmol/mL; $P < 0.001$). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA.

Effects of BCAA supplementation on the serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db *mice.* As shown in Table 3, the serum levels of total cholesterol in the BCAA-supplemented mice were significantly lower than

Table 3. Serum levels of total cholesterol, triglyceride, and leptin in AOM-treated *db/db* mice

Group no.	Treatment	Diet	No. of mice	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Leptin (ng/dL)
1	AOM 15 mg/kg	CRF-1	12	185 ± 34*	244 ± 49	117 ± 18
2	AOM 15 mg/kg	Casein	12	186 ± 40	229 ± 40	133 ± 32
3	AOM 15 mg/kg	BCAA	12	141 ± 48†,‡	187 ± 48†	99 ± 23§

*Mean ± SD.
†Significantly different from group 1 ($P < 0.05$).
‡Significantly different from group 2 ($P < 0.05$).
§Significantly different from group 2 ($P < 0.01$).

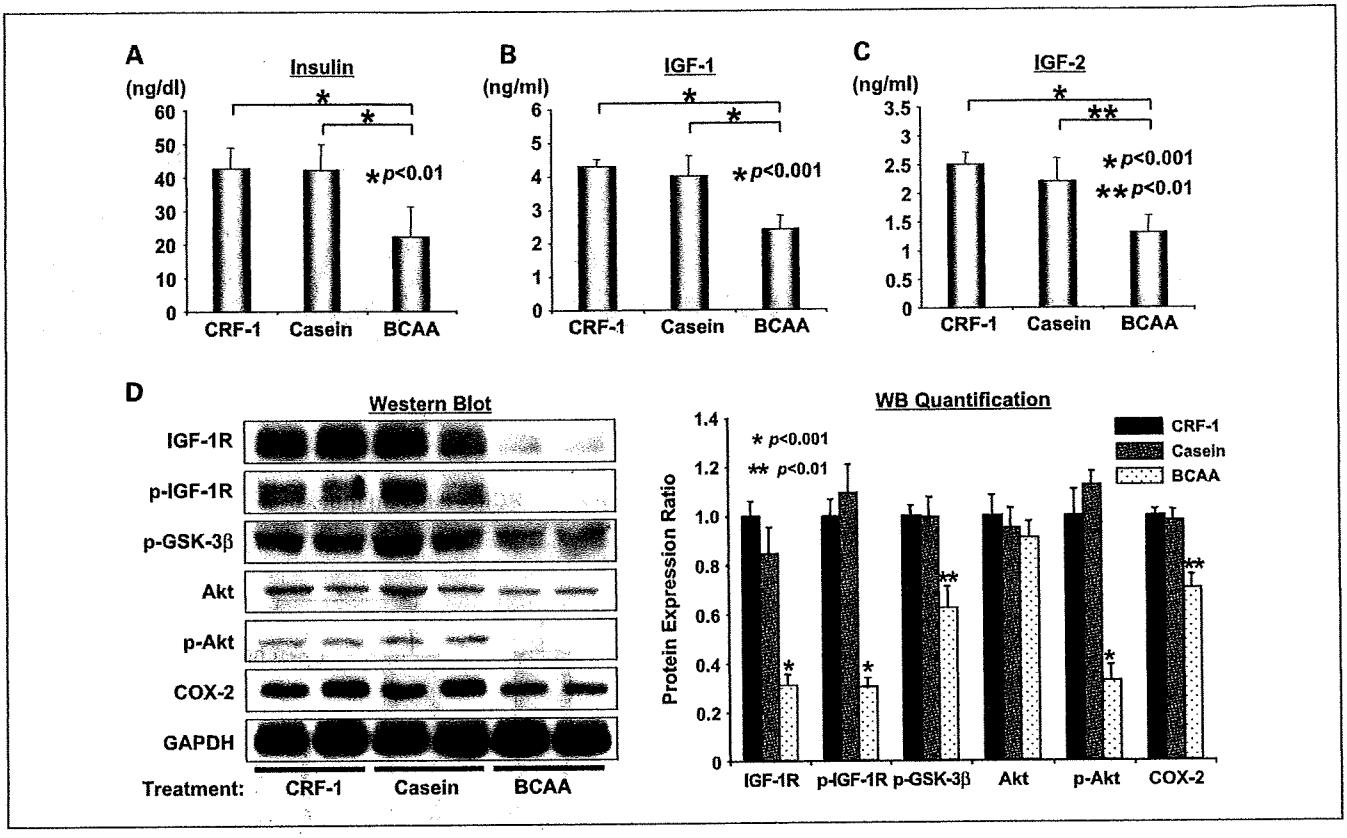

Fig. 2. The effect of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II, and on the expression levels of the IGF-IR, p-IGF-IR, p-GSK-3β, Akt, p-Akt, and COX-2 proteins in AOM-treated *db/db* mice. *A* to *C*, the serum concentration of insulin (*A*), IGF-I (*B*), and IGF-II (*C*) were measured by an enzyme immunoassay. Bars, SD of triplicate assays. *D*, total proteins were extracted from the scraped colonic mucosa, and equivalent amounts of proteins were examined by a western blot analysis as described in Materials and Methods. Lanes, protein samples from two different mice in each group (*left*). The intensities of blots were quantitated by densitometry (*right*). Repeat western blots gave similar results. Values, mean ± SD. *, $P < 0.001$ and **, $P < 0.01$: significant differences obtained by comparison with CRF-1 – treated or casein-treated mice, respectively.

those in the CRF-1–fed ($P < 0.05$) and casein-supplemented mice ($P < 0.05$). The mice supplemented with BCAA showed a significant decrease in the serum levels of triglyceride compared with the CRF-1 fed ($P < 0.05$). The serum leptin level of group 3 (BCAA) was also significantly lower than that of group 2 (casein; $P < 0.01$).

Effects of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II in AOM-treated* db/db *mice. Supplementation with BCAA caused a significant decrease in the serum levels of insulin (Fig. 2A) compared with the CRF-1–fed ($P < 0.01$) and casein-supplemented mice ($P < 0.01$). Similarly, there was a significant decrease in the serum levels of both IGF-I (Fig. 2B) and IGF-II (Fig. 2C) in BCAA-supplemented mice compared with the CRF-1–fed ($P < 0.001$ for each comparison) and casein-supplemented mice ($P < 0.001$ and $P < 0.01$, respectively).

Effects of BCAA supplementation on the expression levels of IGF-IR, p-IGF-IR, p-GSK-3β, p-Akt, and COX-2 proteins, and on cell proliferative activity in the colonic mucosa of AOM-treated* db/db *mice. Hyperinsulinemia and abnormal activation of the IGF/IGF-IR axis play a critical role in obesity-related CRC development (3, 6–10). Therefore, the effects of BCAA on the levels of IGF-IR and the phosphorylated (i.e., activated) form of IGF-IR proteins, and cell proliferation were examined in the colonic mucosa of AOM-treated mice. As shown in Fig. 2D, western blot analyses showed that BCAA supplementation caused a decrease in the levels of IGF-IR ($P < 0.001$ for each comparison) and p-IGF-1R ($P < 0.001$ for each comparison) proteins compared with the CRF-1–fed and casein-supplemented mice. Supplementation with BCAA also decreased the expression levels of the phosphorylated (i.e., inactivated) form of GSK-3β ($P < 0.01$ for each comparison), the phosphorylated (i.e., activated) form of Akt ($P < 0.001$ for each comparison), and COX-2 ($P < 0.01$ for each comparison) proteins compared with the control groups. The finding that BCAA supplementation inhibited the phosphorylation of Akt is considered to be significant because the activation of this protein is one of the critical targets in the constitutive activation of the IGF/IGF-IR axis in colorectal carcinogenesis (30).

In addition, as shown in Fig. 3, the PCNA labeling index of nonlesional crypts in the BCAA-supplemented mice was significantly smaller than that of the CRF-1–fed and casein-supplemented mice ($P < 0.001$ for each comparison), thus indicating that BCAA supplementation significantly inhibits cell proliferation in the colonic mucosa of the AOM-treated *db/db* mice.

Discussion

The present study clearly indicated that dietary supplementation with BCAA effectively suppressed the development of putative precursor lesions, ACF and BCAC (Fig. 1), for CRC

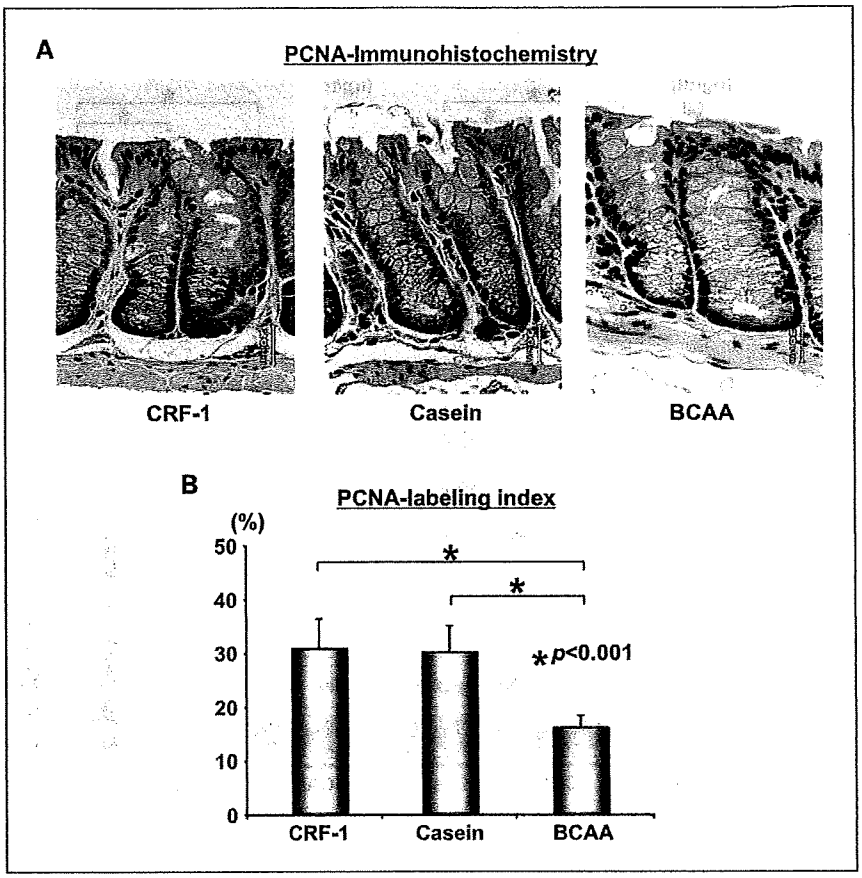

Fig. 3. The effect of BCAA supplementation on colonic epithelial cell proliferation in AOM-treated *db/db* mice. *A*, immunohistochemical staining of the normal crypts in the colon of AOM-treated *db/db* mice with anti-PCNA antibody. Sections of the colon were analyzed from CRF-1 – fed, casein-supplemented, and BCAA-supplemented mice, respectively. They were stained with anti-PCNA monoclonal antibody as described in Materials and Methods. Representative photographs from each group are shown. Bar, 20 μm. *B*, PCNA labeling index in the normal crypts in the colon of AOM-treated *db/db* mice. *Bars*, SD of triplicate assays.

(Table 2) by improving hyperlipidemia and hyperleptinemia in *db/db* mice (Table 3). The suppressive effect of BCAA in the early phase of obesity-related colorectal carcinogenesis was also associated, most likely, with the improvement of hyperinsulinemia (Fig. 2A) and the inhibition of cell proliferation on the colonic mucosa of experimental mice (Fig. 3). BCAA supplementation has also been reported to significantly decrease the incidence of hepatocellular carcinoma in patients with chronic liver disease if they had a body mass index score ≥25, and this effect might be associated with improvement of insulin resistance (15, 16, 31). Thus, BCAA might effectively prevent cancer development, at least in several organs, in obese subjects who are considered to have insulin resistance syndrome (3).

How can BCAA exert chemopreventive effects on obesity-related colorectal carcinogenesis? As described above, insulin resistance might be a critical target of BCAA in this beneficial effect because insulin has oncogenic properties on CRC cells. For instance, insulin stimulates the proliferation of CRC cells and promotes colorectal tumor growth in animal models (6–8). These reports, therefore, suggest that BCAA inhibits the development of colonic premalignant lesions (Table 2) and excessive cell proliferation in the colonic mucosa of AOM-injected *db/db* mice (Fig. 3) by improving insulin resistance (Fig. 2A). Recent studies by others have indicated that BCAA improves glucose tolerance by modulating insulin-independent glucose uptake into skeletal muscle in rodent models (32, 33). An improvement of insulin resistance and glucose tolerance by BCAA has also been shown by certain clinical trials (15, 31).

In addition, it is widely accepted that insulin resistance causes alterations in the IGF/IGF-IR axis, which may be closely associated with the development of CRC (9, 10, 30). For instance, the IGF-IR protein is overexpressed in BCAC compared with the surrounding normal cryptal cells (11). Therefore, the IGF/IGF-IR system is regarded as one of the effective targets with respect to the prevention of CRC (11). Our observations described herein comprise the first report showing that BCAA decreases the serum levels of IGF-I and IGF-II (Fig. 2B and C), thereby inhibiting the expression and activation of IGF-IR on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Our findings suggest that not only the improvement of insulin resistance but the inhibition of IGF/IGF-IR activation by BCAA plays a critical role in suppressing obesity-related and diabetes mellitus–related colorectal carcinogenesis.

The present study revealed that BCAA supplementation in the diet prevents the development of BCAC (Table 2), which is characterized by abundant β-catenin protein expression (23) and also accumulates the IGF-IR protein (11) while decreasing the expression levels of p-Akt and p-GSK-3β proteins on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Recent *in vitro* studies have indicated that insulin and the IGF/IGF-IR axis stabilize and activate the Wnt/β-catenin pathway, which is involved in the development of CRC (34, 35). GSK-3β, which can be phosphorylated by phosphatidylinositol 3-kinase/Akt via insulin or IGF treatment, is considered to be a key kinase for CRC development because the inactivation of GSK-3β leads to the dissociation of the adenomatous polyposis coli/axin/β-catenin complex and cytosolic β-catenin accumulation (36).

Free accumulated β-catenin translocates into the nucleus and forms a complex with the transcription factor T cell factor, thereby activating the transcription of target genes, including cyclin D1 and c-Myc, and thus contributing to abnormal proliferation and tumor progression (37, 38). Therefore, supplementation with BCAA, which targets insulin-associated and IGF-associated β-catenin accumulation by decreasing the levels of p-Akt and p-GSK-3β proteins (Fig. 2D), might be an effective strategy to prevent the development of CRC.

In addition to the beneficial effects mentioned above, BCAA has other physiologic activities that might be useful to prevent the development of CRC. For instance, supplementation with BCAA is capable of reducing the production of oxidative stress and microinflammation in patients with liver cirrhosis, which possibly leads to a decrease in the occurrence of hepatocellular carcinoma (39). In the current study, BCAA caused a decrease in the expression of the COX-2 protein in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). COX-2 is one of the main mediators in the inflammatory signaling pathway and is certainly involved in CRC development; therefore, it might be a critical target for CRC chemoprevention (40). This effect might be explained by the inhibitory effect of BCAA on the IGF/IGF-IR axis because the activation of this axis mediates COX-2 expression (41, 42). Additional studies are required to clarify the direct effects of BCAA on inflammation and their relevance to the antitumor effects of this agent.

In summary, the prevention of CRC by targeting the dysregulation of energy homeostasis, especially insulin resistance and the activation of the IGF/IGF-IR axis, might be a promising strategy for obese people who are at an increased risk of CRC. BCAA seems to be a potentially effective and critical candidate for this purpose because this agent can improve insulin resistance while also exerting a depressant effect on the IGF/IGF-IR axis. The current findings, as well as those from a previous report (11), also suggest the possibility of using specific agents that target insulin resistance as chemopreventive agents for other obesity-related and diabetes mellitus–related malignancies. Therefore, insulin resistance–improving agents, including BCAA, are worthy of being further investigated as candidates for novel chemopreventive agents that may find a potential role in the society today, in which excessive body weight has been found to be associated with the risk of various types of human epithelial malignancies (43, 44).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Giovannucci E, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology 2007;132:2208–25.
2. Frezza EE, Wachtel MS, Chiriva-Internati M. Influence of obesity on the risk of developing colon cancer. Gut 2006;55:285–91.
3. Chang CK, Ulrich CM. Hyperinsulinaemia and hyperglycaemia: possible risk factors of colorectal cancer among diabetic patients. Diabetologia 2003;46:595–607.
4. Berster JM, Goke B. Type 2 diabetes mellitus as risk factor for colorectal cancer. Arch Physiol Biochem 2008;114:84–98.
5. Yang YX, Hennessy S, Lewis JD. Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients. Gastroenterology 2004;127:1044–50.
6. Bjork J, Nilsson J, Hultcrantz R, Johansson C. Growth-regulatory effects of sensory neuropeptides, epidermal growth factor, insulin, and somatostatin on the non-transformed intestinal epithelial cell line IEC-6 and the colon cancer cell line HT 29. Scand J Gastroenterol 1993;28:879–84.
7. Corpet DE, Jacquinet C, Peiffer G, Tache S. Insulin injections promote the growth of aberrant crypt foci in the colon of rats. Nutr Cancer 1997;27:316–20.
8. Tran TT, Medline A, Bruce WR. Insulin promotion of colon tumors in rats. Cancer Epidemiol Biomarkers Prev 1996;5:1013–5.
9. Singh P, Rubin N. Insulin-like growth factors and binding proteins in colon cancer. Gastroenterology 1993;105:1218–37.
10. Durai R, Yang W, Gupta S, Seifalian AM, Winslet MC. The role of the insulin-like growth factor system in colorectal cancer: review of current knowledge. Int J Colorectal Dis 2005;20:203–20.
11. Shimizu M, Shirakami Y, Sakai H, et al. EGCG suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. Cancer Prev Res 2008;1:298–304.
12. Moriwaki H, Miwa Y, Tajika M, Kato M, Fukushima H, Shiraki M. Branched-chain amino acids as a protein- and energy-source in liver cirrhosis. Biochem Biophys Res Commun 2004;313:405–9.
13. Marchesini G, Bianchi G, Merli M, et al. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. Gastroenterology 2003;124:1792–801.
14. Muto Y, Sato S, Watanabe A, et al. Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. Clin Gastroenterol Hepatol 2005;3:705–13.
15. Kawaguchi T, Nagao Y, Matsuoka H, Ide T, Sata M. Branched-chain amino acid-enriched supplementation improves insulin resistance in patients with chronic liver disease. Int J Mol Med 2008;22:105–12.
16. Muto Y, Sato S, Watanabe A, et al. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 2006;35:204–14.
17. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557–76.
18. Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut 2005;54:533–9.
19. Hirose Y, Hata K, Kuno T, et al. Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-db/db mice. Carcinogenesis 2004;25:821–5.
20. Suzuki R, Kohno H, Yasui Y, et al. Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male db/db mice. Int J Cancer 2007;120:252–8.
21. Hayashi K, Suzuki R, Miyamoto S, et al. Citrus auraptene suppresses azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db mice. Nutr Cancer 2007;58:75–84.
22. Bird RP, Good CK. The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. Toxicol Lett 2000;112–113:395–402.
23. Yamada Y, Mori H. Pre-cancerous lesions for colorectal cancers in rodents: a new concept. Carcinogenesis 2003;24:1015–9.
24. Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes 2007;56:1647–54.
25. Hata K, Tanaka T, Kohno H, et al. β-Catenin-accumulated crypts in the colonic mucosa of juvenile ApcMin/+ mice. Cancer Lett 2006;239:123–8.
26. Mori H, Yamada Y, Kuno T, Hirose Y. Aberrant crypt foci and β-catenin accumulated crypts; significance and roles for colorectal carcinogenesis. Mutat Res 2004;566:191–208.
27. Shirakami Y, Shimizu M, Tsurumi H, Hara Y, Tanaka T, Moriwaki H. EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM and DSS. Mol Med Rep 2008;1:355–61.
28. Shimizu M, Shirakami Y, Sakai H, et al. EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. Cancer Lett 2008;262:10–8.
29. Shiraki M, Shimomura Y, Miwa Y, et al. Activation of hepatic branched-chain α-keto acid dehydrogenase complex by tumor necrosis factor-α in rats. Biochem Biophys Res Commun 2005;328:973–8.
30. Adachi Y, Lee CT, Coffee K, et al. Effects of genetic blockade of the insulin-like growth factor receptor in human colon cancer cell lines. Gastroenterology 2002;123:1191–204.
31. Urata Y, Okita K, Korenaga K, Uchida K, Yamasaki T, Sakaida I. The effect of supplementation with branched-chain amino acids in patients with liver cirrhosis. Hepatol Res 2007;37:510–6.
32. Nishitani S, Takehana K, Fujitani S, Sonaka I. Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis. Am J Physiol Gastrointest Liver Physiol 2005;288:G1292–300.

33. Nishitani S,Takehana K. Pharmacological activities of branched-chain amino acids: augmentation of albumin synthesis in liver and improvement of glucose metabolism in skeletal muscle. Hepatol Res 2004;30S:19–24.
34. Sun J, Jin T. Both Wnt and mTOR signaling pathways are involved in insulin-stimulated proto-oncogene expression in intestinal cells. Cell Signal 2008; 20:219–29.
35. Playford MP, Bicknell D, Bodmer WF, Macaulay VM. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of β-catenin. Proc Natl Acad Sci U S A 2000;97:12103–8.
36. Papkoff J, Aikawa M. WNT-1 and HGF regulate GSK3 β activity and β-catenin signaling in mammary epithelial cells. Biochem Biophys Res Commun 1998; 247:851–8.
37. Tetsu O, McCormick F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999; 398:422–6.
38. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998; 281:1509–12.
39. Ohno T, Tanaka Y, Sugauchi F, et al. Suppressive effect of oral administration of branched-chain amino acid granules on oxidative stress and inflammation in HCV-positive patients with liver cirrhosis. Hepatol Res 2008;38:683–8.
40. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 2001;1:11–21.
41. Di Popolo A, Memoli A, Apicella A, et al. IGF-II/IGF-I receptor pathway up-regulates COX-2 mRNA expression and PGE2 synthesis in Caco-2 human colon carcinoma cells. Oncogene 2000;19:5517–24.
42. Stoeltzing O, Liu W, Fan F, et al. Regulation of cyclooxygenase-2 (COX-2) expression in human pancreatic carcinoma cells by the insulin-like growth factor-I receptor (IGF-IR) system. Cancer Lett 2007;258: 291–300.
43. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet 2008;371:569–78.
44. Wiseman M. The Second World Cancer Research Fund/American Institute for Cancer Research Expert Report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Proc Nutr Soc 2008:1–4.

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Linoleic acid metabolite suppresses skin inflammation and tumor promotion in mice: possible roles of programmed cell death 4 induction

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(±)-13-Hydroxy-10-oxo-*trans*-11-octadecenoic acid (13-HOA) is one of the lipoxygenase metabolites of linoleic acid (LA) from corn germ. Recently, we reported that this metabolite suppressed the expression of lipopolysaccharide-induced proinflammatory genes in murine macrophages by disrupting mitogen-activated protein kinases and Akt pathways. In this study, we investigated the inhibitory effects of 13-HOA on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in ears and skin, as well as tumor promotion in female ICR mice. Pretreatment with 13-HOA (1600 nmol) inhibited ear edema formation by 95% ($P < 0.05$) in an inflammation test and reduced tumor incidence and the number of tumors per mouse by 40 and 64% ($P < 0.05$ each), respectively, in a two-stage skin carcinogenesis model. Histological examinations revealed that it decreased epidermal thickness, the number of infiltrated leukocytes and cell proliferation index. Furthermore, 13-HOA (8–40 μM) suppressed TPA-induced anchorage-independent growth of JB6 mouse epidermal cells by 70–100%, whereas LA was virtually inactive. 13-HOA (40 μM) inhibited TPA-induced activator protein-1 transactivation but not extracellular signal-regulated kinase1/2 activation. Interestingly, 13-HOA (40 μM and 1600 nmol in JB6 cells and mouse skin, respectively) induced expression of programmed cell death 4 (Pdcd4), a novel tumor suppressor protein. To our knowledge, this is the first report of a food factor that is able to induce Pdcd4 expression. Collectively, our results indicate that 13-HOA may be a novel anti-inflammatory and antitumor chemopreventive agent with a unique mode of action.

Introduction

A two-stage mouse skin carcinogenesis model is useful for understanding the multistage nature of human neoplasia and ideal for studying a variety of biochemical alternations, changes in cellular functions and histological changes that occur during the different stages of chemical carcinogenesis (1–3). On the other hand, there is a large body of evidence showing that inflammation plays important roles in the promotion and progression of skin tumorigenesis (4). That report also noted that the essential contribution of inflammation to tumor development and progression is supported from observations indicating that tumor promotion originates from exposure of initiated cells to chemical irritants such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). TPA activates transcription factors including activator protein-1 (AP-1) and nuclear factor-κB by upregulation of mitogen-activated protein kinase (MAPK) and Akt (protein kinase B) activities in mouse skin, which have a partial correlation with signaling molecules that participate in lipopolysaccharide (LPS)-treated macrophages (5–7).

The mouse JB6 epidermal variant cell system, which includes transformation-sensitive (P+) and transformation-resistant (P−) cells, is a valuable cell culture model that demonstrates a number of molecular events and pathways involved in the tumor promotion stage of mouse skin carcinogenesis (5,6). This unique cell line responds irreversibly to tumor promoters and growth factors to exhibit tumor phenotypes, including anchorage-independent growth in soft agar (5). Similar to the mouse skin model, TPA activates AP-1 and upstream signaling molecules in JB6 P+ cells, such as MAPKs and Akt (7,8).

Programmed cell death 4 (Pdcd4), also known as MA3 (9) and DUG (10), was recently identified as a novel tumor suppressor. Differential display of the messenger RNA (mRNA) expression profile in JB6 P+ and P− cells showed *pdcd4* to be preferentially expressed in JB6 P− cells, whereas a reduction of Pdcd4 in JB6 P− cells by an antisense approach resulted in acquisition of transformation responses (11). Conversely, stable overexpression of Pdcd4 in JB6 P+ cells produced a transformation-resistant phenotype (12). Moreover, *pdcd4* transgenic mice are resistant to skin tumorigenesis and tumor progression (13). These findings suggest that Pdcd4 is a novel suppressor of tumor promoter-induced transformation. Thus, induction or production of this molecule may be a promising strategy for cancer prevention; however, there are few known compounds capable of inducing Pdcd4 mRNA and protein (14,15).

(±)-13-Hydroxy-10-oxo-*trans*-11-octadecenoic acid (13-HOA) is one of the lipoxygenase metabolites of linoleic acid (LA) found in corn germ, in which LA is oxidized enzymatically or spontaneously to 13-HOA (Figure 1) (16). It was previously reported that 13-HOA showed toxicity toward several cancer cell lines via an unknown mechanism (17). Recently, we reported that 13-HOA significantly inhibited activation of both MAPKs and Akt and reduced the expression of proinflammatory genes in RAW 264.7 murine macrophages (18). Together, these findings suggest that 13-HOA is an effective chemopreventive agent, though there are no known reports of its *in vivo* efficacy.

In the present study, we determined whether 13-HOA inhibits TPA-induced inflammatory and tumor promotion using mouse ear inflammation and 7,12-dimethylbenz(*a*)anthracene (DMBA)/TPA-induced two-stage mouse skin carcinogenesis models and TPA-induced anchorage-independent growth in JB6 P+ cells. In addition, we assessed the effects of 13-HOA on mRNA and protein expression of *pdcd4* in JB6 P+ cells and mouse skin.

Abbreviations: AP-1, activator protein-1; DMBA, 7,12-dimethylbenz(*a*)anthracene; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; EMEM, Eagle's minimum essential medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; Fra-1, fos-like region antigen; 13-HOA, (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid; JNK, c-Jun N-terminal kinase; LA, linoleic acid; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; miR-21, microRNA-21; mRNA, messenger RNA; Myb, v-myb myeloblastosis viral oncogene homolog; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; Pdcd4, programmed cell death 4; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Materials and methods

Animals and cells

Female ICR mice were purchased from Japan SLC (Shizuoka, Japan) at 5–6 weeks of age and maintained according to the Guidelines for the Regulation of Animals, provided by the Animal Experimentation Committee of Kyoto University. All animals were housed under controlled conditions of humidity (60 ± 5%), lighting (12 h light cycle) and temperature (24 ± 2°C). Mouse epidermal JB6 P+ (Cl 41-5a) cells were purchased from American Type Culture Collection (Manassas, VA). JB6 P+ cells and JB6 (P^{+11}) cells transfected stably with

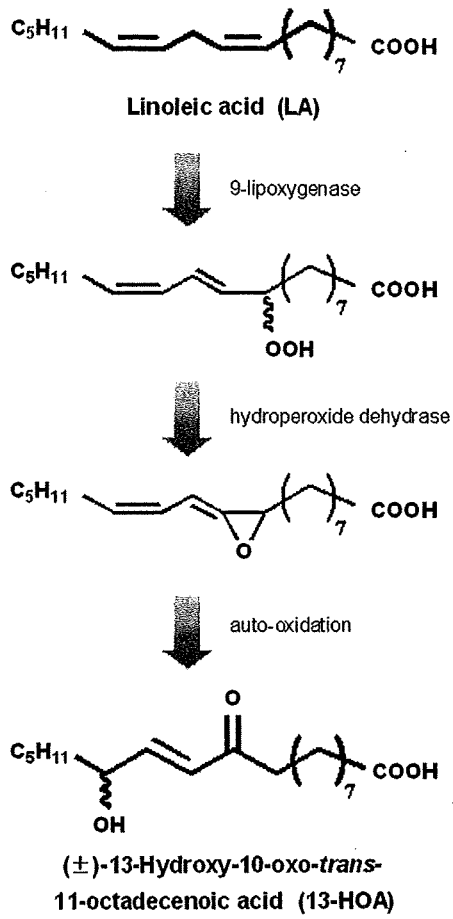

Fig. 1. Biosynthesis pathways of 13-HOA from LA in corn germ.

an AP-1-luciferase reporter (19) were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) or Eagle's minimum essential medium (EMEM) supplemented with 5% heat-inactivated fetal FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and G418 (600 μg/ml), at 37°C under a humidified atmosphere of 95% air and 5% CO_2.

Chemicals

13-HOA was purified as previously reported (>95% purity) (16). DMEM, EMEM and FBS were purchased from Invitrogen (Carlsbad, CA). Antibodies were obtained from the following sources: rabbit anti-phospho-extracellular signal-regulated kinase (ERK)1/2, rabbit anti-ERK1/2 antibody and horseradish peroxidase-conjugated anti-rabbit IgG from Cell Signaling Technology (Beverly, MA); goat anti β-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-α-tubulin antibody from Oncogene (San Diego, CA); horseradish peroxidase-conjugated anti-goat IgG and anti-mouse IgG from Dako (Glostrup, Denmark) and G418 from Sigma–Aldrich (St Louis, MO). Anti-Pdcd4 antibody was generated as previously reported (12). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified.

Mouse ear inflammation test

The inflammation assay was performed as previously reported (20). Briefly, ICR mice at 6–7 weeks old were divided into five groups (n = 5 per group). Each mouse had 0, 160 or 1600 nmol of 13-HOA or 1600 nmol of LA dissolved in 20 μl of acetone applied to the left ear, whereas the other ear received the same volume of the vehicle alone. After 20 min, 1.6 nmol of TPA dissolved in 20 μl acetone was applied to both ears. After 6 h, ear punch biopsies (6 mm diameter) were obtained and weighed to determine the increase in ear weight.

Two-stage mouse skin carcinogenesis test

The antitumor-promoting activities of 13-HOA were examined using a standard initiation–promotion protocol with DMBA and TPA as previously reported, with some modifications (21). The back of each 7-week-old female ICR mouse was shaved with a surgical clipper 2 days before initiation, then DMBA (200 nmol) was dissolved in 200 μl of acetone and applied onto the back of each mouse. One week later, the mice were exposed to a topical application of 0, 160 or 1600 nmol of 13-HOA (n = 25 per group) and 160 or 1600 nmol of LA (n = 15 per group) dissolved in 200 μl acetone 30 min before each TPA or acetone treatment (1.6 nmol), which was done twice a week for 20 weeks. Antitumor-promoting activities were evaluated by the incidence of tumor-bearing mice, the number of tumors (>1 mm in diameter) per mouse and average tumor diameter, which was determined as the average diameter of all papillomas on each mouse.

Histopathological analysis

After week 20 of the two-stage mouse skin carcinogenesis experiment, the mice were euthanized and then tissue biopsies were obtained and subjected to histopathological evaluations. Histopathological examinations were performed using biopsy sections randomly chosen from 12 mice in the none, acetone, TPA-only and DMBA-only groups and from 18 mice in the DMBA–TPA, +160 nmol 13-HOA and +1600 nmol 13-HOA groups. Formalin-fixed and paraffin-embedded skin sections were subjected to deparaffinization and dehydration prior to quenching of endogenous peroxidase activity (1.5% H_2O_2 in absolute methanol for 20 min). An antigen-unmarking step was done by placing the slides in a pressure cooker containing 0.01 M sodium citrate (pH 6.0) for 10 min. The sections were incubated for 60 min with the primary mouse anti-rat proliferating cell nuclear antigen (PCNA) monoclonal antibody (Clone PC-10, DakoCytomation, Glostrup, Denmark, Cat no. M0879) at a dilution of 1:1500 in 10% goat serum. The secondary antibody, biotinylated goat anti-mouse IgG (Cat no. BA-2000, Vector Laboratories, Burlingame, CA), was applied for 30 min in a 1:500 dilution. Slides were processed using Vectastain Elite ABC reatent (Vector Laboratories) with diaminobenzidine as the substrate. Sections were counter stained with hematoxylin (Merck Ltd, Tokyo, Japan). All histological examinations were done by a single pathologist (T.T.) in a blind fashion.

Cell viability

Cell viability was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. JB6 P+ cells at a density of 3×10^4 cells per well were cultured in 96-well plates in DMEM with 5% FBS and then incubated in a CO_2 incubator for 12 h. The cells were rinsed with phosphate-buffered saline and the medium was exchanged with DMEM with 10% FBS containing 0.25% dimethyl sulfoxide (DMSO) as the vehicle, 13-HOA, NS-398 cyclooxygenase-2 inhibitor or LA in DMSO, followed by a 30 min incubation. Ethanol (0.15%) as a vehicle or 30 nM of TPA was added to each well. After treatment for 24 h, Cell Counting Kit-8 (1%) was added and incubated for 2 h at 37°C. Absorbance in each well was determined at 450 nm (reference 650 nm) using a microplate reader (Multiskan JX Ver. 1.1, Thermo Labosystems, Helsinki, Finland). Each experiment was performed in triplicate.

Anchorage-independent transformation test

The effects of 13-HOA on TPA-induced cell transformation in JB6 P+ cells were examined using an anchorage-independent transformation assay; the top agar was 0.33% and the bottom agar was 0.5% according to the procedure described previously (22). Experiments were performed in six-well plates, in which 3×10^3 cells were suspended in 1 ml of 0.33% agar medium, with 4 ml of 0.5% agar medium poured on top. The base agar and that poured on top consisted of DMEM (10% FBS), Bacto Agar (Difco Labs, Detroit, MI) and 13-HOA, NS-398, LA or DMSO (vehicle), along with TPA or ethanol (vehicle). The cells were preincubated with each test sample for 30 min and then incubated for 14 days at 37°C in a CO_2 incubator, with colonies >100 μm in diameter counted. None of the test samples showed notable cytotoxicity for 24 h at the various concentrations. Each experiment was done in triplicate.

Luciferase assay for AP-1-dependent transactivation

JB6 P^{+11} cells (4×10^4) were seeded in 24-well plates in EMEM with 5% FBS for 24 h. The cells were then serum starved in EMEM with 0.1% FBS for 24 h and treated with or without 13-HOA (40 μM) for 30 min before the cells were exposed to TPA (30 nM). The luciferase assay was conducted using a Dual-Luciferase™ Reportor Assay System (Promega, Madison, WI) by slight modification of the manufacturer's protocol. Briefly, after TPA treatment for 12 h, the cells were lysed with Passive Lysis Buffer and the luciferase activity was measured by a luminometer (Lumat LB9507; PerkinElmer, Boston, MA). AP-1 activity was expressed as fold induction relative to the control cells without TPA treatment.

Western blot analysis

The effects of 13-HOA on ERK1/2 activation and Pdcd4 expression in JB6 P+ cells and mouse skin were examined as indicated below. The cells (1×10^6)

were seeded into 60 mm dishes in DMEM with 5% FBS and then incubated in a CO_2 incubator for 12 h. The cells were rinsed with phosphate-buffered saline and the medium was exchanged with DMEM (10% FBS) containing 13-HOA, NS-398, LA or 0.25% DMSO and incubated for 30 min. Next, the cells were treated with 30 nM of TPA for the indicated hours and then the cells were lysed with lysis buffer [protease inhibitor, phosphatase inhibitor (Sigma–Aldrich), 10 mM Tris (pH 7.4), 1% sodium dodecyl sulfate and 1 mM sodium vanadate]. The back of each 7-week-old female ICR mouse was shaved with a surgical clipper before acetone or 13-HOA (1600 nmol dissolved in 200 µl acetone) treatment and then TPA (8 nmol) was dissolved in 200 µl acetone and applied onto the back of each mouse, and the mice were killed by cervical dislocation 6 h later. For the isolation of epidermal protein from mouse skin, fat and dermis were removed on ice, and the collected epidermis was lysed with lysis buffer as above. Lysates from both JB6 P+ cells and mouse skin were sonicated and centrifuged at 15 000 r.p.m. for 5 min. Supernatant was collected and total protein concentration was quantified using a Bio Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and standardized with γ-globulin as the reference.

These lysate samples were denatured in sample buffer containing sodium dodecyl sulfate and mercaptoethanol. An equal amount of protein (20 µg) was loaded for sodium dodecyl sulfate gel electrophoresis and then the samples were electro-transferred to Immobilon-P membranes (Millipore, MA). The transferred protein-bound membranes were blotted with each primary and secondary antibody and then visualized using enhanced chemiluminescence reagents (GE Healthcare UK Ltd, Buckinghamshire, UK). The intensity of each band was analyzed using Scion Image 0.4.0.3 (Scion Corporation, Frederick, MD).

Reverse transcription–polymerase chain reaction

JB6 P+ cells (1×10^6) were seeded into 60 mm dishes in DMEM with 5% FBS and then incubated in a CO_2 incubator for 12 h. The cells were rinsed with phosphate-buffered saline and the medium was exchanged with DMEM with 10% FBS containing the vehicle, DMSO (0.25%), 13-HOA (1.6, 8 or 40 µM), NS-398 (100 µM) or LA (100 µM). After the indicated hours, total RNA was isolated from the cells using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. One microgram of total RNA was then added to a mixture of $MgCl_2$ (Ambion, Austin, TX), 10× polymerase chain reaction (PCR) buffer, deoxynucleoside triphosphate mixture, avian myeloblastosis virus reverse transcriptase (5 U/µl), ribonuclease inhibitor and oligo dT primer (Takara, Kyoto, Japan) in a total volume of 19 µl. Each tube was placed in a thermal cycler (PTC-0100; MJ Research, Watertown, MA) and heated as follows: 10 min at 30°C, 30 min at 55°C, 5 min at 99°C and 5 min at 5°C. Next, 1 µl each of *pdcd4* and *cyclophilin* complementary DNA were amplified with 0.5 µl of each primer (20 µM, Proligo, Kyoto, Japan) and a mixture of 10× PCR buffer, deoxynucleoside triphosphate mixture, $MgCl_2$, *Taq* DNA polymerase (5 U/µl) (Takara) and nuclease-free water. The primers used and PCR conditions were as follows: *pdcd4* (5′-TAATCAgTgCAAgCgAAATTAAggAA-3′ and 5′-CCTTTCCCAgATCTggACCgCCTATC-3′), 27 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 45 s and *cyclophilin* (5′-gCCAggACCTgTATgCTTCA-3′ and 5′-TTgggTCgCgTCTCgTTCgA-3′), 19 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 1 min. Amplified DNA was separated by agarose gel electrophoresis. Image analysis was performed using Scion Image 0.4.0.3. No PCR saturation was confirmed by titrating each complementary DNA amount (data not shown).

Statistical analysis

Data are shown as the mean ± SD from more than three independent experiments and were evaluated by Student's *t*-test or a χ^2-test. Differences were considered statistically significant at the $P < 0.05$ level.

Results

Effects of 13-HOA on TPA-induced edema formation in ICR mice ears

First, we examined the *in vivo* anti-inflammatory activities of 13-HOA using an acute edema formation model, in which TPA was topically applied onto the ears of ICR mice. As shown in Figure 2A, topical application of 13-HOA (160–1600 nmol), 20 min prior to TPA stimulation, significantly inhibited TPA-induced ear edema formation in a dose-dependent manner ($P < 0.05$, at a dose of 1600 nmol), whereas LA, the biosynthetic precursor of 13-HOA, was inactive even at a high dose.

Effects of 13-HOA on tumor promotion in ICR mice

We then examined the inhibitory effects of a topical application of 13-HOA twice a week for 20 weeks on tumor formation in DMBA (200 nmol)-initiated and TPA (1.6 nmol)-promoted mouse skin. One week following DMBA initiation, the dorsal skin of the mice was exposed to TPA in the presence or absence of 13-HOA or LA (160 and 1600 nmol each). No tumors were developed in any of the mice in the none, acetone, DMBA-only and TPA-only groups ($n = 25$ in each group) (data not shown). The results showed that treatment of the animals with 13-HOA decreased tumor incidence by 40% ($P < 0.05$) at a dose of 1600 nmol as well as the number of tumors per mouse in a dose-dependent manner (51–65% inhibition, $P < 0.05$) (Figure 2B–D). LA did not inhibit tumor incidence or size at both doses (160 and 1600 nmol), and there were no differences in regard to tumor diameter among the groups. The mice were euthanized after the assays and

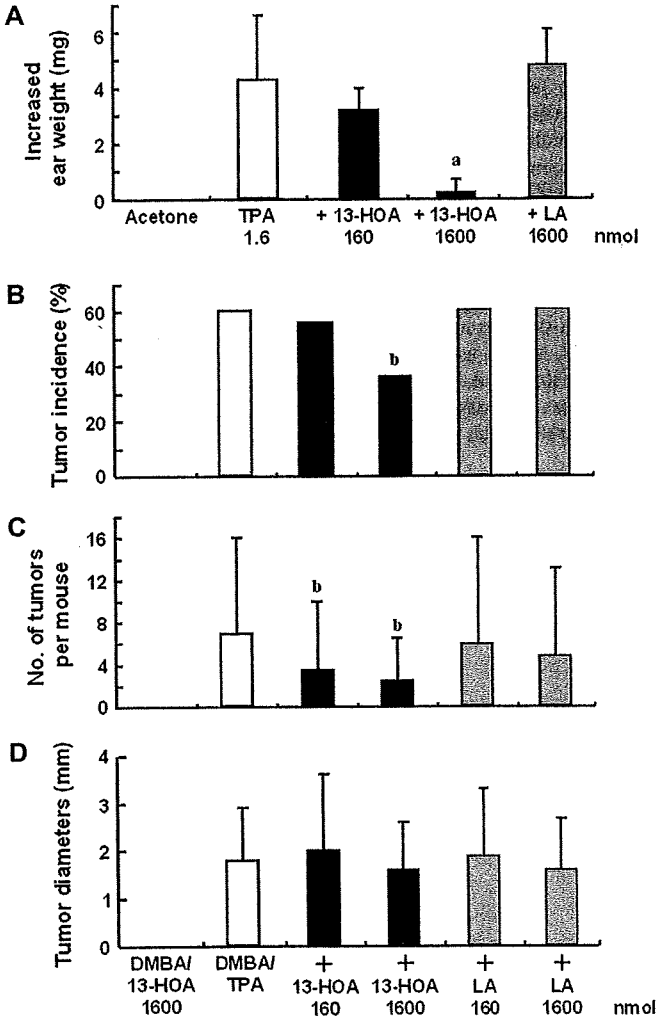

Fig. 2. TPA-induced ear edema formation and tumor promotion in ICR mice were significantly suppressed by topical application of 13-HOA. **(A)** Acetone, 13-HOA (160 or 1600 nmol) or LA (1600 nmol) was applied to an inner area of one ear of ICR mice ($n = 5$ per treatment group). After 20 min, TPA (1.6 nmol) was applied to the same area of both ears. After 6 h, increased ear weight was determined by obtaining a 6.0 mm punch biopsy. Statistical analysis was done by Student's *t*-test. $^aP < 0.05$ versus TPA. **(B–D)** The mice ($n = 15$ in LA treatment group, $n = 25$ in other groups) were given a single application of DMBA (200 nmol) at the beginning of the experiment and then received TPA (1.6 nmol) twice weekly for 20 weeks. The mice were treated with acetone, 13-HOA (160 or 1600 nmol) or LA (160 or 1600 nmol) 40 min prior to each TPA treatment. The antitumor promotion activity of 13-HOA was evaluated by tumor incidence (B), the number of tumors per mouse (C) and tumor diameter (D). None of the mice in the none, acetone, TPA-only and DMBA-only groups developed tumors. To determine significant differences, a χ^2-test was used for tumor incidence, whereas Student's *t*-test was used for both the number of tumors per mouse and tumor diameters. $^bP < 0.05$ versus DMBA–TPA.